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RESEARCH ARTICLE

Stress response of the black coral *Leiopathes glaberrima* when exposed to sub-lethal amounts of crude oil and dispersant

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The 2010 Deep Water Horizon oil well failure released billions of gallons of crude oil into the deep Gulf of Mexico, and, combined with chemical dispersants, this oil caused significant coral mortality. However, the mechanisms by which oil and dispersed oil impact deep marine fauna are not well understood. Here, we investigate the effects of oil and dispersed oil on a black coral common in the deep Gulf of Mexico, *Leiopathes glaberrima.* This coral occurs in several color morphs that show ecological and genetic differences. We hypothesized that dispersed oil would be more detrimental to coral health than oil alone and that this difference would be detectable in the gene expression response of the colonies even at sub-lethal concentrations. In two experiments, four and six colonies of red and white color morphs were exposed to oil, dispersant, and dispersed oil for a minimum of 96 hours. Visual assessment indicated that indeed dispersant and dispersed oil treatments were more damaging than oil alone, for target concentrations of 25 mg L^{-1}. Decline in health was observed for all treatments, independently of color morphotype, but the decline was faster in the white colonies exposed to dispersant. The responses to the treatments were also investigated by monitoring gene expression after 24 hours of sub-lethal chemical exposure. Coral gene expression differed by chemical stressor. Interestingly, the polycyclic aromatic hydrocarbon biomarker gene, cytochrome P450, was only up-regulated in dispersed oil but not oil alone, suggesting that the dispersant increased the availability of such hydrocarbons in the tissue. The gene expression response was apparent at 24 hours when visual impacts were not (yet) detectable. The use of chemical dispersants in oil-spill remediation may cause health declines in deep-water corals and deserves further study.

Keywords: Oil spill; gene expression; polymorphism; corals; dispersant

Introduction

Drilling for hydrocarbons causes physical damage to the seafloor and inevitable habitat disruption to deep-sea communities (Brooke and Schroeder, 2007). Damage to the seafloor results from the installation of infrastructure, drilling discharges (muds and cuttings), and spills (Jones and Gates, 2010; Purser and Thomsen, 2012). Impacts include the loss of physical habitat, decline in species diversity and abundance, and disruption of settlement and recruitment of benthic fauna (Jones and Gates, 2010; Trannum et al., 2010; Järnegren et al., 2017). Oil spills on the outer continental shelf in the Gulf of Mexico have increased in recent years, mainly due to hurricanes and the Deepwater Horizon disaster (Anderson et al., 2012). Although cold-water corals are often found in association with drilling areas (Roberts and Aharon, 1994; Fisher et al., 2007), little is known about the impact of oil exposure on the health and survival of the corals.

An unprecedented opportunity to study the potential consequences of oil spills on deep/cold water corals (reviewed in Fisher et al., 2014) came with the 2010 oil spill in lease block MC252, which released approximately 4.4 million barrels of oil into the Gulf of Mexico (Camilli et al., 2010). Surveys after this spill found oil-impacted coral colonies in at least three coral communities within 25 km of the MC252 wellhead (White et al., 2012; Fisher et al., 2014). In addition to crude oil, corals were exposed to the chemical dispersant Corexit 9500 A (DeLeo et al., 2015). As part of the mitigation strategy, 771,272 gallons of Corexit 9500 A were injected directly into the wellhead at 1544 m and 1,072,514 gallons were sprayed on the surface (Graham et al., 2011). The Macondo oil spill was unique in the amount of oil released, the depth of

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the release and the remediation strategies used (Atlas and Hazen, 2011; Peterson et al., 2012). The mandated natural resource damage assessment (NRDA) revealed a lack of baseline information on the eco-toxicological vulnerability of deep-sea communities (Peterson et al., 2012).

The effects of dispersants and/or crude oil have rarely been tested in deep-sea macro-organisms (DeLeo et al., 2015). Studies on the effects of crude oil on shallow water corals suggest possible damage to the reproductive systems, production of fewer planulae larvae, thinning of cell layers, disruption of cell structure, and damage to stimulus response and feeding mechanisms (Loya and Rinkevich, 1980). Dispersants create smaller oil droplets, making the oil available for biodegradation. However, recent work indicates that dispersants can suppress the activity of oil-degrading microbes (Kleindienst et al., 2015) and this process might also increase the availability of toxic components such as the polycyclic aromatic hydrocarbons (Zuijdgeest and Huettel, 2012). If dispersants enhance the rate of oil dissolution, the chemically dispersed oil might reach more areas of the water column than oil alone, increasing the potential for toxicity to organisms that otherwise would not have been exposed to the oil spill (Goodbody-Gringley et al., 2013). Exposure experiments suggest that the effects of dispersed oil, or dispersant-only mixtures, are more toxic than oil alone (Scarlett et al., 2005; Shafir et al., 2007; Goodbody-Gringley et al., 2013; Rico-Martínez et al., 2013; DeLeo et al., 2015). However, previous experiments focused on chemical concentrations that resulted in visual impacts on organisms.

Here, we assessed the visual impact and gene expression changes to describe and quantify the physiological responses of a deep-sea coral to a sudden but sub-lethal increase in oil, dispersant and dispersed oil. This approach allows for early detection of stress responses to chemical exposure. We focused on *Leiopathes glaberrima*, one of the most common black corals in the northern Gulf of Mexico that acts as an ecosystem engineer in the region (Brooke and Schroeder, 2007). Multiple color variants of *L. glaberrima* have been identified, but systematic studies did not find taxonomical differences among colors (Brugler, 2011; Ruiz-Ramos et al., 2015). Instead, differently colored colonies belong to weakly differentiated populations and show distribution differences on a local scale (Ruiz-Ramos et al., 2015). Studies on color variants in the cold-water scleractinian *Lophelia pertusa* suggest that coral-associated microbial communities may also play a role in color variation (Neulinger et al., 2008). We hypothesized that dispersed oil would be more detrimental to coral health than oil alone and that this difference would be detectable in the gene expression response of the colonies even at sub-lethal concentrations. We exposed two color morphs (red and white) of *L. glaberrima* to different concentrations of oil, dispersed oil, and dispersant solutions to identify a minimum lethal concentration (as reported by DeLeo et al., 2015). Then, we exposed new colony fragments to sub-lethal concentrations of the solutions to characterize the coral's gene expression responses.

Materials and Methods

Coral collections

Experiments were performed during the R/V *Falkor* ECOGIG cruise during November 4–28, 2012, and the E/V *Nautilus* ECOGIG cruise from June 21 to July 5, 2013. Red and white *L. glaberrima* colonies were collected opportunistically at the VK826 site (29°09.5'N, 88°01.0'W) at depths of approximately 500 m with the remotely operated vehicles, Ocean Global explorer MK3 (R/V *Falkor*) and Hercules (E/V *Nautilus*), and placed in an insulated box to keep the corals at a constant environmental temperature close to in situ temperature (5°C) during recovery. On board the ship, corals were kept in a cold room at 6°C. Permitting processes are not established for work on deep-sea corals in the Gulf of Mexico. Letters of acknowledgment were obtained from the National Oceanic and Atmospheric Administration for the research expeditions in accordance with the Magnuson-Stevens Fishery Conservation and Management Act.

Preparation of oil and dispersant solutions for 2012 experiments

Stock and working solution preparations are described in detail in DeLeo et al. (2015). Bulk oil stock solutions were prepared by adding 50 μL of MASS oil (collected from the Macondo well, provided by M. Joye) to 199.95 mL of artificial seawater (ASW) (Instant Ocean™, salinity = 35), for a calculated concentration of 250 parts per million (ppm, volumetrically) or approximately 250 mg L^{-1}. This mix was used to prepare the working solutions. An oil and dispersant stock solution of 250 mg L^{-1} was prepared in a 1:1 ratio, adding 50 μL of oil to 199.90 mL of ASW followed by 50 μL of dispersant Corexit 9500 A (Nalco, TX). The dispersant stock solution of 250 mg L^{-1} was made by adding 50 μL of Corexit 9500 A to 199.95 mL of ASW. The targeted concentrations of the experimental solutions were 25, 7.9, and 0.8 mg L^{-1} for the different treatments. Actual concentrations might be lower due to volatilization, adhesion the glass vials or degradation. Colonies were treated for 96 hours, then transferred to clean ASW for 40 hours of recovery. Results for the different concentrations of oil, dispersed oil and dispersant on *L. glaberrima*, without regard to color morphotype, were presented in DeLeo et al. (2015). Here, we report the results of these experiments with respect to coral color morphotype considering the effects of all treatment concentrations combined because this approach informed the gene expression study (see below).

