Ultraviolet Radiation Significantly Enhances the Molecular Response to Dispersant and Sweet Crude Oil Exposure in *Nematostella vectensis*

Ann M. Tarrant\(^a,\#\), Samantha L. Payton\(^b,\#\), Adam M. Reitzel\(^a,c\), Danielle T. Porter\(^b,d\), Matthew J. Jenny\(^b\)*

From the \(^a\)Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, \(^b\)Department of Biological Sciences, University of Alabama, Tuscaloosa, AL 35487, \(^c\)Department of Biological Sciences, University of North Carolina at Charlotte, Charlotte, NC 28223, \(^d\)Department of Neurobiology and Anatomical Sciences, University of Mississippi Medical Center, Jackson, MS 39216

\(^\#\)These authors contributed equally to this work.

*Address correspondence: Matthew J. Jenny, Department of Biological Sciences, Box 870344, University of Alabama, Tuscaloosa, AL 35487, USA. Tel: 205-348-8225, Fax: 205-348-1786, Email: mjjenny@ua.edu

Running title: *Nematostella* Response to Abiotic Stressors
ABSTRACT

Estuarine organisms are subjected to combinations of anthropogenic and natural stressors, which together can reduce an organisms’ ability to respond to either stress or can potentiate or synergize the cellular impacts for individual stressors. *Nematostella vectensis* (starlet sea anemone) is a useful model for investigating novel and evolutionarily conserved cellular and molecular responses to environmental stress. Using RNA-seq, we assessed global changes in gene expression in *Nematostella* in response to dispersant and/or sweet crude oil exposure alone or combined with ultraviolet radiation (UV). A total of 110 transcripts were differentially expressed by dispersant and/or crude oil exposure, primarily dominated by the down-regulation of 74 unique transcripts in the dispersant treatment. In contrast, UV exposure alone or combined with dispersant and/or oil resulted in the differential expression of 1,133 transcripts, of which 436 were shared between all four treatment combinations. Most significant was the differential expression of 531 transcripts unique to one or more of the combined UV/chemical exposures. Main categories of genes affected by one or more of the treatments included enzymes involved in xenobiotic metabolism and transport, DNA repair enzymes, and general stress response genes conserved among vertebrates and invertebrates. However, the most interesting observation was the induction of several transcripts indicating *de novo* synthesis of mycosporine-like amino acids and other novel cellular antioxidants. Together, our data suggest that the toxicity of oil and/or dispersant and the complexity of the molecular response are significantly enhanced by UV exposure, which may co-occur for shallow water species like *Nematostella*.

KEYWORDS

hydrocarbons, UV radiation, biomarker, chemical pollution, environmental toxicology, RNA sequencing, cnidarian
1. INTRODUCTION

The Deepwater Horizon oil spill in April 2010, resulted in the release of an estimated 4.9 million barrels of sweet crude oil over a period of 87 days, and initial responses included the surface and subsurface application of approximately 1.8 million gallons of chemical dispersant (Ramseur, 2010). As a result, salt marsh organisms were exposed to weathered and dispersed sweet crude oil, and oil-derived compounds (Natter et al., 2012; Stout et al., 2016). This resulted in extensive mortality of saltmarsh plants, particularly in heavily oiled areas (Lin and Mendelssohn, 2012). Benthic invertebrates were impacted both through direct toxicity of the oil, and indirectly through loss of seagrass habitat, and trophic interactions. Observed effects on benthic invertebrates in oiled marsh areas included suppression of crab burrowing (McCall and Pennings, 2012), as well as reduced meiofaunal abundance and diversity (Fleeger et al., 2015), periwinkle abundance (Zengel et al., 2016), and shrimp growth rates (Rozas et al., 2014).

Major constituents of crude oil, a complex mixture of aromatic and non-aromatic hydrocarbons, include the polycyclic aromatic hydrocarbons (PAHs), a group of toxic compounds that pose a significant risk to humans and other animals. Exposure of animals to PAHs can result in oxidative stress and can compromise immune function, endocrine regulation and development (reviewed by Hylland 2006). Among animals, molecular responses to PAH exposure are best understood in vertebrates, where the aryl hydrocarbon receptor (AHR) pathway is the major regulatory pathway. In vertebrates, AHR is a ligand-activated, inducible transcription factor that pairs with its dimerization partner, AHR nuclear translocator (ARNT), to regulate the expression of a battery of phase I and phase II metabolic enzymes. The primary AHR target gene and classic biomarker of vertebrate hydrocarbon exposure is cytochrome P450 1A (CYP1A), a phase I oxidative enzyme that plays a major role in the biotransformation of aromatic hydrocarbons. The AHR pathway also regulates the expression of phase II enzymes that play a role in the conjugative biotransformation of hydrocarbons, such as glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UDPGTs), which facilitate excretion and prevent reabsorption of the hydrocarbons. In addition to these AHR-mediated effects, studies in diverse animal groups have shown that PAH exposure leads to widespread physiological responses; among these, up-regulation of defenses against oxidative stress, including antioxidant genes, non-enzymatic antioxidants, and heat shock proteins is typically observed (e.g., Hannam et al., 2010, Jenny et al., 2016). This cellular response is largely driven by the nuclear factor (erythroid-derived 2)-like 2 (NRF2) transcription factor, often considered to be a master regulator of the oxidative stress response. Activation of this pathway is mainly driven by the potential for cytochrome P450 monooxygenases, e.g. CYP1A, to generate significant amounts of reactive oxygen species, which contributes to toxicity, as a result of inefficient metabolism of cytochrome P450 substrates (Schlezinger et al., 1999, 2006).
In cnidarians, PAH, oil and dispersant toxicity have primarily been studied in tropical reef-building corals, through studies motivated by concerns about potential impacts of oil exposure on reefs. Within natural coral populations, oil exposure has been shown to result in bleaching, tissue retraction, photoinhibition of symbionts, impaired reproduction, and increased occurrence of injuries; however the cellular pathways underlying these effects are poorly known (reviewed by van Dam et al., 2011). Experimental studies have shown that corals can rapidly take up environmentally relevant concentrations of PAHs and polychlorinated biphenyls (PCBs) (Solbakken et al., 1984; Kennedy et al., 1992), both strong activators of the AHR pathway in vertebrate model organisms. However, despite the potential for rapid uptake, corals often concentrate hydrophobic contaminants in their lipid-rich tissues and exhibit poor excretion (Denton et al., 2006; Peters et al., 1997). Some studies have demonstrated low ethoxyresorufin O-dealkylation (EROD) activity, a catalytic measurement of CYP1A activity, in cnidarian (sea anemone) tissues (Heffernan and Winston, 1998; Heffernan and Winston, 2000); while other studies have detected low activity of various phase I and phase II metabolic enzymes in corals, e.g., GST and UDPGT (Gassman and Kennedy, 1992). Thus, the concentration of hydrophobic contaminants in lipid-rich tissues of cnidarians is likely due in part to low rates of xenobiotic metabolism. While a few studies have attempted to characterize dose-response relationships and identify biomarkers of exposure (Rougée et al., 2006; Epstein et al., 2000; Negri and Heyward, 2000; DeLeo et al., 2016; Venn et al., 2009; Goodbody-Gringley et al., 2013), little is known regarding the molecular responses of cnidarians to oil or other petroleum-based pollutants. In addition, many of these studies have also quantified the toxicity of chemical dispersant and enhanced toxicity of dispersant-oil mixtures. Experimental exposure of corals to chemical dispersants alone has resulted in decreased settlement, metamorphosis and survival of coral larvae (Epstein et al. 2000; Negri and Heyward, 2000; Goodbody-Gringley et al., 2013); increased tissue mortality in corals and deepwater octocorals (DeLeo et al., 2016); and induction of a few stress-responsive genes in coral (Venn et al., 2009). Although these studies of dispersant exposure all found that co-exposure to oil and dispersants led to enhanced toxicity (increased severity of the previously mentioned effects) relative to either stressor alone, the associated molecular mechanisms are poorly known.

