1 Exposure to Deepwater Horizon Oil and Corexit 9500 at Low
2 Concentrations Induces Transcriptional Changes and Alters Immune
3 Transcriptional Pathways in Sheepshead Minnows

4

5 ELIZABETH R. JONES¹, CHRISTOPHER J. MARTYNIUK², JEFFREY M.
6 MORRIS³, MICHELLE O. KRASNEC³, AND ROBERT J. GRIFFITT^{1*}

7

8

9

10 ¹Division of Coastal Sciences, School of Ocean Science and Technology, The
11 University of Southern Mississippi

12 ² Department of Physiological Sciences and Center for Environmental and
13 Human Toxicology, UF Genetics Institute, College of Veterinary Medicine,
14 University of Florida

15 ³Abt Associates, Boulder, Colorado

16

17 *Address correspondence to:

18 Robert J. Griffitt

19 The University of Southern Mississippi

20 Division of Coastal Science, School of Ocean Science and Technology

21 703 East beach Drive

22 Ocean Springs, MS 39564

23 joe.griffitt@usm.edu Phone (228) 872-4298.

Abstract: The 2010 Deepwater Horizon (DWH) oil spill caused the release of 4.9 million barrels of crude oil into the Gulf of Mexico, followed by the application of 2.9 million L of the dispersant, Corexit™ to mitigate the spread of oil. The spill resulted in substantial shoreline oiling, potentially exposing coastal organisms to polyaromatic hydrocarbon (PAH) and dispersant contaminants. To investigate molecular effects in fish following exposure to environmentally relevant concentrations of DWH oil and dispersants, we exposed adult sheepshead minnows (*Cyprinodon variegatus*) to two concentrations of high-energy water-accommodated fraction (HEWAF), chemically enhanced water-accommodated fraction (CEWAF) or Corexit 9500™ for 7 and 14 days. Resulting changes in hepatic gene expression were measured using 8 x 15K microarrays. Analytical chemistry confirmed PAH concentrations in HEWAF and CEWAF treatments were low (ranging from 0.26 to 5.98 µg/L), and likely representative of post-spill environmental concentrations. We observed significant changes to gene expression in all treatments (relative to controls), with Corexit and CEWAF having a greater effect on expression patterns in the liver than HEWAF treatments. Sub-network enrichment analysis of biological pathways revealed that the greatest number of altered pathways in high dose HEWAF and CEWAF treatments occurred following a 7-day exposure. Pathways related to immunity comprised the majority of pathways affected in each treatment, followed by pathways related to blood and circulation processes. Our results indicate that oil composition, concentration, and exposure duration all affect molecular responses

46 in exposed fish, and suggest that low-concentration exposures may result in sub-
47 lethal adverse effects.

48

49

50 **Keywords:** PAH, dispersant, Deepwater Horizon, transcriptomics, microarray,
51 immunity

52

1. Introduction

The explosion of the Deepwater Horizon (DWH) drilling rig on April 20, 2010 caused the release of approximately 4.9 million barrels of crude oil into the northern Gulf of Mexico (nGOM), and created the largest oil spill in US history (McNutt et al., 2012). Attempts to mitigate the spread of this oil included skimming and in situ burning, as well as the application of an estimated 2.9 million L of the chemical dispersant, Corexit™, which resulted in large amounts of dispersed oil in the nGOM ecosystem (Kujawinski et al., 2011). Approximately 2100 km of nGOM shoreline from Texas to Florida were oiled, with the most impacted sites located in Louisiana (Michel et al., 2013; Nixon et al., 2016).

It is likely that PAH contamination from the DWH spill persists in heavily-oiled coastal locations, as surveys of visibly contaminated shoreline found that oil remained at 48% of sites after one year, and 39% two years post-spill (Michel et al., 2013). Additionally, submerged oil mats located offshore may be a continued source of contamination via seasonal weathering and storm action that erode and re-deposit sediment along nGOM shorelines (Clement et al., 2012; Michel et al., 2013). This visible oiling of coastal sites post-spill correlates with measurements of sediment PAH contamination, which included increases in some PAH constituents in Louisiana marshes three years after the DWH event (Turner et al., 2014). Such persistent PAH contamination will likely affect nGOM shorelines for years to come, as hypoxic soil conditions (like those present in coastal estuaries) inhibit PAH degradation (Boyd et al., 2005; Hambrick et al., 1980; Turner et al., 2014). This slowed degradation of PAHs in estuarine soils

may contribute to lingering contamination in near shore water columns; for example, although one study detected negligible amounts of PAHs in coastal waters one year after the spill, they also found an instance of post-DWH increases in water PAH concentration due to sediment re-suspension (Allan et al., 2013). The persistence of PAHs in anaerobic sediments coupled with possible re-suspension implies that PAH contamination remains in some coastal waters, and that coastal organisms will contend with the effects of DWH event for the foreseeable future.

Multiple studies demonstrate that exposure to DWH oil has adverse impacts on fish health. Some of these impacts include embryonic and juvenile mortality (Dubansky et al., 2013), reduced fecundity (Hedgpeth and Griffitt, 2016), altered gill and intestinal microbiome and histopathologic changes in gill and liver (Brown-Peterson et al., 2016, 2015), impaired cardiac function (Brette et al., 2014; Incardona et al., 2014, 2013), impaired swimming performance (Mager et al., 2014), DNA damage (Pilcher et al., 2014) and immune dysfunction (Bayha et al., 2017). While many studies have examined phenotypic endpoints to establish the detrimental effects of DWH oil on fish health, fewer have investigated changes in global gene expression that might elucidate the sub-lethal effects of exposure. The majority of information regarding transcriptional changes associated with DWH oil exposure comes from the work of Whitehead and colleagues, who found substantial differences in the transcriptomes of wild Gulf killifish (*Fundulus grandis*) from oiled and pristine locations for up to one year following the spill (Dubansky et al., 2013; Garcia et al., 2012; Whitehead et

al., 2012). Aside from these field studies, there is little information documenting DWH related transcriptional changes in an experimental setting, free from confounding factors that might contribute to field findings. One study by Xu et al. (Xu et al., 2016), used an experimental approach to examine transcriptional changes associated with DWH oil exposure in developing mahi-mahi (*Coryphaena hippurus*), but there remains a need for laboratory data regarding transcriptional changes related to DWH oil exposure in adult fish, particularly using low µg/L concentrations that fish were likely to be exposed to following the oil spill.