Preparation of oil and dispersant solutions for 2013 experiments

For this set of experiments a water-accommodated oil fraction (WAF) was prepared. In an attempt to produce a 1.2 mM WAF, 9.5 mL of surrogate oil was added to 475 mL of ASW. The oil-dispersant treatment was prepared by adding 9.5 mL of oil to 475 mL of ASW followed by the addition of 950 μL Corexit 9500 A (1/10 of the oil concentration, the recommended final ratio of oil to dispersant; USEPA technical product bulletin) to produce a dispersant-enhanced WAF (dispersed oil treatment). The disper-

sant stock solution was made by adding 950 μL of Corexit 9500 A to 475 mL of ASW (mixed at 300 rpm), with an initial dispersant concentration of 848 mg L^{-1}. After mixing oil and oil-dispersant stock solutions in a vortex mixer at high speed (350 rpm) the insoluble oil layer and the WAF were separated using a sterile separation funnel. From the stocks, experimental treatments were diluted to targeted initial total hydrocarbon concentrations of 250 μM (high), 150 μM (medium) and 50 μM (low). The targeted initial concentrations for the dispersant in the oil-dispersant and dispersant treatments were 176.7 mg L^{-1} (high), 106.0 mg L^{-1} (medium) and 35.3 mg L^{-1} (low) (DeLeo et al., 2015). Target concentrations for determining the lethal concentration of WAF were higher than in the 2012 experiments with bulk oil because the 2012 experiments indicated that corals may tolerate somewhat higher concentrations. As with the 2012 experiments, actual concentrations might be lower due to volatilization, adhesion to the glass vials or degradation. Colonies were exposed to the experimental concentrations for 96 hours; no recovery was attempted. Results for the different concentrations of oil, dispersed oil and dispersant on *L. glaberrima*, without regard to color morphotype, were presented in DeLeo et al. (2015). Here, we report the results of these 2013 experiments with respect to coral color morphotype considering the effects of all concentrations combined because, again, this approach informed the gene expression study (see below).

Visual impact assessments (both years)

Colonies ($n_{2012} = 4$, $n_{2013} = 6$) were divided by cutting apical branches at 5–6 cm. We used two fragments per colony per treatment ($n_{2012} = 40$, $n_{2013} = 48$) and added them to capped glass vials. The vials were placed on racks on a tabletop orbital shaker and incubated for 4 days at 200–250 rpm and 6°C, aerating the fragments once every 24 hours (for 5 minutes) by bubbling air into the vials. Every 24 hours colonies were photographed and their health status was assessed visually and recorded. The observations for coral response included: percentage of extended and retracted polyps, percentage of dead polyps (disintegrating) and polyps separating from the skeleton. Swollen polyps, mucus spots and mucus balls on the bottom of the vials were also recorded (**Figures 1** and **2**). For every sampling time point, an overall health rating was assigned on a scale from 0 to 5, where 0 indicates that all polyps of a fragment were dead and 5 indicates that the polyps were healthy (DeLeo et al., 2015). Ratings (phenotypic scores) were assigned based on the percentage of surviving fragment and the following responses: percentage of polyp retraction and/or swelling, presence and persistence of mucus discharge, dead tissue and polyps, sloughing tissue and exposed skeleton (Table S1).

Statistical treatment of visual impact assessments (both years)

Two sets of data were retrieved from the phenotype scores. The first set and their analyses, where all *L. glaberrima* colonies were treated as one species and analyzed with two octocoral species (*Callogorgia americana* and *Paramuricea biscaya*), have been reported by DeLeo et al. (2015). For the second data set, the treatment concentrations were pooled and the *L. glaberrima* colonies were classified as red or white and treated as two experimental groups; these results and their analyses are reported in this study.

The fraction of fragments in which health status declined to a score of 3 or lower was measured with a Kaplan-Meier (K-M) time-to-event survival analysis (IBM® SPSS®, 2013). In this analysis the time point when health ratings dropped to 3 or below (3–0) was considered as "the event", because total mortality (i.e., all polyps dead) was not observed for every treatment and concentration. However, the test did not produce a time-to-event result for the 2012 experiments because all events were considered censored (the event did not occur within the time frame of the study). Mantel-Cox log-rank test was used to quantify differences between the survival curves.

To quantify the decline in health, Cox regressions (IBM® SPSS®, 2013) were performed for treatment (water, oil, dispersant/oil and dispersant), and color (red or white). The time point at which health rating dropped to 3 or below was considered as "the event" in the time-to-event analysis. Hazard ratios were calculated for each factor (all concentrations combined) with respect to water (control) treatment and the red morph of *L. glaberrima*. The red morph was used to calculate the hazard ratio because it was the morph with more healthy appearing polyps at the end of the 2013 experiments (which had higher treatment concentrations than the 2012 experiments).

Because polyp extension and contraction was a prominent trait of coral response, a repeated measures ANOVA was performed to detect differences in polyp extension across treatments (IBM® SPSS®, 2013), with color morphs and treatment (water, oil, dispersant and dispersed oil; all concentrations combined) as between-aquaria factor and time as within-aquaria factor, followed by Tukey post hoc test.

Gene expression experiment (2012)

In situ experiments on reef corals have indicated stress responses to oil and dispersed oil at 0.02 mg mL^{-1} (Knap et al., 1983), while lower concentrations of a water accommodated fraction of oil (0.00065–0.0015 mg mL^{-1}) were lethal to coral larvae (Goodbody-Gringley et al., 2013). Because mortality was not observed during the first 24 hours of exposure to the highest targeted treatment concentrations of 25 mg mL^{-1} in our visual impact study of 2012, we used that concentration for the follow-up gene expression experiments during the same cruise. The gene expression experiments utilized freshly cut fragments from the collected corals, and new stock solutions were prepared as described above (2012 experiments).

The visual health of the fragments during the gene expression experiments was recorded using the same categories as for the visual impact study (Table S1) but monitoring was conducted at 0, 4, 8, 12 and 24 hours. The fragments used to monitor health were preserved in RNAlater (Ambion, TX) in their entirety at the end of the experiment (24 hours) for RNA extraction.