In addition to general toxicity from hydrocarbon and/or dispersant exposures, many marine invertebrates must also deal with the additional cellular stress that occurs from co-exposure to natural abiotic stressors, such as ultraviolet (UV) radiation. Furthermore, UV radiation can enhance PAH toxicity via through two primary mechanisms (reviewed by Neff, 2002). First, PAHs can absorb UV energy that can be transferred to molecular oxygen, resulting in the production of reactive oxygen species (ROS), such as singlet oxygen and superoxide radicals. The second mechanism results from photomodification of the parent PAH into secondary by-
products that can have similar or even greater toxicity. It should also be noted that the
absorption of UV radiation by endogenous cellular compounds can result in the production of
ROS. Finally, cellular DNA can directly absorb UVB radiation (290-320 nm) resulting in the
formation of damaged photoproducts (reviewed in Pfeifer et al., 2005).

In a previous study, we exposed juveniles *Nematostella vectensis* (starlet sea anemone,
“Nematostella” hereafter) to ultraviolet light and PAHs, both separately and in combination, and
we observed greater mortality with the combination of stressors relative to either by itself. We
also measured signification changes in transcription of some candidate biomarker genes
associated with general cellular (heat shock proteins, HSPs) and oxidative stress (superoxide
dismutases, SODs) (Tarrant et al., 2014). *Nematostella* is an estuarine denizen found along the
eastern Atlantic seaboard of the United States and Canada (Reitzel et al., 2008). This species
primarily inhabits sediments in shallow habitats with little flow where individuals can experience
tremendous shifts in abiotic conditions. *Nematostella*’s infaunal distribution also may expose
this species to various pollutants that concentrate into the sediment. Due to ease of laboratory
culture through the full life cycle, tractability of genetic manipulations, and the availability of a
sequenced genome *Nematostella* has become a cnidarian model for cellular and molecular
responses to environmental stress (Goldstone, 2008; Reitzel et al., 2008; Tarrant et al., 2015).

Given the limited information available on the global transcriptomic response to xenobiotic
exposure in cnidarians, we sought to expand the understanding of cnidarian stress responses
beyond the small number of well-characterized biomarkers. This approach provides an
opportunity to investigate the potential of a taxonomically restricted response, which might be
shared with corals or unique to this salt marsh resident. However, since *Nematostella*, as a
cnidarian, belongs to a sister group to the Bilateria, characterization of these responses may
also provide a way to root studies in bilaterian organisms (e.g., fish, oyster, shrimp) to assess
conserved and divergent responses between vertebrates and invertebrates.

In this study we investigate the transcriptome-wide responses of *Nematostella* to dispersant
and/or crude oil exposure alone, or coupled to a physical stressor (UV radiation). We
hypothesized that sweet crude oil exposure would result in up-regulation of genes associated
with xenobiotic metabolism and antioxidant defenses, and that co-exposure to dispersant and/or
UV radiation would result in larger numbers of differentially expressed genes. Because we
sought to gain mechanistic insight into both novel and conserved molecular responses, we
conducted a gene-ontology (GO) enrichment analysis to identify regulated processes in an
unbiased manner and also explicitly examined differential responses of genes with known or
predicted roles in the stress response.
2. MATERIALS AND METHODS

2.1. Animal culture

Adult *Nematostella* were originally collected from Great Sippewissett Marsh, MA USA and propagated in the laboratory over several years. Animals used in these experiments were reared for over one month in common garden at room temperature in glass dishes (12 x 18 x 5.5 cm) containing 500 ml filtered natural seawater, diluted to 20 ppt, referred to subsequently as “seawater” (<100 anemones, ~10 g total wet weight per container). Animals were fed brine shrimp nauplii four times per week, and water was changed weekly. Animals held in these conditions exhibit consistent growth and can be induced to spawn regularly (Reitzel et al. 2013, Stefanik et al. 2013).

2.2. Chemical and UV exposures

For chemical exposures, a water accommodated fraction of Corexit© 9500 dispersant (referred to as DISP) and a water accommodated fraction of Macondo sweet crude oil (referred to as WAF) were prepared by adding dispersant or sweet crude oil into an amber-colored glass bottle containing diluted seawater to give nominal concentrations of DISP (100 ul dispersant diluted into 900 ml distilled water; 2 µl diluted dispersant added to 100 ml seawater; 2 ppm (v:v)) or oil (2 µl of sweet crude oil added to 100 ml seawater; 20 ppm (v:v)). To test for possible effects of Corexit© 9500 chemical dispersant and sweet crude oil, a chemically-enhanced WAF (referred to as CEWAF) was generated by creating a mixture of 2 ppm of dispersant and 20 ppm of sweet crude oil in seawater (2 ul of 1:10-diluted dispersant and 2 ul sweet crude oil added to 100 ml seawater). All three bottles (DISP, WAF and CEWAF) were capped tightly and stirred overnight with a Teflon-coated magnetic stir bar such that a vortex was formed that reached one-third of the way toward the bottom of the bottles (Tarrant et al., 2014). These chemical exposures are consistent with environmentally relevant concentrations (Hamdan and Fulmer, 2001) and are significantly lower than concentrations that have resulted in significant toxicity in other marine invertebrates (Goodbody-Gringley et al., 2013). Furthermore, the concentrations were chosen because they have previously resulted in measurable changes in gene expression (Tarrant et al., 2014).

UV radiation was provided by a UV-B-enriched bulb (Zilla Desert Series fluorescent T5 bulb, purchased at Petco (Falmouth, MA, USA). *Nematostella* were exposed to seawater (experimental control), three chemical treatments (DISP, WAF and CEWAF), seawater with UV
(referred to as UV), or three chemical treatments with the addition of UV (DISP_UV, WAF_UV and CEWAF_UV). For the experimental period, 3-4 adult *Nematostella* were placed into each of three glass dishes (30 mm diameter x 12 mm depth, Electron Microscopy Sciences, Hatfield, PA, USA) per treatment. Dishes were covered either with UV-transparent plastic (UV treatments) or with UV-opaque glass (no UV treatments). Dishes were incubated for 6 h on a shelf 14 cm below the UV bulb (providing 1.6 W m\(^{-2}\) UV-A and 0.66 W m\(^{-2}\) UV-B, measured using an OL 656 UV-vis spectroradiometer as in Tarrant et al. 2014). After 6 h, the UV bulb was turned off, and animals were sampled 18 h later (24 h after the start of the experiment). UV exposure conditions were primarily chosen because we have previously observed effects on gene expression in *Nematostella* with this level of exposure. While the levels are environmentally realistic (lower than noon levels on a cloudless day in July at 42°N), the levels experienced by natural populations of *Nematostella* are reduced to an unknown degree through burrowing into sediments (see Tarrant et al. 2014 for additional discussion).