The purpose of this study was to use a controlled experiment to investigate the effects of exposure to oil, oil-dispersant mixtures, and dispersant on global gene expression profiles in adult estuarine fish. We examined the transcriptional effects of prolonged exposure to low-concentration, environmentally relevant, post-spill concentrations of DWH contaminants, and evaluated these transcriptional changes in the context of altered biological pathways.

2. Methods

2.1 Experimental animals

Adult sheepshead minnows (*Cyprinodon variegatus*) used in this study were obtained from cultures kept at the Shoemaker Toxicology Laboratory at the University of Southern Mississippi Gulf Coast Research Laboratory (Ocean Springs, MS, USA). Prior to the experiment fish were held in 300-L raceways

supplied with recirculating artificial seawater (15 ppt salinity) at temperatures between 26 - 27°C and a 12:12 light/dark photoperiod. Fish were fed *Artemia* nauplii and commercial flake food twice daily.

2.2 Mixture preparations

All oil and dispersant mixtures used in this study were prepared according to methods outlined by Forth et al. (2016). High energy water accommodated fraction (HEWAF) and chemically enhanced water accommodated fraction (CEWAF) were prepared using oil obtained on July 29, 2010 from the hold of barge number CTC02404, which received surface slick oil from multiple skimming vessels operating near the Macondo Well (this oil hereafter referred to as Slick A oil). Oil samples were transferred to the University of Southern Mississippi on March 29, 2011 under chain of custody (sample ID SDG – 10073005), and refrigerated in amber jars until use. To prepare HEWAF stocks, 1 g/L of Slick A oil was added to 15 ppt artificial seawater in a stainless steel blender, and blended at low speed for 30 s. The resulting solution was poured into a foil covered separation funnel and allowed to settle for 1 h. After settling, stock HEWAF was transferred to a flask for use in exposures. To prepare CEWAF stocks, 1 g/L of slick A oil was added to 15 ppt artificial seawater in a glass aspirator bottle. Corexit 9500 dispersant (100 mg; 10 % of oil by volume) was added to the bottle, and the resulting solution mixed on a stir plate at 25% vortex for 24 h. After vortexing, the solution was allowed to settle in the dark for 3 h, and then transferred to a flask for use in exposures. Corexit-only stocks were

prepared by adding 1g Corexit 9500™ to one liter 15 ppt artificial seawater and mixing on a stir plate for 5 minutes. Fresh stocks for all solutions were prepared every 48 hours.

Final test solutions for each treatment were produced by diluting stock HEWAF and CEWAF solutions into 15 ppt artificial seawater at nominal concentrations of 1 ml/L for low concentration exposures, and 10 ml/L for high concentration exposures. Dispersant-only solutions were produced by diluting 1 ml of Corexit stock into 15 ppt seawater. This generated six different test solutions: seawater control, Corexit (1 ml Corexit/L), low CEWAF (1 ml CEWAF/L), high CEWAF (10 ml CEWAF/L), low HEWAF (1 ml HEWAF/L), and high HEWAF (10 ml HEWAF/L).

2.3 Analytical chemistry

For analysis of PAH concentrations, a 250-ml water sample was collected after each stock preparation (for a total of nine samples each for Corexit, CEWAF and HEWAF) and two hours after stock dilution into 15 ppt artificial seawater (one sample for each test solution). Each sample was placed in an amber bottle, and stored at 4°C. For dispersant content analysis, four 10 ml samples were collected from each stock solution (nine samples each for Corexit and CEWAF stocks), and again two hours after dilution of stocks into 15 ppt artificial seawater (one sample for each test solution). Samples were placed in 20 ml glass vials and stored at 4°C. All samples were shipped under chain of custody to ALS Environmental (Kelso WA, USA) and analyzed via gas chromatography with low-

resolution mass spectrometry using selected ion monitoring (8270 C SIM/PAH) as outlined in EPA method 8270D. For each sample a suite of 50 PAH analytes (tPAH50) were measured to quantify total PAH concentrations, and the anionic surfactant, dioctyl sodium sulfosuccinate (DOSS, a component of Corexit 9500), was measured as an estimate of total dispersant concentration.

2.4 Exposure methods

Adult sheepshead minnows (males and females, 120-d post hatch) were exposed to one of six exposure treatments: control, Corexit, low CEWAF, high CEWAF, low HEWAF, and high HEWAF. Fish were exposed for 7- or 14-d using a flow-through system described by Manning et al. (1999). Each treatment consisted of four replicate tanks with eight individual fish per tank (4 males and 4 females). Test solutions within each tank were renewed at a rate of 500 ml every 15 minutes, beginning three days prior to the addition of fish to experimental tanks and continuing for the duration of the experiment. Throughout experiments fish were fed *Artemia* nauplii and commercial flake food twice daily, and maintained under a 12:12 L/D photoperiod. Water was held at 15 ppt salinity and 27°C and monitored daily for temperature, salinity, pH, and dissolved oxygen. After 7- or 14-d fish were sacrificed using MS-222 (Sigma-Aldrich, St. Louis, MO, USA). Liver tissue from each fish was collected, placed in 500-µL RNA Later (Invitrogen, Carlsbad, CA, USA), and stored at -80°C until further analysis.

2.5 RNA extraction

Liver RNA was extracted from four individuals in each treatment (one from each replicate tank). Total RNA was isolated using Qiagen RNeasy kit (Hilden, Germany) according to the manufacturer's protocol. RNA was quantified spectrophotometrically using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA), and RNA integrity assessed using a 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA integrity scores for all samples were ≥ 8 , therefore all RNA samples were deemed of high quality and appropriate for inclusion in microarray analyses.