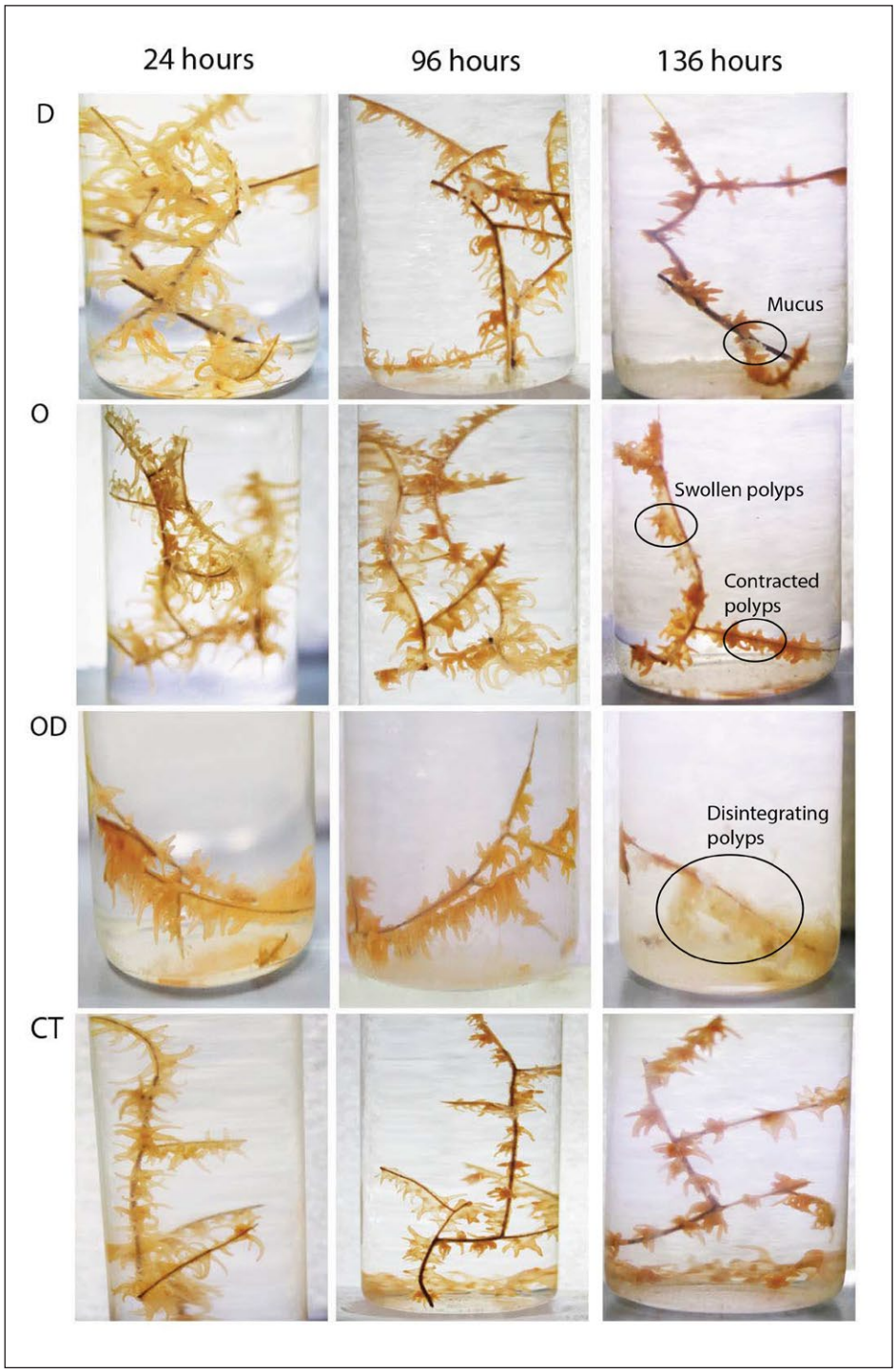

Figure 1: Health changes in a red *Leiopathes glaberrima* colony during 2012 visual impact studies. Photographic images of a red colony in treatment concentrations of 25 mg L^{-1} during 2012 experiments. Columns from left to right: after exposure to treatment for 24 hours; after exposure to treatment at 96 hours; and at end of 40-hour recovery period in ASW (136 hours). Rows from top to bottom: D = dispersant, O = bulk oil, OD = bulk oil and dispersant and CT = controls. In the dispersant-only treatment, polyps were lost from the tips of the fragments, tissue retracted between the polyps so that the black skeleton was more apparent and tentacles were contracted. Polyps in the OD treatment had disintegrated by 136 hours and the vials were cloudy with mucus. Polyps in the oil treatment show a characteristic swollen appearance beginning at 96 hours. Control fragments looked comparatively healthy throughout the experiment. DOI: https://doi.org/10.1525/elementa.261.f1

RNA extraction and sequencing

Total RNA was extracted using the modified Trizol extraction protocol from Polato et al. (2011) and then applied to a Qiagen RNeasy mini spin column (Qiagen, CA). Quality and concentration of total RNA was quantified on an Agilent 2100 Bioanalyzer (Agilent Technologies, CA). RNA samples were pooled by the color of the colonies and treatment. A total of 8 libraries (control red, control white, oil red, oil white, dispersant red, dispersant white, oil-dispersant red,

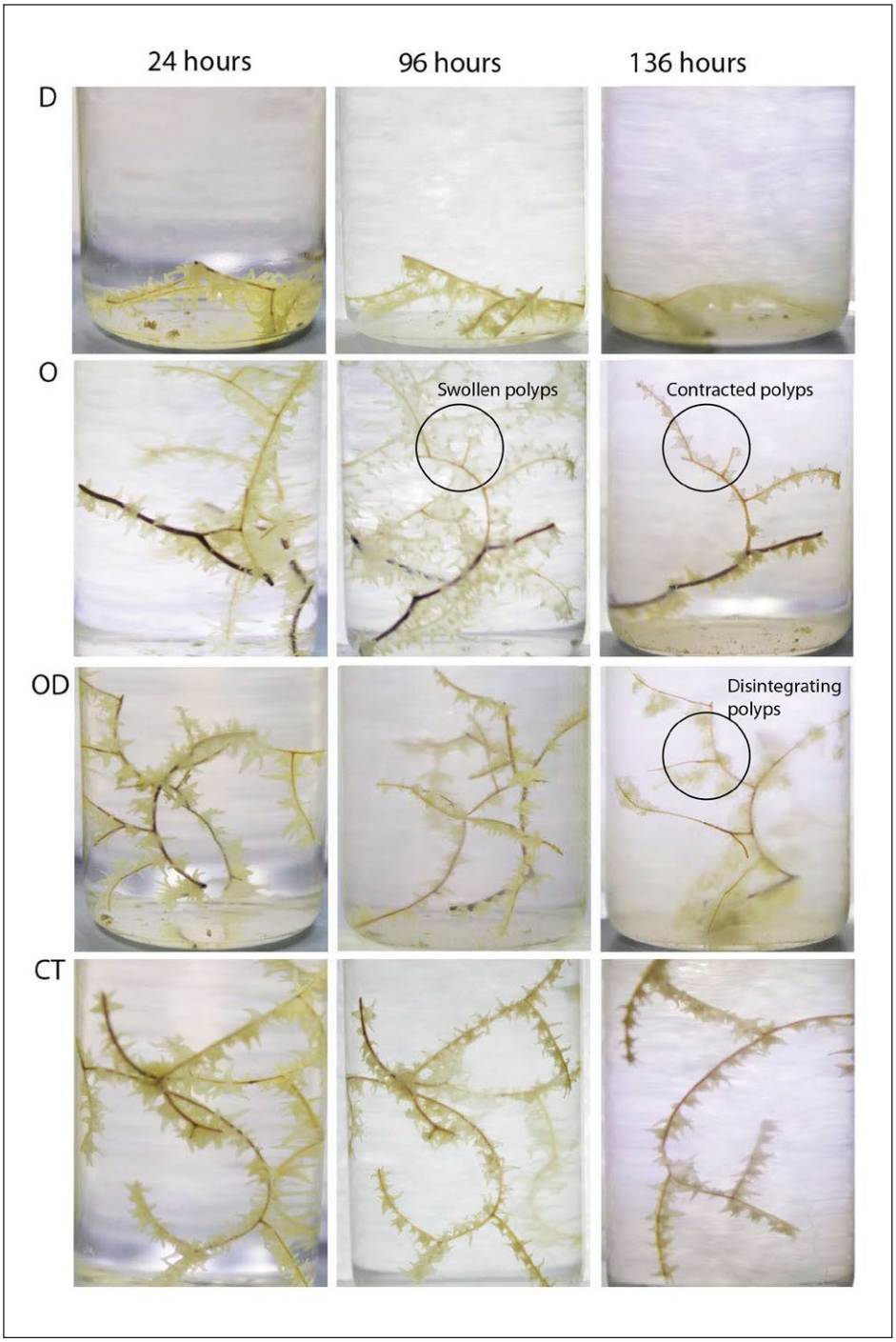

Figure 2: Health changes in a white *Leiopathes glaberrima* colony during 2012 visual impact studies. Photographic images of a white colony in treatment concentrations of 25 mg L^{-1} during 2012 experiments. Columns from left to right: after exposure to treatment for 24 hours; after exposure to treatment for 96 hours; and at end of 40-hour recovery period in ASW (136 hours). Rows from top to bottom: D = dispersant, O = bulk oil, OD = bulk oil and dispersant and CT = controls. The fragments in the D and OD treatments had disintegrated by 136 hours and the vials were cloudy with mucus. Polyps in the oil treatment showed a characteristic swollen appearance beginning at 96 hours, and at 136 hours (recovery) polyps in the oil treatment appeared contracted. Control fragments looked comparatively healthy throughout the experiment. DOI: https://doi.org/10.1525/elementa.261.f2

oil-dispersant white) were prepared using the TruSeq Stranded mRNA Sample Prep Kit according to the manufacturer's protocol. This preparation included a poly-A tail selection step. Libraries were single-end sequenced for 150 cycles on one lane of an Illumina HiSeq 2500 in RapidRun mode at the Penn State Genomics Core Facility (Table S2).