All 8 combinations of controls and chemical and/or UV treatments were characterized by global transcriptional profiling (RNA-seq). At the end of the exposure periods, all animals were immediately placed in microcentrifuge tubes containing a 10-fold excess (w:v) of RNAlater using plastic transfer pipettes. Samples in RNAlater were gently inverted and stored at -20°C until total RNA isolation. Single biological replicates were generated by pooling all of the adult *Nematostella* from a single dish into one sample; thus, the three dishes in each treatment resulted in three biological replicates.

### 2.3. Library preparation and next generation sequencing

Total RNA was extracted from the pooled animals within each glass dish using the Aurum Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules CA), according to the manufacturer’s protocol, including DNAse treatment. RNA quantity and purity were assessed using a Nano Drop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Quality of the total RNA was confirmed prior to library preparation with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using the Agilent RNA 6000 Nano assay (average RNA Integrity Number (RIN) of 9.225; minimum RIN of 8.2 and maximum RIN of 9.7). The Illumina library preparation and sequencing was performed by the Genomics Service Laboratory at the HudsonAlpha Institute for Biotechnology (Huntsville, AL) using the HiSeq 2500 platform to generate a minimum target of 25 million, 50 base-pair, paired-end reads per sample. RNA-seq was performed on three individual biological replicates per control or treatment (total of 24 samples).
2.4. Reference mapping, statistical analyses of differential expression and gene ontology

The software pipeline optimized by Helm et al. (2013) was used for all reference mapping and statistical analysis of differential gene expression. A summary of the methods is included here, but all supplementary reference files, including source codes, are available through Helm et al. (2013). A reference transcriptome consisting of 26,514 transcripts, including the mitochondrial genome, and 18S and 28S ribosomal RNA, was used for mapping all reads that passed the Illumina chastity filter. The sequence reads were mapped using bowtie 2.2.0 with the following parameters: –very-sensitive-local (increases sensitivity at the cost of computational resources) and –a flags (returns all possible mappings for a single read). Counts were generated from the bowtie2 map file using the script generated by Helm et al. (2013). Any reads that mapped to more than one reference sequence were not counted, and multiple mappings to the same reference sequence are only counted once. The R library edgeR version 3.0.4 (Robinson et al., 2009) was used for determining the significance of the differential gene expression using the parameters as previously described (Helm et al., 2013). Differentially expressed genes were identified by comparing all seven chemical and/or UV treatments to the Seawater/No UV control. P-values were corrected for multiple testing using p.adjust and Bonferroni correction, and only genes with an adjusted p-value ≤ 0.05 (equivalent to an unadjusted p-value ≤ 2.84 \times 10^{-6}) and a fold change of ≥ 1.5 were considered statistically significant.

Differentially expressed genes were analyzed using Gene Ontology (GO) annotations (cellular component, molecular function and biological processes) with GOseq version 1.10.1, a software package that performs GO enrichment analysis for RNA-seq data by properly incorporating the effect of selection bias (Young et al., 2010). GO term enrichment was determined by comparing the list of differentially expressed genes against all of the transcripts in the reference transcriptome. An adjusted p-value of ≤ 2 \times 10^{-4} was used as the cutoff for consideration of enrichment of the GO terms.

2.5. Confirmation of differential gene expression by real-time RT-PCR

An identical parallel experiment was conducted to provide material for RT-PCR measurements. For this, total RNA was extracted from the pooled animals within each glass dish and the quality of the total RNA was assessed as previously mentioned. cDNA was synthesized from 1 µg of total RNA in a 20 µl reaction using an iScript cDNA synthesis kit (Bio-Rad). Expression of genes of interest was measured using a MyCycler Real-Time PCR
detection system and a 20 µl reaction mixture consisting of 10 µl of SsoFast EvaGreen Supermix (Bio-Rad), 500 nmol l\(^{-1}\) gene-specific primers and 0.8 µl of cDNA (0.25 µl in the 18S assay). Sequences of gene-specific primers were previously provided (Tarrant et al., 2014). Expression was calculated by comparing the threshold cycle of amplification against a standard curve constructed from a serially diluted plasmid standard containing the amplicon of interest. PCR conditions were as follows: 95°C for 2 min followed by 40 cycles of 95°C for 5 s and 60°C for 10 s. After 40 cycles, the products were subjected to melt curve analysis to ensure that only a single specific product was amplified.

Expression for each gene of interest was normalized to 18S expression, and fold-change was then calculated by dividing expression for each sample by the mean expression for the control (no UV or chemical exposure). Normality was evaluated by the Kolmogorov–Smirnov test, and equality of variance was evaluated through visual inspection of residuals. Data were either left untransformed or transformed by the square root or base-10 logarithm to satisfy these assumptions. Expression of each gene was analyzed using a two-way analysis of variance with UV exposure and chemical treatments as fixed factors. Posthoc comparisons for significant effects were conducted using Tukey’s test. Transformation, evaluation of residuals and statistical tests were conducted using SYSTAT 13 (Systat Software Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1. Sequencing and mapping coverage statistics

A total of 812,627,568 PE DNA sequencing reads were generated from 24 samples (3 biological replicates, 8 treatments) from which an average overall alignment rate of 64.01 ± 0.96% (mean ± 1 standard deviation) per sample was achieved after quality filtering. This overall rate included alignments to protein-coding genes and the mitochondrial genome, as well as 18S and 28S ribosomal RNA. A summary of the total number of reads and the various alignments is provided in Supplemental Table 1. On average, ~16.35 million PE reads per sample aligned to protein coding genes, representing an average coverage of 48.3 ± 1.1% of reads. All Illumina read files have been deposited in the NCBI Sequence Read Archive (BioProject ID: PRJNA427809).

3.2. Nematostella exhibits modest molecular responses to oil and/or dispersant exposure

We first assessed differential gene expression in response to water-accommodated fractions of dispersant (DISP), oil (WAF) or oil & dispersant (CEWAF) without any UV exposure. A
complete summary of results (including p-values, counts, fold change, and transcript and gene ontology annotations) is included as Supplementary File #1. The DISP treatment produced the strongest molecular response with a total of 91 transcripts differentially expressed, 6 up-regulated and 85 down-regulated (Figure 1A). Based on a comparison of all three treatments with the seawater control, a total of 12 transcripts were up-regulated in at least one of the three treatments while 98 transcripts were down-regulated (Figure 1B). DISP resulted in the up-regulation of 2 unique transcripts, while WAF resulted in the up-regulation of 6 unique transcripts. Of the remaining 3 transcripts, one was up-regulated in both DISP and WAF, while the last two were up-regulated in all three exposures. Interestingly, there was no overlap in down-regulated transcripts between all three exposures. DISP resulted in the strongest unique signature with a total of 74 unique transcripts down-regulated. These relatively modest changes in gene expression may appear to contrast with previous studies that have reported severe deleterious effects on corals (e.g., tissue mortality, reduced larval survival and settlement) following exposure to Corexit® 9500 dispersant (DeLeo et al., 2012; Goodbody-Grinling et al., 2013); however, these previous studies used much higher concentrations (35-100 ppm) than those used here (2 ppm). These differences in results and exposure conditions point to the need for more detailed dose-response studies; for cnidarians, such studies have only been conducted with other dispersants (e.g., Corexit® 9527; Venn et al. 2009).