2.6 Microarray and pathway analyses

Changes in gene expression were measured using custom designed oligonucleotide 8 x 15k microarrays manufactured by Agilent Technologies (Santa Clara, CA, USA). For 7-d exposures, all treatments were analyzed via microarray, while only high CEWAF, high HEWAF and Corexit treatments were analyzed from 14-d exposures. Total liver RNA from four biological replicates (one fish per tank) from each treatment was used in Cyanine 3 (Cy3) labeled cRNA synthesis according to Agilent's One-Color Microarray-Based Gene Expression protocol (Santa Clara, CA, USA). Dye incorporation for each sample was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Dye specific activity for all samples exceeded 6.0 pmol Cy3/ μ g, with the exception of two 14-day control samples, which were excluded from further analysis due to low dye incorporation. Hybridization was performed

214 according to Agilent's One-Color Microarray-Based Gene Expression protocol
215 (Santa Clara, CA, USA), and arrays scanned using an Agilent G2505B scanner.
216 Expression data were extracted from TIFF images using Agilent Feature
217 Extraction Software (v10.7.3.1), and data was quality assessed via manual
218 inspection of software generated quality control reports. Based upon inspection,
219 all array data were determined to be of high quality and acceptable for inclusion
220 in gene expression analysis. Raw expression data were imported into JMP
221 Genomics v 5.1, log₂ transformed and quantile normalized. Data were filtered to
222 assign all data with intensities ≤ 4.5 a value of 4.5 (7-d samples) or ≤ 5.5 a value
223 of 5.5 (14-d samples) as these values were determined to represent the limits of
224 detection for microarrays analyses and were a conservative assessment (based
225 on limits of detection for Agilent RNA spike-in and negative controls). After data
226 filtering, differentially expressed genes (DEGs) were identified by one way
227 analysis of variance (ANOVA) followed by a false discovery rate (FDR) of 5.0%.

228 To gain insight into biological pathways responsive to exposure to Corexit,
229 CEWAF or HEWAF, enrichment analyses for all transcripts were performed using
230 Pathways Studio 9.0 software (Elsevier Inc., Rockville, MD, USA). Prior to
231 enrichment analyses, 14,000 of the sheephead minnow probes were mapped to
232 human homologs using official gene symbols + aliases. Gene set enrichment
233 analysis (GSEA) was performed using 1000 permutations of the Kolmogorov-
234 Smirnov algorithm, and enrichment was set at $\alpha = 0.05$. Gene sets used in
235 the GSEA were based upon existing gene ontology knowledge as well as
236 Pathways Studio curated pathways. Sub-network enrichment analysis (SNEA)

was performed to identify affected gene networks in exposed liver tissues. Enrichment for SNEA was $\alpha = 0.05$. All enrichment analyses were performed using the function “highest magnitude of fold change, best p-value” for duplicate probes. This method utilizes all probes regardless of p-value to build distributions based on fold changes to statistically test for enrichment of processes. Because it leverages the entire data set, this method provides a robust estimate of exposure effects.

3. Results

3.1 Exposure results

Water quality parameters in experimental tanks remained relatively constant throughout the duration of exposures, with no differences among treatments. Both salinity and total ammonia were stable over the experiment at 15 ppt and < 0.1 mg/L in all tanks. Mean ($\pm$ SD) tank temperature was $26.8 (\pm 0.7)$ °C, mean pH $8.6 (\pm 0.1)$, and mean dissolved oxygen $5.8 (\pm 0.7)$ mg/L.

Stocks of HEWAF and CEWAF as well as all test solutions were analyzed for a suite of 50 PAH constituents (listed in Table S1). Stocks of HEWAF contained higher concentrations of PAHs than CEWAF stocks (Table 1); additionally tPAH50 concentrations were more stable in HEWAF stocks than CEWAF stocks (Table 1). For test solutions, analyses revealed low tPAH50 concentrations in both CEWAF and HEWAF treatments, with high treatments having concentrations of $5.98 \mu\text{g/L}$ for HEWAF solutions and $1.10 \mu\text{g/L}$ for CEWAF solutions, and low treatments having concentrations of $0.26 \mu\text{g/L}$ for

HEWAF solutions and 0.35 ug/L for CEWAF solutions. Interestingly, low CEWAF treatments exhibited a slightly higher tPAH50 concentration than low HEWAF treatments (a pattern opposite that of stock and high concentration test solutions); this effect is possibly due to measurement error at low concentrations. Measurements and limits of detection for each PAH analyte in all test solutions are listed in Table S1.

Dispersant concentrations for CEWAF and Corexit stocks, as well as all test solutions, were determined using measurement of DOSS content as an estimation of dispersant concentration. Estimates regarding the proportion of DOSS present in Corexit are varied, ranging from 21% (Ramirez et al. 2013) to 15% (Ferguson and Choyke, personal communication) of the total composition of Corexit 9500A™. Our data are more similar to the latter estimate, as DOSS concentrations for both Corexit and CEWAF stocks in this study were approximately 15% the total Corexit used in stock preparation (Table 1). However, for clarity's sake, henceforward we will report DOSS measurements when discussing dispersant concentrations in CEWAF and Corexit experiments. In test solutions, DOSS was not present in control or HEWAF treatments, while in CEWAF treatments, DOSS concentrations measured 10 µg/L for low CEWAF treatments (nominal concentration of 1 mg CEWAF/L) and 100 µg/L for high CEWAF treatments (nominal concentration of 10 mg CEWAF/L). Dispersant-only (Corexit) treatments had a measured DOSS concentration of 92 µg/L. While measured DOSS concentrations in our test solutions were slightly lower than expected based on estimates of 15 % DOSS (w/w) in Corexit, DOSS

concentrations were similar in high CEWAF and Corexit only treatments, thus allowing comparison of the effects of dispersant alone with the combined effects of dispersant and oil.

3.2 Fish health and mortality

Fish were monitored daily for signs of distress and none were noted. There was no mortality or evidence of distress in any of the treatments.

3.3 Gene expression and pathway analysis

Microarray analysis revealed a relatively small number of probes displaying significant (FDR, $p < 0.05$) changes in expression following exposure to experimental treatments. All expression data are provided in Appendix A, and have been deposited into NCBI Gene Expression Omnibus (GEO) database (accession number **GSE98348**). For 7-day exposures, 49 and 34 probes were affected by low and high CEWAF treatments, while 37 probes were affected by Corexit treatment; and 4 and 5 probes affected by the low and high HEWAF treatments, respectively (FDR $p < 0.05$) (Table 2). These results indicate that there was a subtle effect of PAH dose on gene expression at the low concentrations tested in this study, although the addition of dispersant to oil resulted in a marked increase in the number of genes affected. For 14-day exposures, CEWAF again had the greatest effect on gene expression (109 altered genes), followed by Corexit (44 altered genes) and HEWAF treatments (altered genes) (Table 2). A hierarchical cluster analysis was performed on the

subset of significantly altered probes to assess the similarity of gene expression profiles among treatment groups. This analysis revealed a clear similarity in expression profiles for groups with dispersant (CEWAF and Corexit) vs. groups without (HEWAF and control) for both 7- and 14-d exposures (Figure 1 A-D).