Differential gene expression

Transcripts were normalized using Trinity's in-silico read normalization with targeted maximum coverage of 30 (Haas et al., 2013) and assembled *de novo* with Trinity (version r2013-10-11) (Haas et al., 2013) with a minimum contig length of 250 base pairs and minimum k-mer coverage of 2. For details on quality control

and transcriptome assembly see Supplemental Materials (Text S1, Table S3). Trimmed transcripts were mapped back to the assembled transcriptome with Bowtie2 (Langmead et al., 2009) using default parameters (summary results in Table S4). Only sequences that uniquely mapped to the reference transcriptome were used for gene expression analysis. Mapped reads were sorted and duplicates were removed using MarkDuplicates in the Picard package (Li et al., 2009). Samtools (Li et al., 2009) were used to filter the best quality reads (reliable quality of ≥10) and select the uniquely mapped reads (see sequence quality and assembly statistics in Text S1). Count data (from the reads that map uniquely to each contig or gene) from the alignment files were obtained with the eXpress package (Roberts and Pachter, 2013). Transcripts with less than 10 counts were removed, and a differential expression analysis was run with the DESeq package (Anders, 2010; 1/15/17 10:09 PM in R; R Development Core Team, 2008). Read counts were normalized by the effective library size, and variance of the data was estimated. Because technical replicates of the colors were pooled for sequencing, color morphs could not be compared directly. Therefore the variance was calculated by treating all samples as replicates of the same condition using the method per condition option in DESeq (Anders, 2010). Negative binomial testing was used to compare differences in genes expression between the treatments; only those genes with an adjusted p-value ≤ 0.05 were considered as significant.

To enable a comparison between color morphs despite the pooling strategy used for sequencing, statistical solutions were applied. To visualize patterns of gene expression by color and treatment, the overall mean dispersion of the data was calculated as before and transformed using the variance stabilizing transformation function. When read counts are small, log2 fold changes can be highly variable; the variance stabilizing transformation moderates the fold change estimates, facilitating the clustering of the data (Anders, 2010). Heatmaps of the 100 most highly differentially expressed genes were generated using heatmap.2 function (gplot package) with the default hclust cluster algorithm (Warnes et al., 2009). The gene rows and sample columns were bootstrapped (10,000 iterations) with the pvclust function (Suzuki and Shimodaira, 2006) to obtain support values for the hierarchical clustering.

Functional analysis

First, differentially expressed genes were searched against the NCBI nr database (Altschul et al., 1997). The results from the *BLASTx* query were then annotated with Blast2GO (Conesa et al., 2005). Second, protein-coding transcripts were blasted against the UniProt database and the annotated gene list was used as the background list for functional annotation. Gene enrichment tests and functional annotation analyses were performed on the differentially expressed genes and the background list using the functional annotation tools within DAVID Bioinformatics Resources 6.7 (Dennis Jr et al., 2003).

Results

Visual impact in 2012 studies

The coral's responses to chemical exposure ranged from mucus discharge to polyp retraction and/or swelling and sloughing tissue. Based on these responses, a health rating was assigned to each fragment (see methods for details). Overall, polyp health deteriorated with time for all treatments but occurred earlier in the bulk dispersed oil treatment (**Figure 3A** and **C**). The Cox regression did not show differences in survival between the red and white fragments during the 96 hours of exposure ($p = 0.52$, Wald test; Table S5). The decline in health associated with the exposure to any concentration of the oil-dispersant mixture was 15 times faster than exposure to water ($p < 0.00$, Wald test; Table S5). The decline associated with any concentration of dispersant was 6.67 times faster than water ($p < 0.01$, Wald test), and decline in any concentration of bulk oil was 7.5 times faster than water ($p < 0.00$, Wald test; Table S5). Control fragments remained healthy to the end of the experiment. The interaction of treatments and length of exposure influenced the percentage of extended polyps in the fragments (Repeated measures ANOVA, Lower Bound, $F = 4.6$, $df = 3$, $p < 0.006$, Table S6). Differences between treatments may be caused by the differences in the percentages of extended polyps in the oil and dispersed oil treatments (Tukey HSD mean difference 16.35, $p < 0.002$). There was no difference in polyp extension between oil and control treatment (water) (Tukey HSD mean difference of −10.56, $p = 0.16$; Table S7).

Visual impact in 2013 studies

The two color morphs showed significant differences in health ratings when exposed to the WAF-oil treatments (Kaplan-Meir analysis, $p = 0.01$, Log-rank test; Table S8, **Figure 4**; mean time to decline in health rating to <3 was 73 hours). The decline in health associated with the exposure to any concentration of dispersant and dispersed WAF-oil was 2.41 and 2.33 times faster than exposure to water ($p < 0.00$, Wald test; Table S5). The decline in health associated with any WAF-oil treatment was 0.22 times faster than exposure to water ($p < 0.00$, Wald test; Table S5). Note that by the end of the experiments (between 72 and 96 hours), control fragments showed a decline in health (**Figure 3**) but fragments exposed to WAF treatments had a relatively faster decline in health. The interaction of time and treatments influenced the percentage of extended polyps in the fragments (Repeated measures ANOVA, Lower Bound, $F = 7.91$, $df = 3$, $p < 0.001$; Table S9). Differences between treatment were significant for the dispersant (Tukey HSD mean difference 35, $p < 0.01$) and the dispersed oil treatment (Tukey HSD mean difference of 47.30, $p < 0.000$; Table S10). There was no difference in polyp extension between oil and control treatment (water) (Tukey HSD mean difference of –0.81, $p = 1.00$; Table S10).

Gene expression study – 2012

As predicted from the 2012 visual impact study, mortality was not observed during the 24 hours of exposure at 25 mg L^{-1} at which point gene expression was meas-