The WAF treatment resulted in the differential expression of 25 transcripts (9 up-regulated and 16 down-regulated), while the CEWAF treatment produced the slightest change in gene expression with 11 transcripts differentially expressed (2 up-regulated and 9 down-regulated) (Figure 1A). Furthermore, WAF treatment resulted in the down-regulation of 4 unique transcripts, while CEWAF resulted in the down-regulation of 8 unique transcripts. Of the remaining 12 transcripts, 11 were up-regulated in both DISP and WAF, while the last one was up-regulated in WAF or CEWAF (Figure 1B). Thus, relative to dispersant alone Nematostella had minimal shifts in gene expression when exposed to WAF and surprisingly even fewer changes in gene expression in the combination treatment (CEWAF) (Figure 1). However, the low number of differentially expressed genes in response to WAF or CEWAF exposure is particularly important to note because similar exposures to crude oil in vertebrates have shown significantly greater numbers of differentially expressed genes (one or two orders of magnitude greater than observed in the current Nematostella data) (Zhu et al., 2016; Xu et al., 2016; Garcia et al., 2012). However, these large changes in gene expression in vertebrates result largely from the activation of the AHR pathway, as well as the significant amount of cross-talk with the NRF2 pathway (reviewed in Wakabayashi et al., 2010).
Figure 1. Summary of differential gene expression in response to DISP, WAF and CEWAF exposure. A) Total number of differentially expressed genes in response to DISP, WAF or CEWAF exposure for 24 hours (p-value ≤ 0.05 and a fold change of ≥ 1.5). B) Venn diagrams of differentially expressed genes up-regulated (left) or down-regulated (right) after exposure to DISP, WAF or CEWAF for 24 hours.

3.3. UV exposure elicits a strong molecular response and synergizes the molecular response to dispersant and/or oil exposure

We next evaluated changes in gene expression following exposure to dispersant and/or sweet crude oil in combination with a 6-hour UV exposure at the start of the 24-hour period. A complete summary of these results (including p-values, counts, fold change, and transcript and
gene ontology annotations) is included as Supplementary File #2. UV exposure resulted in a significant change in gene expression with a total of 605 transcripts being differentially expressed, 434 up-regulated and 171 down-regulated (Figure 2A). DISP_UV exposure resulted in the up-regulation of 480 transcripts and the down-regulation of 221 transcripts, while WAF_UV exposure resulted in an even stronger response with 541 and 248 transcripts up- or down-regulated, respectively. Finally, CEWAF_UV exposure produced the strongest response with 592 transcripts up-regulated, while 278 transcripts were down-regulated (Figure 2B).

A combined total of 1,133 unique transcripts were differentially expressed by all four UV treatments, 726 up-regulated and 407 down-regulated (Figure 2B). Of the 726 transcripts up-regulated by the combination of treatments, 292 transcripts were unique to DISP_UV, WAF_UV or CEWAF_UV exposure. Of the 434 transcripts up-regulated by UV exposure, only 21 were unique to UV exposure alone while 333 transcripts were up-regulated in all four UV treatment combinations. DISP_UV, WAF_UV and CEWAF_UV resulted in the up-regulation of 26, 50 or 89 unique transcripts, respectively, while 58 up-regulated transcripts were shared between DISP_UV, WAF_UV and CEWAF_UV. Of the total number of 407 transcripts down-regulated by all four UV treatment combinations, 236 transcripts were unique to DISP_UV, WAF_UV and CEWAF_UV exposure (Figure 2B). Of the 171 transcripts down-regulated by UV exposure, only 21 were unique to UV exposure alone while 103 transcripts were down-regulated in all four UV treatment combinations. DISP_UV, WAF_UV and CEWAF_UV exposure resulted in the down-regulation of 24, 56 or 57 unique transcripts, respectively, while 31 down-regulated transcripts were shared between DISP_UV, WAF_UV and CEWAF_UV treatments (Figure 2B).
Figure 2. Summary of differential gene expression in response to UV, DISP_UV, WAF_UV and CEWAF_UV exposure. A) Total number of differentially expressed genes in response to UV, DISP_UV, WAF_UV or CEWAF_UV exposure (p-value ≤ 0.05 and a fold change of ≥ 1.5).

B) Venn diagrams of differentially expressed genes up-regulated (left) or down-regulated (right) after exposure to UV, DISP_UV, WAF_UV or CEWAF_UV.

Expression patterns for four transcripts that were differentially expressed in the Illumina study were confirmed via real-time RT-PCR (Figure 3). In each case, a two-way ANOVA revealed a statistical interaction between responses to UV and chemical exposure. For one of the Cu/Zn SODs (v1g234825), post-hoc testing did not distinguish any statistically distinct comparisons, but all three Cu/Zn SODs showed the same pattern of up-regulation with UV.
exposure alone or in the CEWAF_UV treatment. These results are consistent with the Illumina results: with both methods, three transcripts (the Cu/Zn SODs, Figures 3a-3c) were up-regulated in multiple UV treatments, and the Hsp70 transcript (Figures 3d) was down-regulated in several UV treatments.

Figure 3. Fold-change in gene expression from qPCR measurements of selected genes. Nematostella were exposed to seawater alone, crude oil, or crude oil with dispersant. Grey bars indicate animals exposed to UV radiation, and black bars indicate no UV radiation. Data were analyzed using two-way ANOVA, and post-hoc analyses were conducted using Tukey’s test. Different letters over bars indicate statistically different values. These genes correspond to (A) NvCuZnSOD2 (v1g165732), (B) NvCuZnSOD3 (v1g3582), (C) NvCuZnSOD1 (v1g234825) and (D) NvHsp70 (v1g234533) from Tarrant et al. (2014).

To further investigate the functional annotation of differential gene expression in response to chemical and UV treatments, we performed a GO enrichment analysis to identify the GO categories that are overrepresented in the sets of differentially expressed transcripts. Due to the low number of differentially expressed transcripts, there were no significantly enriched GO categories in the DISP, WAF or CEWAF treatments. However, consistent with the observed
changes in differential gene expression, there was a concomitant increase in the total number of
associated GO terms in the combined UV treatments that exhibited more robust changes in
global transcript levels (Figure 4). A total of 33 GO categories were significantly enriched in the
transcripts up-regulated in one or more of the UV treatments (Table 1), while a total of 23 GO
categories were significantly enriched in the transcripts down-regulated by one or more of the
four UV treatment combinations (Table 2).