Pathways Studio 9 was used to identify gene sets and gene sub-networks significantly affected by treatment groups. Appendices B and C present all significantly affected pathways ($p < 0.05$) identified using Pathways Studio GSEA and SNEA bioinformatic approaches. Both GSEA and SNEA approaches represent methods to biologically characterize experimental changes in gene expression; GSEA analyzes gene sets in known functional categories that are over represented in the experimental set, while SNEA analyzes networks built around gene hubs determined through analysis of known relationships between genes, such as expression, binding and common pathways (Alexeyenko et al., 2012). The simultaneous use of both approaches provides a robust estimate of changes in biological function associated with experimentally altered gene expression.

The combined GSEA and SNEA results indicated that treatment composition (oil, dispersed oil, or dispersant), PAH concentration, and exposure length all affected biological pathways in the liver. Both analyses revealed a substantial number of altered pathways present in all treatment groups, including Corexit-only treatments (Table 2, Fig 2 C-D). However, the greatest number of altered pathways identified by both analyses was in the 7-d high HEWAF treatment, which had the highest PAH concentration of all the treatment groups

(Table 2, Fig. 2). Shorter exposure duration also had a marked effect on biological processes, as both analyses identified a higher number of altered pathways for 7-d than 14-d exposures (Table 2, Fig. 3 A-C). It is interesting to note that while high HEWAF treatments exhibited the greatest number of altered pathways, CEWAF and Corexit treatments resulted in the greatest number of differentially expressed genes (Table 2). These results are likely an artifact of HEWAF affected genes being present in multiple pathways and sub-networks.

Significantly affected sub-networks identified via SNEA (p -values < 0.05 and median changes greater or less than zero) were manually sorted into broad functional categories to gain further insight into biological processes associated with alteration of gene networks. When examined in this manner, sub-networks affected by each treatment converged into a suite of 17 biological themes shared among all treatments (Table 3). Sub-networks associated with immunity comprised the majority of pathways altered in each treatment, followed by those related to blood and circulation processes, transcription and translational processes and DNA replication and repair (Table 3).

Immune related sub-networks were impacted across all experimental treatment groups, with the greatest effects at the highest PAH concentration (high HEWAF) for both 7- and 14-d exposures (Table 3). Overall, there were a greater number of altered immune pathways for 7-d treatments than 14-d treatments. In 7-d treatments, the majority of altered immune sub-networks were down-regulated (Fig. 4). However, individuals from the 14-d treatment exhibited many up-regulated immune pathways, particularly in CEWAF and Corexit

treatments (Appendix D: Table S2). For all treatments, innate immune processes were most affected, with the greatest number of altered pathways related to macrophage function, followed by those related to granulocyte function (Table 4, Appendix D: Table S2). Although affected to a lesser extent, all treatments exhibited some perturbations in pathways related to adaptive immune processes, with pathways related to T cell function most affected, followed by those associated with B cell functioning (Appendix D: Table S2).

Pathways related to blood and circulation were also impacted across all treatment groups (Appendix D: Table S3). The 7-d high CEWAF treatment exhibited the greatest number of altered pathways in this category, and, like immunity, there was a greater number of altered pathways in 7-d exposures than 14-d exposures (Table 4). These pathways included sub-networks associated with blood clotting, cardiac function, endothelial cell function, erythrocyte function and degradation, and hematopoiesis (Appendix D: Table S3). Sub-networks associated with blood clotting and endothelial cells were consistently down-regulated, however sub-networks associated with other blood and circulation functions were both up- and down-regulated and exhibited no clear treatment related pattern.

4. Discussion

This study characterizes the molecular changes in adult sheepshead minnows associated with low concentration PAH exposures. Analytical chemistry determined that tPAH50 concentrations used in this experiment were

low in both HEWAF (0.26 and 5.98 $\mu\text{g/L}$ tPAH50) and CEWAF (0.35 and 1.10 $\mu\text{g/L}$ tPAH50) treatments. While low concentrations may explain the relatively modest effect on the overall number of genes differentially expressed, the concentrations used here are environmentally relevant given our knowledge of aqueous PAH concentrations in estuaries following the release event (Allan et al., 2013; Whitehead et al., 2012). Direct comparison of PAH concentrations from this study with those from field studies is difficult due to variation in analytical methods and number of analytes measured, however there is evidence that our exposure concentrations are representative of environmentally relevant, post-spill levels. For example, on August 30, 2010 approximately one month after the DWH wellhead was capped, Whitehead et al. (2012) measured 70 PAH analytes (tPAH70) totaling 0.49 $\mu\text{g/L}$ in water from an oiled site in Louisiana. The lowest concentrations measured in this study were tPAH50 0.26 $\mu\text{g/L}$ for low HEWAF and tPAH50 0.35 $\mu\text{g/L}$ for low CEWAF. Post-spill analysis of water chemistry at oiled sites also suggests that such low PAH concentrations may persist due to sediment re-suspension. Allan et al. (2013) found that while PAH concentrations in waters off Grand Bay, AL returned to pre-spill levels 11 months after the DWH spill (March 2011), they increased to peak-oil levels two months later (May 2011) in conjunction with increased wind and coastal clean-up activities. The authors hypothesized that this increase in water PAH content was due to sediment perturbation. Given the meager degradation of PAHs measured in coastal sediments three years post DWH spill (Turner et al., 2014), the

possibility of continued low-level water-borne PAH exposure due to re-suspension of contaminated sediments remains an environmental concern.

Our results illustrate a substantial molecular response to Corexit 9500™ exposure in adult sheepshead minnows. These effects are interesting, given that prior studies document low to moderate Corexit toxicity in fish. Acute toxicity studies have reported LC₅₀ values for Corexit 9500 ranging from 27 to 400 ppm (George-Ares and Clark, 2000; Hemmer et al., 2011). Additionally, in vitro tests demonstrated no endocrine disruptive effects and only weak cytotoxic effects for Corexit 9500 (Judson et al., 2010). Aside from these studies, the specific physiological and molecular effects of Corexit 9500A in fish are poorly characterized. Recent investigations have revealed cardiovascular impacts associated with Corexit exposure in rats (Krajnak et al., 2011), however, while our pathway analysis of gene sub-networks showed alteration of pathways related to circulation in treatment groups containing dispersant (Corexit and CEWAF treatments), treatments without dispersant (HEWAF) elicited similar responses (Table 2). Overall, the mechanisms driving fish molecular responses to dispersant exposure remain unclear.