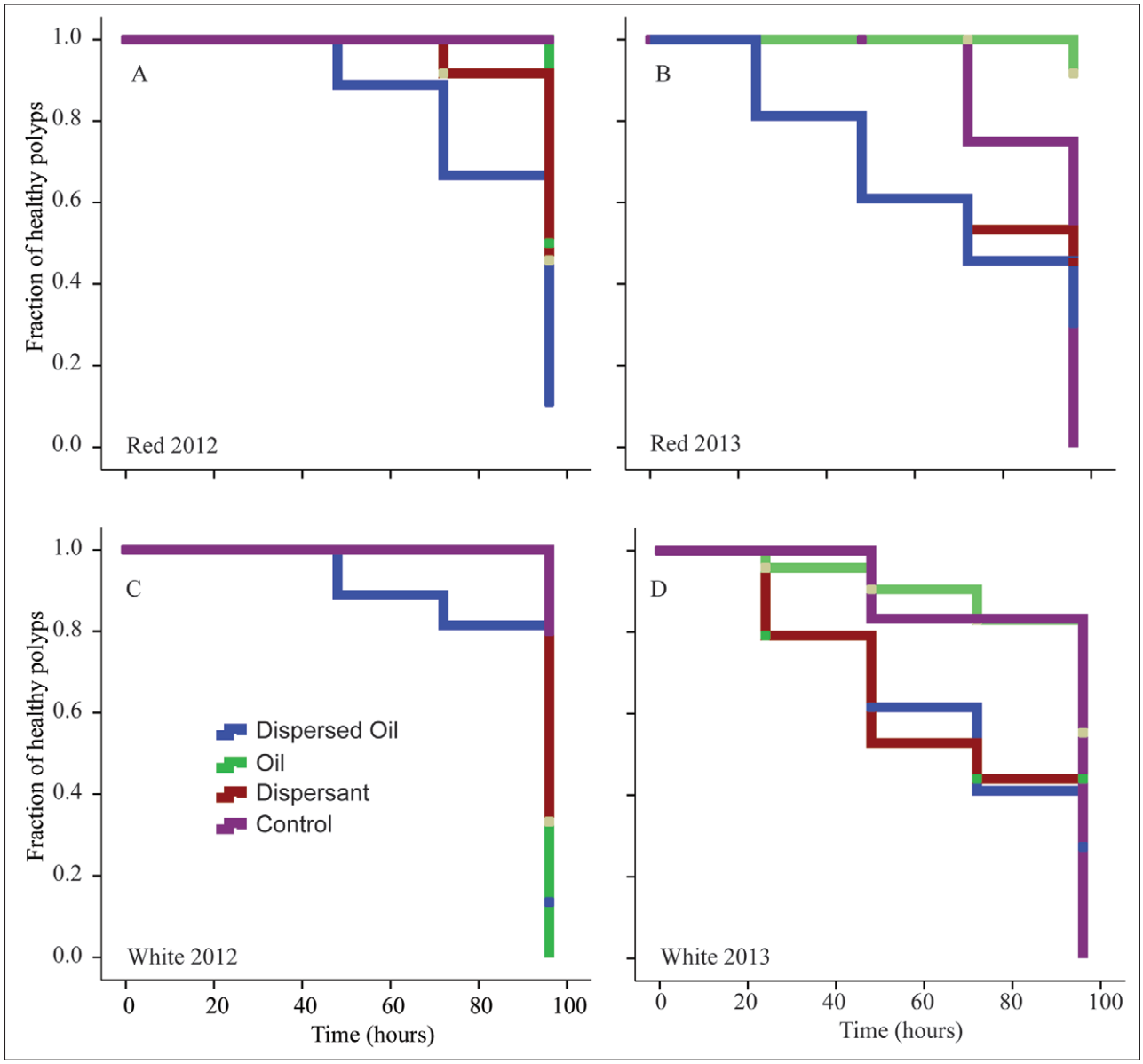

Figure 3: Decline in health scores for *Leiopathes* fragments during the 2012 and 2013 visual impact studies. Kaplan-Meier time-to-event curves of red and white fragments in response to the chemical treatments at concentration of 25 mg mL^{-1} for 2012 **(A, C)** and 2013 **(B, D)** visual impact studies. The event was a decline in health rating to ≤3. In 2012, control fragments (purple lines) were healthy throughout the experiment, but in 2013 control fragments declined in health between 72 and 96 hours. DOI: https://doi.org/10.1525/elementa.261.f3

ured. In some fragments, mucus spots formed at the tips where the sampling cuts were made, and a slight decline in health (from a score of 5 to 4) was observed. Because health ratings changed little, Kaplan-Meier time-to-event analysis was not performed. At 24 hours of exposure there was no significant difference between the percentage of extended polyps in the treatments (compared to the controls, **Figure 4A**; One-way ANOVA, F = 0.94, df = 3, p = 0.43; Table S11).

Functional annotation of the 100 highest expressed genes of *Leiopathes* in DAVID identified gene sets associated with ion transport (enrichment score = 1.03), cell adhesion (enrichment score = 0.67), ribosomal constituents (enrichment score = 1.16), zinc-finger proteins (enrichment score = 0.90), and actin-binding proteins (enrichment score = 1.50) (Tables S12–S15). See Results section in Text S1 for sequence quality (Figure S1), transcriptome completeness and taxonomic and functional annotations.

Because reads may map to multiple reference sequences and therefore affect the estimation of gene expression, we considered only the reads that mapped to just one place in the reference transcriptome (Wit et al., 2012). Only 30 to 40% of the reads mapped to a unique reference sequence. Nevertheless, differential expression among treatments and between color morphs was observed (**Figure 5**).

At first, the libraries of the red and white *L. glaberrima* fragments were used as treatment replicates. The hierarchical clustering of the 100 most highly expressed genes (after variance stabilization) grouped the samples by color.

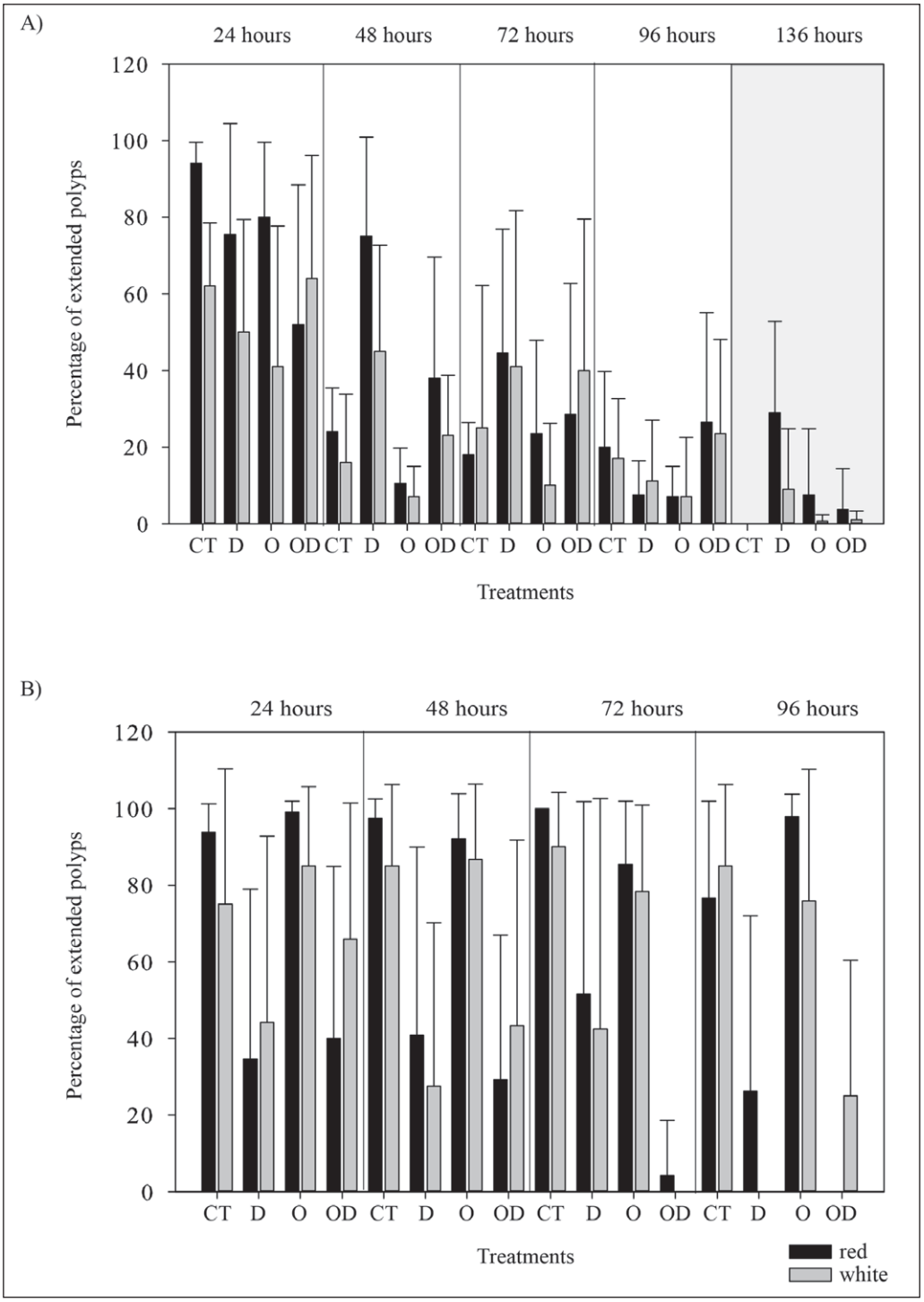

Figure 4: Average percentage of extended polyps during the 2012 and 2013 visual impact studies. A) Average percentage of extended polyps in the 2012 visual impact study. After 96 hours the colonies were transferred to artificial seawater for recovery; 136 hours marks the end of the recovery period (shaded panel). **B)** Average percentage of extended polyps in the 2013 visual impact study; there was no recovery period. Black = red corals, grey = white corals, error bars = standard deviation. DOI: https://doi.org/10.1525/elementa.261.f4