**Figure 4. Summary of the total number of gene ontology annotations from differentially
expressed transcripts in response to cellular stress.**

Many of the enriched GO terms from all three functional categories are related to
mitochondrial respiration (e.g., oxidative phosphorylation, mitochondrial electron transport,
electron carrier activity, cytochrome-c oxidase activity, ubiquinone biosynthetic process).
Additional GO categories in transcripts up-regulated by the various UV treatment combinations
are consistent with effects on protein degradation and the unfolded protein response (e.g.,
chaperone binding, ATP-dependent peptidase activity, misfolded or incompletely synthesized
protein catabolic process) (Table 1). Many of the GO categories enriched in down-regulated
transcripts from the various UV treatment combinations are related to extracellular matrix
components and functions that are consistent with basic morphogenetic and homeostatic roles,
e.g., myosin filament assembly, calcium ion binding, extracellular matrix structural constituent,
actin filament assembly and maintenance, and notochord morphogenesis and skeletal system
development (Table 2).

Interestingly, our GO results for both the up- and down-regulated genes strongly mirrored
experimental observations from a microarray analysis of normal human fibroblast cells exposed
to UV-B (Tsai et al., 2009). In this study, genes involved in protein turnover and mitochondrial respiration were up-regulated and genes involved in cytoskeletal structure and cellular adhesion were down-regulated. A second earlier microarray study utilizing primary human keratinocytes also observed the significant induction of genes related to proteasomal degradation and protein translation, while also observing the down-regulation of genes associated with cellular adhesion (Sesto et al., 2002). However, this particular oligonucleotide microarray only had coverage for 6,000 genes and there was no mention of genes involved in mitochondrial respiration.

Furthermore, a related study utilizing primary murine fibroblast skin cells demonstrated that UV-B exposure resulted in a significant increase in the production of both superoxide anion radicals and hydrogen peroxide, most likely the result of increased mitochondrial respiration (Masaki and Sakurai, 1997). The later study by Tsai et al. (2009) confirmed that mitochondrial activity significantly increases in a time-dependent manner during continuous UV-B exposure.
Table 1. Gene Ontology Enrichment Summary for Up-Regulated Genes

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Table 2. Gene Ontology Enrichment Summary for Down-Regulated Genes

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<th>Gene Ontology</th>
<th>GO Accession #</th>
<th>Description</th>
<th>UV</th>
<th>DISP_UV</th>
<th>WAF_UV</th>
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3.4. Nematostella defensome response

We next specifically examined differential responses of genes with known or predicted roles in the stress response (Figure 5), including members of the Nematostella defensome (Goldstone, 2008), components of the shikimate synthesis pathways (Starcevic et al., 2008; Shinzato et al., 2011), and genes differentially expressed following exposure to metals (Elran et al., 2014). We observed differential expression of many classic stress response genes between...
the various UV treatments, as well as the expression of some novel cellular antioxidants and genes involved in the synthesis of UV protective compounds (Figure 5).

**Figure 5.** Heat map of the *Nematostella* “defensome” response to cellular stress. The “defensome” is being represented by six groups: A) phase I and phase II metabolic enzymes, B) membrane transporters, C) DNA repair enzymes, D) general stress response genes, E) cellular signaling proteins, and F) proteins involved in the production of UV protective compounds and
cellular antioxidants. Fold change is based on a log2 scale. Blue: up-regulated genes, yellow:
down-regulated genes.

3.4.1. Differential expression of genes involved in xenobiotic metabolism and membrane transport

Although the AHR pathway is well characterized in vertebrates and AHR/ARNT genes have
been identified in diverse invertebrate groups (Goldstone et al., 2006; Liu et al., 2010; Reitzel et al.,
2014; Jenny et al., 2016), previous studies have suggested the lack of an AHR-dependent
response to hydrocarbon exposure in multiple invertebrate species (reviewed in Hahn et al.,
2017). While an AHR gene has been identified in Nematostella, the NvAHR protein fails to bind
prototypical AHR ligands and shows no sign of physical interaction with the NvARNT protein (Reitzel
et al., 2014). Furthermore, there is no evidence in the Nematostella genome of a CYP1A gene,
although a large number of other CYP genes belonging to the xenobiotic-metabolizing Clans 2
through 4 are present (Reitzel et al., 2008; Goldstone, 2008). Even with this in mind, we were
surprised that a number of specific classes of genes expected to be up-regulated with any
xenobiotic exposure, such as phase I and II enzymes, were strongly up-regulated in the UV
alone treatment (Figure 5A and 5B). Several cytochrome P450 (CYP) genes, mostly in Clans 2
and 3, as well as NADPH-cytochrome P450 reductase (NCPR) were induced by one or more of
the UV treatments, but there was little evidence of additive or synergistic changes in the
expression levels between the combined UV treatments. Of the 11 CYP genes differentially
expressed, 4 of them were down-regulated in one more of the UV treatments. The only
divergent change in CYP expression was observed with one CYP Clan 2 gene (v1g22420)
which was down-regulated in the DISP_UV treatment, but up-regulated in the WAF_UV
treatment. Other phase I enzymes, such as the flavin-dependent monooxygenase (FMO), two
aldehyde dehydrogenase (ALDH) genes and short-chain dehydrogenases (D42E1 and D39U1)
were also strongly induced in all four UV treatments. Finally, three phase II conjugating
enzymes belonging to the GST and UDPGT were differentially expressed, with the UDPGT and
one GST transcript significantly up-regulated only in the UV treatment, while a second GST
class was significantly up-regulated in all four UV treatments (Figure 5A).

Although previous studies have demonstrated the up-regulation of CYP1 genes in
vertebrates in response to UV-B exposure, this activation is mediated through the AHR-
dependent binding of the UV-B activated tryptophan photoproduct 6-formylindolo[3,2-
b]carbazole (FICZ) (Wei et al., 1998; Wei et al., 1999; Fritsche et al., 2007). Interestingly, a
large repertoire of Clan 2 and Clan 3 CYP genes has been demonstrated to be up-regulated in
the copepod (Tigriopus japonicas) in response to UV-B exposure (Puthumama et al., 2017). At this time we have no specific mechanism to explain the induction of invertebrate CYP genes in response to UV treatment, but other transcription factors such as the nuclear receptor hepatocyte nuclear factor 4α (HNF4α, NR2A1), as well as pregnane X receptor (PXR) and constitutive androstane receptor (CAR) play major roles in the regulation of CYP2 and CYP3 genes (Tirona et al., 2003; Chen et al., 2005; Jover et al., 2009) in vertebrates. Thus similar nuclear receptors may play a role in the regulation of invertebrate CYP genes in response to cellular stress. These current data do highlight the utility of investigations in early diverging animal phyla to achieve a greater understanding of the evolution of transcriptional regulation of the stress response in metazoans.