The alteration of pathways related to immune function was, by far, the greatest effect noted for all treatment groups in this study. Hydrocarbons are well known to alter immune function, and several immune impacts related to DWH oil exposure have been noted in fish, such as external lesions and increased susceptibility to pathogens (Bayha et al., 2017; Murawski et al., 2014). Prior work on fish from other (non DWH related) PAH contaminated sites has related

such lesions to decreased macrophage function, particularly phagocytic and chemotactic activities (Reynaud and Deschaux, 2006; Seeley and Weeks-Perkins, 1991; Weeks et al., 1986; Weeks and Warinner, 1986). While the fish in this study exhibited no signs of external lesions or infections, results from our pathway analysis indicate a decrease in macrophage function for all treatment groups, suggesting suppressive effects on innate immunity similar to those seen in previous studies.

PAH exposure is also known to affect adaptive immunity via alterations in B and T cell functioning, however, studies investigating fish adaptive immune responses to DWH contamination reveal contradictory results. For example, Garcia et al. (2012) found up-regulation of the gene for immunoglobulin mu (IgM), the major systemic antibody found in fish in livers from *Fundulus grandis* sampled at DWH oil-impacted sites. Up-regulation of this gene is typically associated with an active immune response mediated by B cell activity. However, Baya et al. (2017) found that fish exposed to DWH oil exhibited down-regulation of IgM transcripts, indicating immune suppression. Song et al. (2012) also reported down-regulation of IgM related to heavy oil exposure. Our data support the latter results, as they indicate suppression of B cell activity with PAH exposure, which would likely be associated with decreased antibody secretion (Table D1). Unlike B cells, few studies have examined the effect of DWH oil on T cell function in fish; however, earlier work has described suppressed T cell proliferation associated with PAH exposure (Faisal and Huggett, 1993; Reynaud et al., 2003). Our pathway analysis indicates impacts to T cell function across all

treatments, including those without PAHs (Corexit groups). While T cell pathways were altered in each treatment group, the direction of change (up- or down-regulation) varied for each of the sub-networks, and exhibited no treatment related trend; therefore the biological outcomes associated with these changes is unclear. Although there is substantial evidence that oil and dispersant treatments alter the adaptive immune response of fish, it is apparent that additional research into the mechanisms mediating these changes is needed.

In addition to immune effects, our results demonstrate significant alteration of pathways related to blood and circulation. In particular, our analysis showed down-regulation of blood clotting pathways in all treatments. Some treatments also exhibited down-regulation of pathways associated with vascular endothelial cells, erythrocyte integrity, and cardiac function (Table D2). Overall, our pathway analysis results indicate general adverse effects on circulatory processes associated with DWH oil exposure, and are consistent with physiological results reported in the literature. Prior studies have noted pericardial edema and intracranial hemorrhage in developing fish exposed to DWH oil (Incardona et al., 2014, 2013; Mager et al., 2014), as well as hepatic vascular congestion in juvenile fish exposed DWH contaminated sediments (Brown-Peterson et al., 2016, 2015). These all represent effects elicited by decreases in blood clotting, compromised vascular endothelia, and erythrocyte damage such as those documented by our pathway analysis. Much of the previous work examining adverse effects of DWH oil exposure on circulatory processes in fish has focused on early life stages, however our results suggest the possibility that adult fish

may also be susceptible to the cardio-toxic and hemorrhagic effects of DWH oil exposure.

Overall, our findings provide an important link between field and laboratory studies examining the molecular effects of DWH oil exposure in estuarine fish. In particular, our data corroborate the findings of Whitehead and colleagues, who published a series of studies using microarrays and RNA seq to document alterations to gene expression and biological pathways in *Fundulis grandis* collected from oiled sites following the DWH spill (Dubansky et al., 2013; Garcia et al., 2012; Whitehead et al., 2012). Similar to our findings, each of these field studies revealed enrichment of pathways related to immunity. It is interesting to note that the fish from these field studies were collected at a heavily oiled site immediately after peak oil landfall, where tPAH70 concentrations were measured as 214 µg/L (Whitehead et al., 2012). The similarity between the data in prior field studies, and our data in an unrelated fish species exposed in the laboratory to much lower PAH concentrations, suggests that immune impacts are a conserved and pervasive effect of DWH oil exposure.

5. Conclusion

In this study, we report a substantial molecular response mounted in reaction to low concentration PAH and dispersant exposure in adult sheepshead minnows. These effects illustrate significant changes in gene expression as well as alteration of pathways governing key biological processes such as immune, cardiovascular, and blood related functions in all treatments, including

dispersant-only groups. Our results imply that the sub-lethal effects associated with dispersant exposure are greater than indicated by previous toxicity tests. Additionally, the environmental relevance of our test organism coupled with the low PAH doses used in this study suggest our results are representative of real-world responses occurring in resident fish at oil-impacted sites. The magnitude of these responses further implies that the impacts of oil exposure on fish health may be widespread and persistent at sites oiled during the DWH spill.

Acknowledgements: The authors thank B. Bailey, N. Brown-Peterson, B. M. Hedgepeth, J. A. Javonovich Avilar, A. A. Karels, and J. P. Lindsay for assistance in the laboratory. Funds for this work were provided as part of the Natural Resource Damage Assessment (NRDA) for the Deepwater Horizon Oil Spill. Culture and exposure of fish were carried out under University of Southern Mississippi IAUCUC protocol number 11092204. Data presented here are part of a larger toxicological database that is being generated as part of the Deepwater Horizon Natural Resource Damage Assessment. Therefore these data may be subject to additional analysis and interpretation, which may include interpretation in the context of additional data not presented here.

References

Alexeyenko, A., Lee, W., Pernemalm, M., Guegan, J., Dessen, P., Lazar, V.,

512 Lehtiö, J., Pawitan, Y., 2012. Network enrichment analysis: extension of
 513 gene-set enrichment analysis to gene networks. *BMC Bioinformatics* 13,
 514 226. doi:10.1186/1471-2105-13-226

515 Allan, S.E., Smith, B.W., Anderson, K. a, 2013. Impact of the Deepwater Horizon
 516 oil spill on bioavailable polycyclic aromatic hydrocarbons in Gulf of Mexico
 517 coastal waters. *Env. Sci Technol* 46, 2033–2039.
 518 doi:10.1021/es202942q.Impact

519 Bayha, K.M., Ortell, N., Ryan, C.N., Griffitt, K.J., Krasnec, M., Sena, J., Ramaraj,
 520 T., Takeshita, R., Mayer, G.D., Schilkey, F., Griffitt, R.J., 2017. Crude Oil
 521 Impairs Immune Function and Increases Susceptibility to Pathogenic
 522 Bacteria in Southern Flounder. *PLoS One* 1–21.