The binomial test for differential expression revealed a total 220 significantly different transcripts, distributed as 191 transcripts from the comparisons between treatments and controls (**Figure 6**), and 29 from the between treatment comparisons (not presented in **Figure 6**). One hundred and two transcripts were expressed in response to dispersed oil of which only 19 were annotated. Fifty-five genes (13 annotated) were expressed in response to oil, with 51 of these transcripts also expressed in the dispersed oil treatments (treatment against control, DESeq analysis, Benjamini-Hochberg (BH) adjusted p-value ≤ 0.05; Table S16, **Figure 6**). Thirty-four transcripts (eight annotated) were expressed in response to dispersant (treatment against control, DESeq analysis, BH adjusted p-value ≤ 0.05; Table S16, **Figure 6**). Additionally, 11 transcripts (two annotated) were significantly different between oil and dispersant, 16 transcripts were different between dispersant and dispersed oil (three annotated),

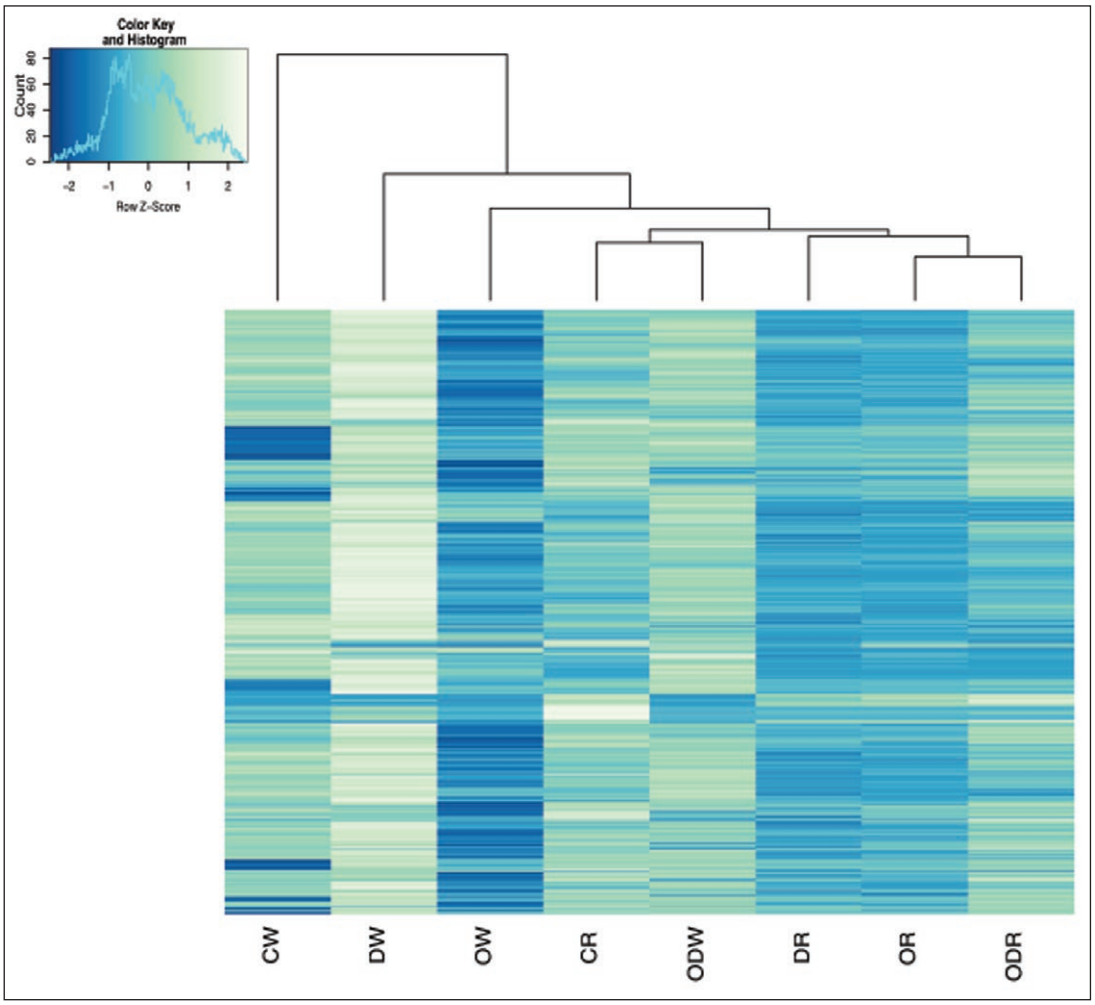

Figure 5: Patterns of gene expression in the two color morphs of *L. glaberrima.* Hierarchical clustering of the 1000 most highly expressed genes across treatments for the two color morphs of *L. glaberrima*: DW = white fragments in dispersant, CW = white fragment in control, ODW = white fragments in oil and dispersant, OW = white fragments in oil, DR = red fragments in dispersant, ODR = red fragments in oil and dispersant, CR = red fragments in control, and OR = red fragments in oil. Highest expressed genes in light green, lowest in blue. The z-score shows how many standard deviations a measured expression is above or below the mean. DOI: https://doi.org/10.1525/elementa.261.f5

and two transcripts (no annotations) were differentially expressed between oil and dispersed oil treatments (DESeq analysis, BH adjusted p-value ≤ 0.05, Table S16).

Of the annotated transcripts, genes for the latent-transforming growth factor beta binding protein 2 (*LTBP2*), serine/arginine repetitive matrix protein (Srrm2), protein phosphatase (*Ppm1d*), EGF-like proteins (*Svep1* and *Egfem1*) and ER lumen protein-retaining receptor 3 (*Kdelr3*) were up-regulated in the oil and the dispersed oil treatments. Genes for gamma-butyrobetaine dioxygenase (*Bbox1*) were down-regulated in the oil treatment. Peroxidaxin and Alpha-1 A adrenergic receptor genes were up-regulated in the dispersant treatments. Cytochrome P450 and fibrillin-2 genes were up-regulated in the dispersed oil treatment. The gene for protein Abcc4 was down-regulated in the dispersant and dispersed oil treatment. Additionally, 11 transcripts (three annotated) were significantly expressed across treatments compared to the controls, the gene for protein Nynrin was down-regulated in all treatments while ATP-dependent DNA helicase Q5 and GTPase IMAP family member 4 genes were up-regulated in all treatments.

The difference in gene expression between the two color morphs might obscure the interpretation of the experimental treatments. Therefore, we explored our results by regrouping data in two different ways. First, we compared expression between treated (six replicates) and untreated (two replicates) corals regardless of color and treatment. We recovered a similar set of expressed genes as with the previous approach (see Table S9). Then, we separated the sequencing data into two datasets, from now on referred to as red library (four red libraries), and white library (four white libraries), and compared treatment effects within the color morphs (no replication).

Differential gene expression between red and white colonies

After discarding those transcripts with zero counts for any of the treatments (filter cut-off was ≥ 10 counts), 180 transcripts were differentially expressed between the color morphs. Seventy-five transcripts were significantly different between red and white control colonies; 19 were annotated (Table S18). In the oil treatment, 30 transcripts were differentially expressed (14 annotated). Thirty-eight transcripts (13 annotated) were significantly differenti-