Several solute carrier and ABC transporter transcripts significantly induced by one or more of the UV treatment combinations (Figure 5B). A total of 10 transcripts encoding solute carriers or ABC transporters were induced in all four UV treatment combinations, with no sign of any significant difference between treatments. Interestingly, previous studies have demonstrated the induction of ATP-binding cassette (ABC) transporters in response to UV exposure (Uchiumi et al., 1993; Hu et al., 2000). Furthermore, ABC transporters have been demonstrated to play a protective role in developing sea urchins exposed to UV-B (Leite et al., 2014). A single solute carrier, SLC35G1, encoding a putative calcium-transporting ATPase was induced in all four UV treatment combinations, but the induction was modestly enhanced in DISP_UV, WAF_UV and CEWAF_UV, resulting in a ~1.5 to 1.8-fold increase in transcript abundance. Two additional solute carriers (SLC34A2 and SLC25A36) were not induced by UV treatment alone, but were induced by DISP_UV exposure, while SLC34A2 was also induced by CEWAF_UV exposure. Finally, a copy of an ABCA1 transcript, as well as copies of ABCB9 and ABCF transcripts, were not induced by UV treatment alone, but were induced by UV treatment combined with one or more dispersant and/or oil exposures (Figure 5B). The induction of these transporters in the combined UV treatments is indicative of enhance toxicity of photo-activated hydrocarbons and is consistent with their functions in the cellular export of xenobiotics.

3.4.2. Differential expression of genes involved in DNA repair

One of the most common forms of cellular damage associated with UVB exposure is the formation of cyclobutane pyrimidine dimers (CPDs), which results in the covalent linking between pairs of thymine or cytosine bases on the same strand of DNA through the formation of a cyclobutane bridge (Niggli and Cerutti, 1983; Douki and Cadet, 2001). A second common type of DNA lesions associated with UVB exposure are pyrimidine-pyrimidone (6-4)
photoproducts, although the relative abundance of CPDs and (6-4) photoproduct formation is dependent on specific wavelengths (Matsunaga et al., 1991). Two transcripts (v1g163561 and v1g223118) encoding deoxyribodipyrimidine photolyases were consistently up-regulated in all four UV treatments (Figure 5C). The photolyase/cryptochrome superfamily of proteins, recognized by the conserved photolyase homology region (PHR) domain, represents a group of photoreceptor proteins that play various roles in DNA repair and regulation of circadian rhythms (reviewed in Sancar, 2003). Deoxyribodipyrimidine photolyases are light-dependent DNA-repair enzymes that remove these UV-light induced lesions through the process of photoreactivation. They contain a two-electron reduced flavin FADH\(^{-}\) cofactor that is activated by light energy and serves as an electron donor to the pyrimidine dimer resulting in a radical anion that splits into two pyrimidines (reviewed in Sancar, 2003).

Differential expression was also observed for several cryptochrome/photolyase members; for these genes, more detailed phylogenetic analyses have been conducted, leading to supplementary annotations (Reitzel et al., 2010; Shoguchi et al., 2013). Two transcripts encoding cryptochrome family members (CRY1 v1g168581 = NvCry1a; CRY1 v1g194898 = CRY-DASH-like) were induced in all four combined UV treatments, while a third cryptochrome (v1g219650 = NvCry2) transcript was only induced in the UV or WAF_UV treatments and a final family member (v1g203127 = 6-4 photolyase-like) was only induced in the WAF_UV treatment (Figure 5C). Cry-DASH proteins also have the conserved PHR domain found in both photolyase and cryptochrome proteins. Although the significant enrichment of the GO annotation for deoxyribodipyrimidine photolyase activity (Table 1) was observed in all four UV treatment combinations, the enrichment of a related molecular function, DNA (6-4) photolyase activity, in the WAF_UV treatment is directly related to the differential gene expression of multiple cryptochrome transcripts (Figure 5C). While, cryptochromes are believed to no longer play a role in eukaryotic DNA repair; Cry-DASH proteins retain some DNA repair ability (reviewed by Chaves et al., 2011).

Additional differentially expressed genes include those involved in nucleotide excision repair (XPC, ERCC6) and base excision repair (NEIL1), as well as two enzymes involved in DNA replication and double-strand break repair via nonhomologous end joining transcripts (XRCC5 and WRN) which were only induced in the DISP_UV treatment (Figure 5C). While nucleotide excision repair enzymes are also associated with the repair of bulky DNA lesions such as CPDs and (6-4) photoproducts (Shah and He, 2015), the induction of the other types of DNA repair enzymes may be indicative of other types of oxidative damage to nucleotide bases (Shafirovich et al., 2016).
3.4.3. Differential expression of genes involved in general stress response and cell signaling

UV treatment alone or combined with dispersant and/or oil treatment did result in the induction of some classic oxidative stress-response genes, including a single heat shock protein 70 transcript (HSP70) and multiple copper/zinc superoxide dismutases (Cu/Zn SODs) (Figure 5D), consistent with our previous findings (Tarrant et al., 2014). However, we also observed the down-regulation of a second HSP70 transcript, as well as multiple HSP20 transcripts, in multiple treatments (Figure 5D). Interestingly, a single glutathione peroxidase (GPX) transcript and transcript encoding a phytochelatin synthase enzyme (PCS2) were only induced in the CEWAF_UV treatment. UV treatment alone or combined with dispersant and/or oil treatment also resulted in the induction of multiple transcripts encoding proteins involved in heme homeostasis, including biliverdin reductase A (BVRA), biliverdin reductase B (BVRB), and multiple heme-binding proteins (HEBP2). Combined chemical and UV exposure resulted in a synergistic induction, 4-fold and 16-fold, of two transcripts encoding thiosulfate sulfurtransferases, TSTD1 and TSTD3 respectively, in the CEWAF_UV treatment. We also observed a significant up-regulation of the pleckstrin homology domain transcript (PHD) previously identified as a potential stress response gene by Elran et al. (2014). Finally, two peroxidasin transcripts (PXDN and PXDNL) were significantly down-regulated in all four UV treatments, while a peroxinectin transcript (POXA) was up-regulated in the WAF_UV and CEWAF_UV treatments (Figure 5D). Peroxidasin is a heme-containing peroxidase enzyme that is involved with extracellular matrix formation (Papageorgiou and Heymans, 2014), while peroxinectin is cellular adhesion protein (Lin et al., 2007). The altered expression of these genes is consistent with the significant enrichment of GO terms involved in extracellular matrix and morphogenesis (Table 2).