523 Boyd, T.J., Montgomery, M.T., Steele, J.K., Pohlman, J.W., Reatherford, S.R.,
 524 Spargo, B.J., Smith, D.C., 2005. Dissolved oxygen saturation controls PAH
 525 biodegradation in freshwater estuary sediments. *Microb. Ecol.* 49, 226–235.
 526 doi:10.1007/s00248-004-0279-0

527 Brette, F., Machado, B., Cros, C., Incardona, J.P., Scholz, N.L., Block, B.A.,
 528 2014. Excitation-Contraction Coupling in Fish 1681.

529 Brown-Peterson, N.J., Krasnec, M., Takeshita, R., Ryan, C.N., Griffitt, K.J., Lay,
 530 C., Mayer, G.D., Bayha, K.M., Hawkins, W.E., Lipton, I., Morris, J., Griffitt,
 531 R.J., 2015. A multiple endpoint analysis of the effects of chronic exposure to
 532 sediment contaminated with Deepwater Horizon oil on juvenile Southern
 533 flounder and their associated microbiomes. *Aquat. Toxicol.* 165, 197–209.
 534 doi:10.1016/j.aquatox.2015.06.001

535 Brown-Peterson, N.J., Krasnec, M.O., Lay, C.R., Morris, J.M., Griffitt, R.J., 2016.
536 Responses of juvenile southern flounder exposed to Deepwater Horizon oil-
537 contaminated sediments. *Environ. Toxicol. Chem.* 9999, 1–10.
538 doi:10.1002/etc.3629

539 Clement, T.P., Hayworth, J., Mulabagal, V., Yin, F., 2012. Research Brief-II
540 Impact of hurricane Isaac on mobilizing Deepwater Horizon oil spill residues
541 along Alabama’s coastline - a physicochemical characterization study 11.

542 Dubansky, B., Whitehead, A., Miller, J.T., Rice, C.D., Galvez, F., 2013.
543 Multitissue Molecular, Genomic, and Developmental Effects of the
544 Deepwater Horizon Oil Spill on Resident Gulf Killifish (*Fundulus grandis*).
545 *Environ. Sci. Technol.* 47, 5074–5082. doi:10.1021/es400458p

546 Faisal, M., Huggett, R.J., 1993. Effects of polycyclic aromatic hydrocarbons on
547 the lymphocyte mitogenic responses in spot, *Leiostomus xanthurus*. *Mar.*
548 *Environ. Res.* 35, 121–124. doi:10.1016/0141-1136(93)90024-T

549 Forth, H.P., Mitchelmore, C.L., Morris, J.M., Lipton, J., 2016. Characterization of
550 oil and water accommodated fractions used to conduct aquatic toxicity
551 testing in support of the *Deepwater Horizon* oil spill natural resource damage
552 assessment. *Environ. Toxicol. Chem.* doi:10.1002/etc.3672

553 Garcia, T.I., Shen, Y., Crawford, D., Oleksiak, M.F., Whitehead, A., Walter, R.B.,
554 2012. RNA-Seq reveals complex genetic response to deepwater horizon oil
555 release in *Fundulus grandis*. *BMC Genomics* 13, 474. doi:10.1186/1471-
556 2164-13-474

557 George-Ares, A., Clark, J.R., 2000. Aquatic toxicity of two Corexit® dispersants.

558 Chemosphere 40, 897–906. doi:10.1016/S0045-6535(99)00498-1

559 Hambrick, G.A., Delaune, R.D., Patrick, W.H., 1980. Effect of Estuarine

560 Sediment pH and Oxidation-Reduction Potential on Microbial Hydrocarbon

561 Degradation. Appl. Environ. Microbiol.

562 Hedgpeth, B.M., Griffitt, R.J., 2016. Simultaneous exposure to chronic hypoxia

563 and dissolved polycyclic aromatic hydrocarbons results in reduced egg

564 production and larval survival in the sheepshead minnow (*Cyprinodon*

565 *variegatus*). Environ. Toxicol. Chem. 35, 645–651. doi:10.1002/etc.3207

566 Hemmer, M.J., Barron, M.G., Greene, R.M., 2011. Comparative toxicity of eight

567 oil dispersants, Louisiana sweet crude oil (LSC), and chemically dispersed

568 LSC to two aquatic test species. Environ. Toxicol. Chem. 30, 2244–2252.

569 doi:10.1002/etc.619

570 Incardona, J.P., Gardner, L.D., Linbo, T.L., Brown, T.L., Esbaugh, A.J., Mager,

571 E.M., Stieglitz, J.D., French, B.L., Labenia, J.S., Laetz, C. a, Tagal, M.,

572 Sloan, C. a, Elizur, A., Benetti, D.D., Grosell, M., Block, B. a, Scholz, N.L.,

573 2014. Deepwater Horizon crude oil impacts the developing hearts of large

574 predatory pelagic fish. Proc. Natl. Acad. Sci. U. S. A. 111, E1510-8.

575 doi:10.1073/pnas.1320950111

576 Incardona, J.P., Swarts, T.L., Edmunds, R.C., Linbo, T.L., Aquilina-Beck, A.,

577 Sloan, C.A., Gardner, L.D., Block, B.A., Scholz, N.L., 2013. Exxon Valdez to

578 Deepwater Horizon: Comparable toxicity of both crude oils to fish early life

579 stages. Aquat. Toxicol. 142–143, 303–316.