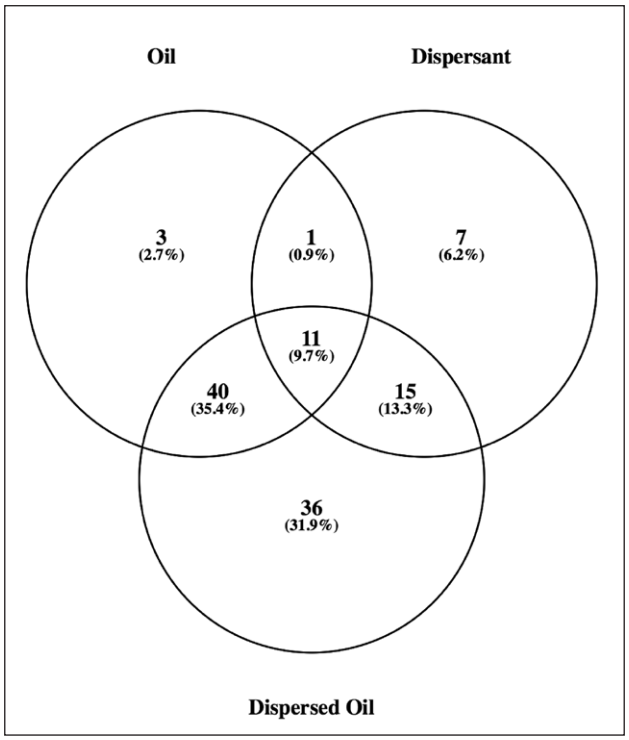

Figure 6: Number of differentially expressed genes in *L. glaberrima* by treatment. Venn diagram of the number (and percentage) of significantly expressed genes in the oil, dispersant and dispersed oil treatments when compared against control and using color morphs as treatment replicates (BH adjusted p-value ≤ 0.10). DOI: https://doi.org/10.1525/elementa.261.f6

ated between the red and white fragments in the dispersant treatments. The comparison between the fragments in the dispersed oil treatments recovered 37 differentially expressed transcripts, of which 18 had matches to UniProt.

Genes for hemicentins (*Hmcn1* and *Hmcn2*) were down-regulated in red fragments exposed to dispersant and dispersed oil. Oncoprotein-induced transcript 3 (*Oit3*) genes were up-regulated in the red library for the controls and the dispersant treatments. Beta-carotene 15 (*Bco1*) genes were down-regulated in all treatments.

Gene expression in red colonies

Ninety-five transcripts were significantly different between controls and the dispersant treatments in red colonies (34 in which base mean or the mean of normalized counts, averaged over all samples from both conditions, was larger than 0, with three annotated; Table S19, **Figure 7**). Between the controls and the oil treatments, 602 transcripts were differentially expressed (166 in which base mean was >0, with 17 annotated). Between the controls and the dispersed oil, 168 transcripts (96 in which base mean was >0, with 17 annotated) were differentially expressed (Table S19, **Figure 7**). Of the annotated genes, the collagen alpha-6(VI) chain protein gene was up-regulated in the dispersant and dispersed oil treatments. The gene for leucine-zipper-like transcriptional regulator 1 (*LZTR*-1) was down-regulated in the dispersant treatment, while the genes for RNA-binding protein 28 (*Rbn28*), WD-repeat-containing proteins (*Wdr3*, *Wdr38*, *Wdr61*) and Catenin-beta 1 (*Ctnnb1*) were up-regulated in the oil treatments.

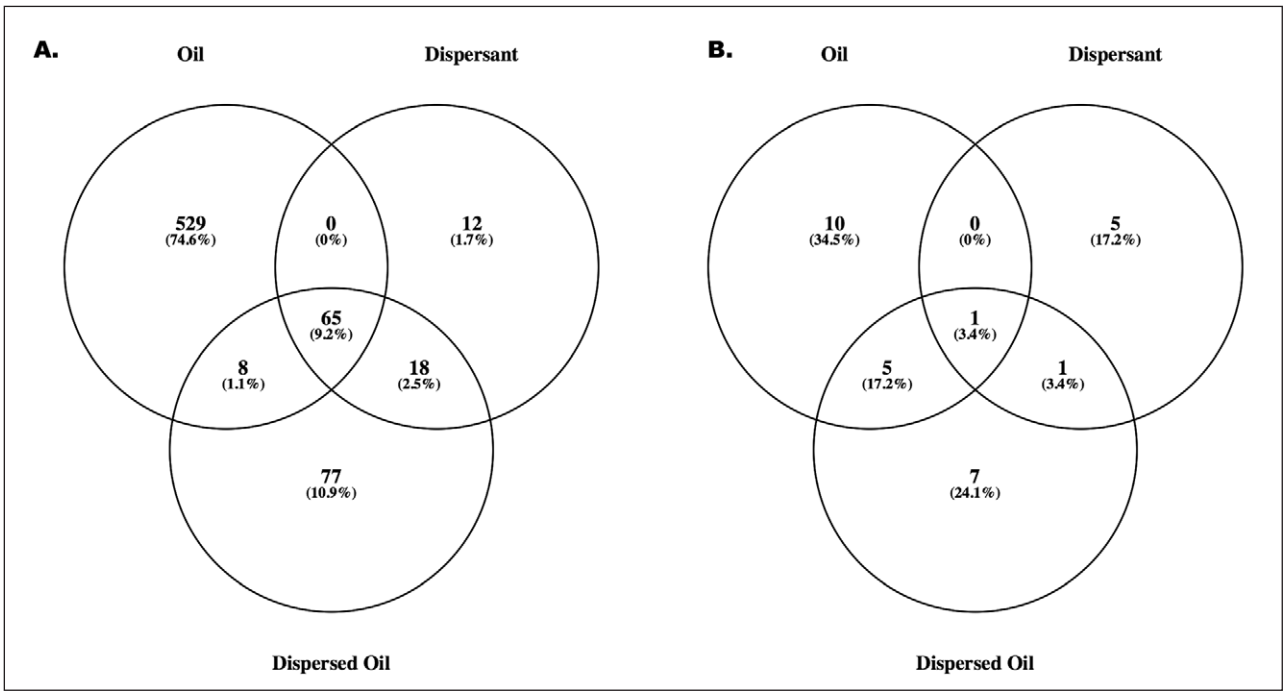

Figure 7: Number of differentially expressed genes in *L. glaberrima* by treatment in red and white morphs. Venn diagram of the number (and percentage) of significantly expressed genes in the oil, dispersant and dispersed oil treatments when compared against control for **(A)** red colonies and **(B)** white colonies (BH adjusted p-value ≤ 0.10). DOI: https://doi.org/10.1525/elementa.261.f7

Gene expression in white colonies

After adjusting for multiple testing, 16 transcripts (three annotated) were differently expressed between the oil treatment and the controls in white colonies. Fourteen transcripts (two annotated) were differentially expressed between dispersed oil and control treatments, and seven (one annotated) between dispersant and control (Table S20). The genes for protein dispatched homolog 3 (*Ptchd2*) and mRNA-decapping enzyme 1B (*Dcp1b*) were down-regulated in the oil treatments. The golgin B1 gene was up-regulated in both oil and dispersed oil treatments. The pro-protein convertase 5 gene (*Pcsk5*) was also up-regulated in the dispersed oil treatments. The ankyrin Repeat Domain 60 gene (*Ankrd60*) was up-regulated in the samples exposed to the dispersant treatments compared to the controls.

Discussion

Leiopathes glaberrima showed visible signs of stress and health deterioration (e.g., mucus production, tissue disintegration) in response to hydrocarbon and dispersed hydrocarbon exposure at the tested concentrations, as reported previously (DeLeo et al., 2015). Here we documented that the decline in health was also reflected in gene expression changes, with the onset of this response beginning before visible signs of health deterioration were detected. Dispersed oil treatments resulted in gene expression changes related to immune response, wound healing, and oxidative stress. Genes with these functions had previously been associated with a coral stress response (Reitzel et al., 2008; Polato et al., 2010; Voolstra et al., 2011), and the biomarker gene cytochrome P450 for polycyclic aromatic hydrocarbon exposure (Rougée et al., 2006; Arukwe et al., 2008; Amutha et al., 2009; Hook and Osborn, 2012; Fodrie et al., 2014) was only up-regulated in this treatment. Phenotypic and gene expression differences were observed between the two color morphs (**Figures 3** and **5**). Compared to the white colonies, the red colonies had more gene expression variation during the early response to sub-lethal chemical exposure. At these sub-lethal concentrations, there was no difference in the visible impact of the chemical exposure with color morphotype but the white colonies deteriorated faster than red colonies at higher chemical concentrations (**Figure 3**, Tables S5 and S6). Higher stress tolerance in genotypes with a more vigorous change in gene expression was similarly observed in shallow-water corals (Parkinson et al., 2015). This study adds to the growing evidence of the impact of oil spills and mitigation efforts on benthic ecosystems and highlights the use of gene expression assays to detect sub-lethal stress responses.