We observed differential expression in several genes related to cell signaling pathways, many of which play prominent roles in the stress response (Figure 5E). Most interesting was the down-regulation of heat shock factor (HSF), the transcription factor responsible for regulating transcription of heat shock genes, in three different treatments (DISP, WAF, and WAF_UV). Hypoxia inducible factor 1-alpha (HIF1A) was induced in all four UV treatments, and there was an enhanced induction (1.5- to 1.7-fold increase) in the DISP_UV and CEWAF_UV treatments. Furthermore, the hypoxia-inducible vascular endothelial growth factor A (VGFAA), as well as the HIF1A regulatory enzyme Egl nine homolog 1 (EGLN1), were also induced in all four UV treatment combinations. Two transcripts encoding extracellular sulfatase 2 (SULT2), an enzyme that modulates signaling molecule binding sites on heparin sulfate proteoglycans, were
strongly induced in CEWAF_UV. Sequestosome-1 (SQSTM1), a cargo protein involved in selective autophagy, was significantly induced in the CEWAF treatment, as well as all four UV treatment combinations. While UV exposure alone was not enough to induce a significant change in expression, UV exposure combined with dispersant and/or oil treatment did result in the significant induction of growth arrest and DNA damage-inducible protein GADD45 gamma (GA45G), as well as two transcripts encoding proteins involved in apoptotic signaling (Bcl-2 homologous antagonist/killer, BAK; Fas apoptotic inhibitory molecule 1, FAIM1). Three additional transcripts encoding proteins involved in apoptotic cell death (cell death regulator AVEN; programmed cell death 6-interacting protein, PDC6I; programmed cell death 2-like, PDD2L) were up-regulated in all four UV treatments. Surprisingly, transcripts encoding putative c-fos and c-jun, two proteins that dimerize together to form the UV-responsive AP-1 transcription factor, were down-regulated in some of the UV treatment combinations. Finally, differential expression of multiple nuclear receptors (NR2E = NvNR6, a TLL subfamily member; COT1 = NvNR10, COUP-TF-like; RXRAB = NvNR2, a cnidarian-specific subfamily member) and other nuclear transcription factors (HEY) was observed in multiple UV treatments (Figure 5F; supplemental annotation of nuclear receptors based on Reitzel and Tarrant, 2009). The "HEY" transcript corresponds to a basic-helix-loop-helix family member (Hes/Hey-like) that has a strong diel expression pattern in both *Nematostella* and the coral *Acropora millepora* (Oren et al., 2015).

### 3.4.4 Differential expression of a gene involved in the production of novel cellular antioxidants

The ergothioneine biosynthesis protein 1-like (EGTB) transcript (v1g169317), up-regulated in all four UV-related treatments (Figure 5F), is an interesting gene for future research consideration. Ergothioneine is an alternative amino acid with putative antioxidant effects (Paul and Snyder, 2010). The Joint Genome Institute (JGI) Genome Portal for *Nematostella* currently has this gene annotated as sulfatase-modifying factor enzyme 1 (SUMF1). However, the highest BLAST results for this protein yield matches to sequences annotated as EGTB-like proteins, but the protein also has two conserved domain regions. While the C-terminal region contains a methyltransferase domain with strongest homology to a putative 4-mercaptohistidine N1-methyltransferase (ovoA_Cterm), the N-terminal region of the protein shares homology to the SUMF1/formylglycine-generating enzyme (YfmG) domain, as well as a 5-histidylcysteine sulfoxide synthase (ovoA_Nterm) domain. Comparisons of EGTB proteins also show similarity between the sulfoxide synthase domain of EGTB and the YfmG domain in the *Nematostella* EGTB-like protein (v1g169317). As previously mentioned, ergothioneine is an alternative amino
Acid with putative antioxidant effects (Paul and Snyder 2010). However, the EGTB enzymes are generally thought to be restricted to microorganisms such as fungi, bacteria and cyanobacteria. Thus, if the Nematostella gene did indeed encode an EGTB enzyme, it would likely represent another example of horizontal gene transfer. This gene is located on scaffold 124 of the Nematostella genome and contains many introns, as well as a GC content of 49% which is consistent with the 47% GC content of the overall genome. Thus, this gene does appear to be a true nuclear encoded gene and not a contaminating sequence from any potential proteobacterial endosymbiont. Furthermore, the highly conserved ovoA_Nterm and ovoA_Cterm domains within this protein suggest that it could very well be a 5-histidylcysteine sulfoxide synthase enzyme involved in ovothiol biosynthesis (Braunshausen and Seebeck, 2011), and this is further supported by the 47% shared protein sequence identity to a 5-histidylcysteine sulfoxide synthase enzyme from the sea urchin Paracentrotus lividus (Castellano et al., 2016) identified by BLASTp analysis. The strong homology between the ergothioneine and ovothiol synthesizing enzymes is explained by the observation that both compounds are thiol-histidine natural products that are formed from the oxidative coupling of cysteine to histidine or a histidine derivative, respectively, with reactions catalyzed by sulfoxide synthase domains (Song et al., 2013; Mashabela and Seebeck, 2013; Goncharenko et al., 2015). Ovothiols are mercaptophistidine molecules with very strong reducing antioxidant functions, and are commonly found in the eggs, ovaries and biological fluids of marine invertebrates (Turner et al., 1987; Castellano et al., 2016), and have been demonstrated to protect sea urchin eggs from the oxidative stress associated with the respiratory burst of fertilization (Turner et al., 1986; Shapiro and Turner, 1988). The induction of this transcript in all four UV treatments supports previous observations of ovothiol induction in response to cellular stress (Castellano et al., 2016) and suggests a more expanded role as a cellular antioxidant.

3.4.5. Expression of genes involved in the production of UV-protective compounds

One of the most interesting observations was the induction of transcripts encoding putative proteins related to amino acid synthesis and metabolism, as well as vitamin B6 biosynthesis (Figure 5F). Of particular interest was the very strong induction of several enzymes that participate in the shikimic acid pathway, which is involved in the biosynthesis of aromatic amino acids (phenylalanine, tyrosine and tryptophan) in microorganisms, fungi and plants, and biosynthesis of UV-protective mycosporine-like amino acids (MAAs). MAAs are small water-soluble compounds derived from aromatic amino acids that are capable of absorbing UVA and UVB radiation, and they have also been implicated in roles as cellular antioxidants (reviewed by
Shick and Dunlap, 2002, Oren and Gunde-Cimerman, 2007). Aromatic amino acids and MAAs in metazoans, including marine organisms, are generally hypothesized to be of dietary origin since most metazoans lack genes coding for the enzymes of the shikimic acid pathway. However, previous studies suggest that cnidarians, including nonsymbiotic corals and anemones, are capable of synthesizing these essential amino acids (Fitzgerald and Szmant, 1997; Shick et al., 2002; Starcevic et al., 2008). Starcevic et al. (2008) provided evidence for the horizontal transfer of both bacterial and dinoflagellate ancestral genes of the shikimic acid pathway into the genome of Nematostella. Of those genes, 3-dehydroquinate synthase (DHQS; v1g70416), the enzyme responsible for catalyzing the second step of the shikimic acid pathway, was significantly up-regulated by UV treatment and this up-regulation was significantly enhanced with DISP_UV, WAF_UV and CEWAF_UV treatments (Figure 5F). However, the remaining genes encoding a chorismate synthase (AroC, v1g223774), two 5-enolpyruvylshikimate-3-phosphate (EPSP) synthases (AroA, v1g202287; AroA, v1g75553), and a second DHQS-like transcript (AroB, v1g62604) did not significantly change in expression. Despite recent evidence that some genes in the Nematostella genome assembly likely resulted from bacterial contamination (Artamonova and Mushegian, 2013), AroA and AroB are well supported as real products of horizontally transferred. AroC is more likely to be a bacterial contamination.