580 doi:10.1016/j.aquatox.2013.08.011

581 Judson, R.S., Martin, M.T., Reif, D.M., Houck, K.A., Knudsen, T.B., Rotroff, D.M.,
 582 Xia, M., Sakamuru, S., Huang, R., Shinn, P., Austin, C.P., Kavlock, R.J., Dix,
 583 D.J., 2010. Analysis of eight oil spill dispersants using rapid, in vitro tests for
 584 endocrine and other biological activity. *Environ. Sci. Technol.* 44, 5979–
 585 5985. doi:10.1021/es102150z

586 Krajnak, K., Kan, H., Waugh, S., Miller, G.R., Johnson, C., Roberts, J.R.,
 587 Goldsmith, W.T., Jackson, M., McKinney, W., Frazer, D., Kashon, M.L.,
 588 Castranova, V., 2011. Acute Effects of COREXIT EC9500A on
 589 Cardiovascular Functions in Rats. *J. Toxicol. Environ. Heal. Part A* 74,
 590 1397–1404. doi:10.1080/15287394.2011.606795

591 Kujawinski, E.B., Kido Soule, M.C., Valentine, D.L., Boysen, A.K., Longnecker,
 592 K., Redmond, M.C., 2011. Fate of dispersants associated with the
 593 Deepwater Horizon oil spill. *Environ. Sci. Technol.* 45, 1298–1306.
 594 doi:10.1021/es103838p

595 Mager, E.M., Esbaugh, A.J., Stieglitz, J.D., Hoenig, R., Bodinier, C., Incardona,
 596 J.P., Scholz, N.L., Benetti, D.D., Grosell, M., 2014. Acute embryonic or
 597 juvenile exposure to deepwater horizon crude oil impairs the swimming
 598 performance of mahi-mahi (*Coryphaena hippurus*). *Environ. Sci. Technol.*
 599 48, 7053–7061. doi:10.1021/es501628k

600 Manning, C., Schesny, A., Hawkins, W., Barnes, D., Barnes, C., Walker, W.,
 601 1999. Exposure methodologies and systems for long-term chemical
 602 carcinogenicity studies with small fish species. *Toxicol. Methods* 9, 201–217.
 603 doi:10.1080/105172399242708

604 McNutt, M.K., Camilli, R., Crone, T.J., Guthrie, G.D., Hsieh, P.A., Ryerson, T.B.,
 605 Savas, O., Shaffer, F., 2012. Review of flow rate estimates of the Deepwater
 606 Horizon oil spill. *Proc. Natl. Acad. Sci. U. S. A.* 109, 20260–7.
 607 doi:10.1073/pnas.1112139108

608 Michel, J., Owens, E.H., Zengel, S., Graham, A., Nixon, Z., Allard, T., Holton, W.,
 609 Reimer, P.D., Lamarche, A., White, M., Rutherford, N., Childs, C., Mauseth,
 610 G., Challenger, G., Taylor, E., 2013. Extent and Degree of Shoreline Oiling:
 611 Deepwater Horizon Oil Spill, Gulf of Mexico, USA. *PLoS One* 8, 1–9.
 612 doi:10.1371/journal.pone.0065087

613 Murawski, S.A., Hogarth, W.T., Peebles, E.B., Barbeiri, L., 2014. Prevalence of
 614 External Skin Lesions and Polycyclic Aromatic Hydrocarbon Concentrations
 615 in Gulf of Mexico Fishes, Post-Deepwater Horizon. *Trans. Am. Fish. Soc.*
 616 143, 1084–1097. doi:10.1080/00028487.2014.911205

617 Nixon, Z., Zengel, S., Baker, M., Steinhoff, M., Fricano, G., Rouhani, S., Michel,
 618 J., 2016. Shoreline oiling from the Deepwater Horizon oil spill. *Mar. Pollut.*
 619 *Bull.* 107, 170–178. doi:10.1016/j.marpolbul.2016.04.003

620 Oliveros, J. C., 2007-2015. Venny. An interactive tool for comparing lists with
 621 Venn's diagrams. <http://bioinfogp.cnb.csic.es/tools/venny/index.html>

622 Pilcher, W., Miles, S., Tang, S., Mayer, G., Whitehead, A., 2014. Genomic and
 623 genotoxic responses to controlled weathered-Oil exposures confirm and
 624 extend field studies on impacts of the deepwater horizon oil spill on native
 625 killifish. *PLoS One* 9. doi:10.1371/journal.pone.0106351

626 Ramirez, C.E., Batchu, S.R., Gardinali, P.R., 2013. High sensitivity liquid

627 chromatography tandem mass spectrometric methods for the analysis of
 628 dioctyl sulfosuccinate in different stages of an oil spill response monitoring
 629 effort. *Anal. Bioanal. Chem.* 405, 4167–4175. doi:10.1007/s00216-013-6841-
 630 1
 631 Reynaud, S., Deschaux, P., 2006. The effects of polycyclic aromatic
 632 hydrocarbons on the immune system of fish: A review. *Aquat. Toxicol.* 77,
 633 229–238. doi:10.1016/j.aquatox.2005.10.018
 634 Reynaud, S., Duchiron, C., Deschaux, P., 2003. 3-Methylcholanthrene inhibits
 635 lymphocyte proliferation and increases intracellular calcium levels in
 636 common carp (*Cyprinus carpio* L). *Aquat. Toxicol.* 63, 319–331.
 637 doi:10.1016/S0166-445X(02)00188-1
 638 Seeley, K.R., Weeks-Perkins, B.A., 1991. Altered Phagocytic Activity of
 639 Macrophages in Oyster Toadfish from a Highly Polluted Subestuary. *J.*
 640 *Aquat. Anim. Health* 3, 224–227. doi:10.1577/1548-
 641 8667(1991)003<0224:APAOMI>2.3.CO;2
 642 Song, J.Y., Ohta, S., Nakayama, K., Murakami, Y., Kitamura, S.I., 2012. A time-
 643 course study of immune response in Japanese flounder *Paralichthys*
 644 *olivaceus* exposed to heavy oil. *Environ. Sci. Pollut. Res.* 19, 2300–2304.
 645 doi:10.1007/s11356-012-0737-z
 646 Turner, R.E., Overton, E.B., Meyer, B.M., Miles, M.S., Hooper-Bui, L., 2014.
 647 Changes in the concentration and relative abundance of alkanes and PAHs
 648 from the Deepwater Horizon oiling of coastal marshes. *Mar. Pollut. Bull.* 86,
 649 291–297. doi:10.1016/j.marpolbul.2014.07.003

Weeks, B.A., Warinner, J.E., 1986. Functional evaluation of macrophages in fish from a polluted estuary. *Vet. Immunol. Immunopathol.* 12, 313–320. doi:10.1016/0165-2427(86)90135-2

Weeks, B.A., Warinner, J.E., Mason, P.L., McGinnis, D.S., 1986. Influence of toxic chemicals on the chemotactic response of fish macrophages. *J. Fish Biol.* 28, 653–658. doi:10.1111/j.1095-8649.1986.tb05200.x