Xenobiotic stress response triggered by dispersed oil

The stress response, in terms of quantity of expressed genes, was strongest in response to dispersed oil at the tested concentration of 25 mg L^{-1}. The dispersed oil treatment elicited a 2-fold stronger differential gene expression response than the oil treatment (Tables S16 and S17). Only the dispersed oil treatment caused up-regulation of the genes for cytochrome P450 (*CYP P450*) and fibrillin-2, two important markers of stress response. *CYP P450* is often used as a biomarker for xenobiotic stress in the presence of polycyclic aromatic hydrocarbons (Rougée et al., 2006; Arukwe et al., 2008; Hook and Osborn, 2012; Fodrie et al., 2014) due to its central role in the oxidative metabolism of exogenous and endogenous substances (Arukwe et al., 2008). The up-regulation of *CYP P450* in response to the dispersed oil treatments but not in the bulk oil treatments might indicate that the addition of dispersant increases the availability of oil inside the coral tissue (Zuijdgeest and Huettel, 2012). The fibrillin-2 gene and other genes associated with wound healing (Hemicentins) respond to stress in *Nematostella* (Reitzel et al., 2008) and other invertebrates (Riesgo et al., 2012). Although experimental cutting of colonies might have also introduced stress (Stossel et al., 2001) and activated genes involved in wound-healing, any general effects of experimental cutting would have been invisible to the differential gene expression analysis because both control and treatment fragments were cut.

Thirty-five percent of the genes responded to both oil and the dispersed oil treatments (**Figure 6**). Of the genes expressed in both treatments, the genes for EGF-like domain-containing proteins are associated with immune and stress responses in cnidarians (Reitzel et al., 2008; Barshis et al., 2013). The genes for EGF-like domain proteins Svep1 and Egfem1 were up-regulated in the oil and the dispersed oil treatments. However, there were also differences associated with the color of the colonies. Although *Svep1* was up-regulated in oil and dispersed oil treatments, it was down-regulated in the controls and dispersant treatments of the red colonies but not in the white colonies. Differential expression of other EGF-like proteins (OIT3 and TLL2) was also observed between the red and white libraries (next section).

Fewer genes were up-regulated in the dispersant treatments compared to the dispersed oil treatment, perhaps as a consequence of the rapid deterioration of the polyps. However, studies on *Caenorhabditis elegans* have also reported aberrant gene expression in response to oil and dispersed oil treatments, with lack of response to exposure to dispersant alone (Zhang et al., 2013). Nevertheless, the eight annotated genes that responded to the dispersant treatments were also detected in other experiments evaluating coral response to stress. For example, two isoforms of peroxidasin were up-regulated in the fragments exposed to the dispersant treatment, and have been detected in multiple coral stress studies (Reitzel et al., 2008; Polato et al., 2010; Voolstra et al., 2011). The *in vivo* functions of peroxidasins are uncertain, but they might be involved with innate immune defense (Gotenstein et al., 2010), response to oxidative stress and programmed cell death (Horikoshi et al., 1999).

Highly variable gene expression response among colonies of L. glaberrima

Hierarchical clustering showed that the two color phenotypes (red and white) differed in their gene expression response to experimental treatments (Figure S2). Thirty-nine genes had

an opposite response in red and white colonies (Table S18). One of these functional differences was the up-regulation of EGF-like domain-containing proteins (OIT3, TLL2) in the red fragments. The differentiation in expression of *OIT3* between the color morphotypes is noteworthy, as *OIT3* is one of a set of genes that are "frontloaded" (i.e., have high constitutive expression) in thermal tolerant corals (Barshis et al., 2013). Coral colonies in which these genes were up-regulated in control conditions appear to be more resilient to environmental stress (Barshis et al., 2013). Here, *OIT3* was up-regulated in red but not in white control treatments. The expression of *OIT3* also increased in the red fragments exposed to the dispersant treatment (Table S18). As with the EGF-like proteins, the gene for transcription factor Srrm2 was up-regulated in the experimental treatments but the color comparisons suggest an increase in the expression of *Srrm2* specifically in *Red* when exposed to the dispersant (Table S16 and Table S18). Red colonies of *L. glaberrima* showed more expression changes in response to the experimental treatments and had better tolerance to oil than white colonies despite the fact that the white library had 30% more sequences than the red library. In the shallow water coral, *Acropora palmata*, colonies more tolerant of cold temperature stress similarly changed their gene expression to a larger degree than colonies more sensitive to the stress (Parkinson et al., 2015). Whether the enhanced ability of corals to respond to stress with gene expression changes is due to more sensitive stress detection or a wider dynamic range of gene expression needs further study.

Both colors morphotypes suffered from a decline in health when exposed to the 25 mg L^{-1} concentration of oil, dispersant and dispersed oil (**Figure 4**). The lower survival of the white fragments in oil, coupled with the low expression of stress-related genes, might indicate that the white colonies are more susceptible to oil spills than their red counterparts. However, increased replication for each color morphotype is necessary before the large variability in gene expression and visible stress response among colonies can be attributed to morphotype.

Greater visible impact of dispersant and dispersed oil on corals than oil alone

We reported previously that dispersant and the dispersed oil were more detrimental to cold-water corals than oil alone, regardless of whether solutions were prepared as bulk oil (2012 experiments) or WAF-oil (2013 experiments) fractions (DeLeo et al., 2015). For both oil dissolution methods (bulk and WAF) the concentrations were likely overestimated due to volatilization, adherence to the containers, and degradation. In this study, visual health impacts were recorded at both 1:1 (2012 experiments) and 1:10 (2013 experiments) ratios of dispersant to oil (**Figures 1** and **2**). Increased susceptibility to dispersant and chemically dispersed oil has also been observed in other organisms (Epstein et al., 2000; Scarlett et al., 2005; Shafir et al., 2007; Milinkovitch et al., 2011; Rico-Martínez et al., 2013), particularly in invertebrates lacking the protection of shells and exoskeletons (Scarlett et al., 2005; Rico-Martínez et al., 2013).

One might expect that corals from the deep Gulf of Mexico would be somewhat tolerant to oil exposure because naturally occurring hydrocarbon seeps are widespread in the Gulf of Mexico and carbonates produced at these sites provide the primary substrate for corals in the deep Gulf (Fisher et al., 2007; Orcutt et al., 2010). Although some corals have a distribution correlated with active seepage (Quattrini et al., 2013), *L. glaberrima* does not, and analyses of the stable isotope content of its tissue indicate that *L. glaberrima* does not obtain significant nutrition from seep primary production (Becker et al., 2009). Adult colonies of *L. glaberrima* are therefore unlikely to interact directly with hydrocarbons in their natural environment.

Conclusions

Changes in gene expression associated with stress response were detected before *L. glaberrima* colonies showed visual signs of health deterioration. This early stress response was strongest in response to dispersed oil at the targeted test concentration (25 mg mL^{-1}). While predicting point concentrations of oil and dispersant during a spill is difficult, impact assessments of oil spills should consider the effects of prolonged exposure to both oil and the diluted toxicants used in the chemical dispersion of oil (Goodbody-Gringley et al., 2013; White et al., 2014).

Data Accessibility Statement

Transcriptome alignments and phenotype scores: GRIIDC R1.x132.136:0024 at: https://data.gulfresearchinitiative.org (DOI: https://doi.org/10.7266/N73T9F5N).

Supplemental Files

The supplemental files for this article can be found as follows:

- **Text S1.** Methods for assembly and transcriptome completeness. Results on sequence quality, assembly, transcriptome completeness and taxonomic and functional annotations. Tables S1–S20: Results tables for the visual impact studies and the Differential expression analysis. DOI: https://doi.org/10.1525/elementa.261.s1
- **Tables.** Functional annotations and enrichment scores for expressed genes. DOI: https://doi.org/10.1525/elementa.261.s2

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Competing interests

The authors have no competing interests to declare.

Author contributions

DVRR and IBB conceived the experiments with contributions from CF. DVRR carried out the experiments, analyzed the data and led the writing of the manuscript. IBB and CF oversaw the research, secured funding and co-wrote the manuscript with DVRR.

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