While DHQS participates in the synthesis of the aromatic amino acids, it also plays a significant role in the synthesis of shinorine, a photo-protective MAA (Rahman et al., 2014). Interestingly, a second route for MAA biosynthesis relies on the conversion of the metabolites of the pentose-phosphate pathway, specifically sedoheptulose 7-phosphate to 4-deoxygadusol, which serves as a precursor of MAAs (Balskus and Walsh, 2010). Shinzato et al. (2011) demonstrated that both Acropora digitifera and Nematostella contain homologs of a four-gene cluster of enzymes (DHQS, O-MT, ATP-grasp, NRPS) also found in the cyanobacterium Anabaena variabilis which is sufficient for the conversion of pentose-phosphate metabolites to shinorine. Three of those four genes were significantly up-regulated in all four UV-related treatments. In addition to the previously discussed DHQS transcript, we also observed the significant induction of the O-methyltransferase transcript (O-MT; v1g167288) in all four UV treatments (Figure 5F). The DHQS and O-MT proteins have been demonstrated to work together to convert sedoheptulose 7-phosphate into 4-deoxygadusol (Balskus and Walsh, 2010). We also observed the significant induction of a transcript (v1g206757) encoding an enzyme in the ATP-grasp superfamily (Figure 5F), which include proteins that catalyze the ATP-assisted kinase/ligase reaction of carboxylic acid with a nucleophile to produce an
acylphosphate intermediate (reviewed in Fawaz et al., 2011). The ATP-grasp enzyme homolog in cyanobacteria has been demonstrated to be capable of converting 4-deoxygadusol and glycine into mycosporine glycine (Balskus and Walsh, 2010). In contrast, we did not observe induction of the nonribosomal peptide-synthetase (NRPS)-like transcript (v1g221241), which putatively encodes an enzyme that is capable of converting mycosporine glycine and serine into shinorine (Balskus and Walsh, 2010). However, we did observe induction of a linear gramicidin synthase subunit C (LGRC) transcript (v1g247078), which has some homology to the NRPS-like gene in Hydra magnipapillata, in the DISP_UV and CEWAF_UV treatments. Interestingly, both of these genes (v1g221241 and v1g247078) contain a conserved adenylation domain found in NRPS proteins (A_NRPS domain). However, the top BLASTp results of the putatively encoded NRPS-like protein (v1g221241) demonstrated strong homology (50-70% identity) to linear gramicidin synthase subunit D-like genes from several different invertebrate species, including genes from multiple cnidarian species, e.g., Acropora digitifera, Orbicella faveolata and Exaiptasia pallida. In contrast, the top BLASTp results of the LGRC sequence (v1g247078) had the strongest homology (33-38% identity) to NRPS proteins of bacterial origin. Taken together, these data suggest that Nematostella is capable of synthesizing MAAs as a protective mechanism in response to cellular stress. However, some exciting questions remain regarding whether or not the potential synthesis of these MAAs is primarily driven by genes encoded in the Nematostella genome due to horizontal gene transfer, the result of gene expression from a proteobacterial endosymbiont, or some form of symbiotic interplay between Nematostella and a proteobacterial endosymbiont.

Some additional supporting evidence for the de novo synthesis of MAAs in Nematostella is present in the induction of other genes related to amino acid metabolism and vitamin B6 synthesis. The significant induction of 4-hydroxyphenylpyruvate dioxygenase (HPDL, v1g13173) in response to all four UV treatments is particularly interesting because HPDL catalyzes the second reaction in the catabolism of tyrosine, an amino acid that serves as a feedback inhibitor of the shikimic acid pathway in cyanobacteria (Portwich et al., 2003). Furthermore, we also observed the significant induction of pyridoxine 5′-phosphate oxidase (PNPO, v1g80869) and pyridoxal 5′-phosphate synthase (PdxST, v1g194582), two key enzymes in the metabolism of vitamin B6, in all four UV treatments (Figure 5F). While pyridoxal 5′-phosphate, the active form of vitamin B6, can function as a co-enzyme in a variety of enzymatic reactions, it can also be used as a Schiff base in the production of aromatic amino acids in the shikimic acid pathway (Seigler, 1998). Interestingly, we also observed the significant up-regulation of a transcript encoding a protein with a conserved pyridoxal-dependent
decarboxylase domain (DDC, v1g204120) found in aromatic L-amino acid decarboxylases, enzymes responsible for multiple decarboxylation reactions that convert aromatic amino acids into neuromodulators, and are also responsible for the conversion of L-3,4-dihydroxyphenylalanine (L-DOPA) and 5-hydroxytryptophan (5-HTP) into dopamine and serotonin, respectively (Ebadi and Simonneau, 1991; Paiardini et al., 2017). However, the second to last step of the shikimic acid pathway does require a decarboxylase enzyme, so perhaps this enzyme is possibly playing a role in the production of aromatic amino acids. Further characterization of this DDC enzyme would be required to determine its phylogenetic relationship and functional role in Nematostella.

4. CONCLUSIONS

Coastal marine species experience a multitude of abiotic, biotic, and anthropogenic conditions that can exert cellular stress in complex ways when combined. While PAHs and other organic pollutants can result in severe toxicity for many species, cnidarians, like Nematostella, have a limited response to these substances at the organismal and the molecular level. Contrary to our prediction, Nematostella did not upregulate any phase I or phase II metabolic genes in response to dispersant and/or oil alone. However, when combined with ecologically relevant exposure to UV light, the toxicity of sweet crude oil and chemicals designed to minimize the environmental impact of oil spills (dispersant) leads anemones to mount an extensive molecular response as protection from cellular damage. This response included well-described components of the chemical defensome, as well as genes involved in DNA repair, and the production of UV-protective compounds and novel antioxidants. However, many questions remain regarding the activation of the “defensome” by hydrocarbon exposure in cnidarians, as well as the specific mechanisms by which they metabolize and excrete xenobiotics. Together, these results emphasize the need to consider environmental stressors in combination when determining unsafe levels in the environment and potential biomarkers to assess the health of natural populations.

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References


Goldstone, J.V., Hamdoun, A., Cole, B.J., Howard-Ashby, M., Nebert, D.W., Scally, M.,
Dean, M., Epel, D., Hahn, M.E., Stegeman, J.J., 2006. The chemical defensome:
environmental sensing and response genes in the Strongylocentrotus purpuratus
Goldstone, J.V., 2008. Environmental sensing and response genes in cnidarian: the chemical
Toxicity of Deepwater Horizon source oil and the chemical dispersant, Corexit 9500, to coral
larvae. PLoS One 8, e45574/
synthase EgtB from the ergothioneine biosynthetic pathway. Angew. Chem. Int. Ed. 54, 2821-
2824.
Grzanka, D., Domaniewski, J., Grzanka, A., Zryn, A., 2006. Ultraviolet radiation (UV) induces
reorganization of actin cytoskeleton in CHOAA8 cells. Neoplasma 53, 328-332.
Hahn, M. E., Karchner, S. I., Merson, R. R., 2017. Diversity as opportunity: insights from 600
PAH phenanthrene on immune function and oxidative stress in the haemolymph of the
temperate scallop Pecten maximus. Chemosphere 78, 779-784.
microsomal mixed-function oxidase system of the sea anemone (phylum Cnidaria). Comp.


• Crude oil exposure elicits a modest transcriptomic change in *Nematostella*.
• Dispersant produces a stronger transcriptomic response compared to crude oil.
• UV radiation exposure produces dramatic changes in global gene expression.
• UV exposure synergizes crude oil and dispersant toxicity in *Nematostella*.
• Data suggest that *Nematostella* is capable of *de novo* MAA synthesis.