Whitehead, A., Dubansky, B., Bodinier, C., Garcia, T.I., Miles, S., Pilley, C., Raghunathan, V., Roach, J.L., Walker, N., Walter, R.B., Rice, C.D., Galvez, F., 2012. Genomic and physiological footprint of the Deepwater Horizon oil spill on resident marsh fishes. *Proc. Natl. Acad. Sci. U. S. A.* 109, 20298–302. doi:10.1073/pnas.1109545108

Xu, E.G., Mager, E.M., Grosell, M., Pasparakis, C., Schlenker, L.S., Stieglitz, J.D., Benetti, D., Hazard, E.S., Courtney, S.M., Diamante, G., Freitas, J., Hardiman, G., Schlenk, D., 2016. Time- and Oil-Dependent Transcriptomic and Physiological Responses to *Deepwater Horizon* Oil in Mahi-Mahi (*Coryphaena hippurus*) Embryos and Larvae. *Environ. Sci. Technol.* 50, 7842–7851. doi:10.1021/acs.est.6b02205

Legends

Table 1: tPAH50 and DOSS concentrations ($\pm$ SD) for stock solutions prepared throughout the course of the experiment (n=9 samples for each stock).

Table 2: Summary of differentially expressed genes (DEGs) identified by one-

way ANOVA, differentially expressed genes corrected using a False Discovery Rate of 5% (DEGs FDR), and altered pathways identified via sub network enrichment analysis (SNEA) for each treatment. Pathways reported include those with a median fold change greater or less than 0. All analyses are relative to 15 ppt sea water control.

Table 3: Biological processes or themes categorizing altered sub-networks. Only sub-networks with mean changes greater or less than zero were included in analysis.

Table 4: Common immune sub-networks significantly affected in the liver of sheepshead minnow.

Figure 1: Effects of HEWAF, CEWAF, and COREXIT exposure on differential gene expression in sheepshead minnow liver. (A-B): Venn diagrams showing relatedness among genes differentially expressed in 7 day (A) and 14 day (B) exposures. (C-D): Hierarchical clustering analysis of DEGs from 7 day (C) and 14 day (D) exposures. Note that for both groups gene expression profiles of CEWAF treatments cluster with COREXIT, while HEWAF treatments cluster with control.

Figure 2: Effects of HEWAF, CEWAF, and COREXIT exposure on pathway sub-networks as determined by SNEA. (A-B): Effect of PAH doses on sub-networks

in (A) CEWAF and (B) HEWAF treatments. (C-D): Effect of high PAH dose on sub-networks in (C) 7 day and (D) 14 exposures.

Figure 3: Effect of exposure time on sub-networks for high PAH doses of (A) CEWAF, (B) HEWAF, and (C) COREXIT.

Figure 4: Representative innate (A) and adaptive (B) immune pathways significantly affected by 7 d high dose HEWAF treatment. Green indicates down-regulation of genes and pathways; red indicates up-regulation. A: Neutrophil degranulation via FPR 1 signaling in 7 d High HEWAF treatments. B: Th2 cell differentiation in 7 d High HEWAF treatments. Key to entity abbreviations can be found in Appendix E.

740 **Table 1**

Solution	tPAH50	DOSS
Control	0.04 ± 0.08	1.12 ± 0.63
Corexit	N/A	148555.56 ± 25617.92
HEWAF	2467.39 ± 199.91	N/A
CEWAF	535.84 ± 297.00	14555.56 ± 1236.03

741

742

743

744 **Table 2**

Treatment	Exposure Duration	DEGs	DEGs (FDR)	Pathways GSEA	Pathways SNEA
Low HEWAF	7 days	850	4	121	199
High HEWAF	7 days	730	5	238	337
Low CEWAF	7 days	1318	49	107	231
High CEWAF	7 days	1157	34	89	284
Corexit	7 days	1207	37	83	208
High HEWAF	14 days	508	7	63	162
High CEWAF	14 days	1490	109	62	180
Corexit	14 days	1488	44	30	119

745 **Table 3**

Biological process	Low HEWAF 7 days	High HEWAF 7 days	Low CEWAF 7 days	High CEWAF 7 days	Corexit 7 days	High HEWAF 14 days	High CEWAF 14 days	Corexit 14 days
Immunity	55	150	60	61	108	6	42	40
Blood and circulation	22	26	16	46	10	16	20	16
DNA replication / repair	6	39	24	42	12	2	3	1
Transcription / translation	11	1	23	10	13	10	22	9
Energy metabolism	13	13	15	13	3	9	8	6
Lipid processing	13	13	13	13	4	10	6	3
Brain function	9	5	9	5	4	6	1	3
Digestion	3	8	8	9	6	3	4	3
Cell death / damage	12	14	7	13	0	2	6	2
Protein processing	4	5	7	8	3	1	3	5
Muscle function	4	4	6	3	3	2	0	3
Connective tissue	5	7	5	4	4	1	4	1
Mitochondrial function	0	4	5	2	1	0	0	2
Liver function	4	4	4	3	0	2	2	0
Xenobiotic clearance	6	8	3	6	0	2	7	4
Reproduction	3	7	2	11	2	2	4	2
Kidney function	2	2	1	12	2	0	2	2

746 **Table 4**

7 day exposure						
Biological process	Common cell process Gene set seed	Low HEWAF	High HEWAF	Low CEWAF	High CEWAF	Corexit
		Median change				
Immunity	Macrophage infiltration	-1.11	-1.14	1.06	-1.05	-1.06
	Macrophage apoptosis	-1.08	-1.04	-1.06	1.03	-1.04
	Macrophage polarity	-1/04	-1.19	-1.08	-1.08	-1.15
	Macrophage activation	-1.01	-1.14	-1.12	-1.08	-1.15
14 day exposure						
Biological process	Common cell process Gene set seed	High HEWAF		High CEWAF		Corexit
		Median change				
Immunity	Neutrophil homeostasis	-1.21		-1.39		-1.44
	Encapsidation	-1.30		-1.34		-1.11
	Plasmacytoid dendritic cell differentiation	-1.19		-1.20		-1.11
	Immune complex clearance	-1.35		-1.12		-1.01
	Disease resistance	-1.01		1.04		1.01

747

Figure 1

751 **Figure 2**
752

753

754
755
756

Figure 3

757

Figure 4