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MOLECULAR RESPONSES TO DEEPWATER HORIZON OIL SPILL CONTAMINANTS IN ESTUARINE FISH

by

Elizabeth R. Jones

A Dissertation
Submitted to the Graduate School,
the College of Arts and Sciences
and the School of Ocean Science and Engineering
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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ABSTRACT

The 2010 Deepwater Horizon (DWH) oil spill resulted in the oiling of approximately 2100 km of shoreline. During that time, resident organisms had to contend with the effects of multiple contaminants and suboptimal environmental conditions. This research examines the molecular effects of DWH oil spill contaminants in estuarine fish (*Cyprinodon variegatus* and *Fundulus grandis*) across multiple life stages, contaminant concentrations, and in conjunction with environmental stressors.

Our results indicate that:

1. Oil elicits substantial transcriptional effects across life stages in *C. variegatus*. In adults, exposure to low concentrations of oil and dispersant results in transcriptional effects related to immunity, circulation processes, and DNA replication and repair. In early life-stage *C. variegatus*, oil exposure elicits different effects across developmental windows. Embryos mount a muted transcriptional response to oil, while larval stages mount a larger response dominated by dysregulation of transcription related to cholesterol biosynthesis, cardiac development, and immunity. In particular, the magnitude of transcriptional response in larval stages suggests examination of larvae may provide the most sensitive assessment of oil spill impacts.

2. Oil elicits differing transcriptional effects in ecologically similar species. We compared oil-induced transcription in larval *C. variegatus* and *F. grandis* and found distinct gene expression patterns in the two species, including opposing expression of genes involved in cholesterol biosynthesis. These results suggest that nuanced differences in molecular effects exist among fish, and should be considered when predicting the ecosystem-level effects of oil contamination.

3. Oil elicits altered DNA methylation patterns in larval *C. variegatus*. We found that oil exposure resulted in altered methylation at several genomic loci, and that simultaneous exposure to oil and hypoxia results in much greater effects to methylation than oil alone. We also determined that differentially methylated loci were correlated with differentially expressed genes, suggesting that altered methylation influences transcriptional responses following oil and hypoxia exposure.

Overall, these results suggest that the sub-lethal impacts of oil spill contaminants constitute a substantial insult to cellular and molecular functions in estuarine fish, and imply that effects to fish health following oil spills could be more widespread and persistent than previously thought.

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I would like to thank Dr. Joe Griffitt for his help and guidance throughout this process. When I found myself without an advisor, Joe didn't hesitate to take me on and has worked hard to enable my success. During my tenure at GCRL, Joe allowed me to develop my research ideas and provided me the resources to follow through with them. He has mentored me in every aspect of an academic career by including me in research, grant writing, and mentoring opportunities. I feel that he has worked to cultivate me as a colleague, and I owe him a huge debt of gratitude.

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Throughout this project I benefited from association with a number of collaborators from other institutions, including Dr. Dana Wetzel (Mote Marine Laboratory) who assisted me with analytical chemistry, Dr. Christopher Martynuik (University of Florida) who assisted with microarray hybridizations, and Dr. Marisol Sepulveda (Purdue University), who collaborated with our lab on several projects.

This work would not have been possible without the substantial help I received from members of the Griffitt lab (past and present): Dr. Maria Rodgers, Danielle Simning, Laura Moncrieff, Jeremy Johnson, and Dr. Natalie Ortell. They have been on call during experiments, acted as sounding boards, and even fed me from time to time. I would not have finished without them.

Finally, I would like to acknowledge the funding agencies who supported this work: The Gulf of Mexico Research Initiative and the Natural Resource Damage Assessment. Thank you.

DEDICATION

I dedicate this work to my family: my husband, Stephen Jones, and my children, Austin, Sara, and Anna Jones. You have all sacrificed so much to help me accomplish my goals. This work is as much yours as it is mine.

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A NOTE ON GENE SYMBOLS

Throughout this document I have followed conventional rules regarding use of protein and gene symbols. In mammals and fish, protein names are abbreviated in CAPITAL letters. For genes, mammalian orthologs are abbreviated using *ITALICIZED CAPITAL* letters, while fish genes are indicated by *lower case italicized* abbreviations. For example, cytochrome p450 1A is abbreviated as:

CYP1A	Protein (mammals and fish)
CYP1A	Mammalian gene
cyp1a	Fish gene

CHAPTER I – GENERAL INTRODUCTION

1.1 Oil spill threats to coastal ecosystems

Coastal habitats are among the most ecologically and economically valuable ecosystems worldwide, yet estimates suggest that nearly 50% of salt marshes are lost or degraded globally (Barbier et al. 2011). Petroleum contamination from oil spills is one of the many threats to the health of these regions (Harfoot et al. 2018). A recent example of such contamination is the Deepwater Horizon (DWH) spill, which released 4.9 million barrels of oil into the Gulf of Mexico from April through September of 2010 (McNutt et al. 2012). Attempts to mitigate the spread of oil included skimming, burning, and the use of dispersants to break up oil droplets (Kujawinski et al. 2011); however, despite these efforts, oil reached approximately 2100 km of shoreline from Florida to Texas (Michel et al. 2013; Nixon et al. 2016). The timing of shoreline oiling during spring and summer months coincided with the spawning period of many estuarine fish, thus vulnerable early life stages of resident organisms were exposed to oil contaminants (Talbot and Able 1984; Rooker et al. 2013; Dubansky et al. 2013).

Oil is known to elicit many toxic effects in fish ranging from immune dysfunction and hepatic damage in adult fish, to mortality, reduced hatch, and teratogenic effects in developing fish (Murawski et al. 2014; Incardona et al. 2015; Brown-Peterson et al. 2015; Xu et al. 2016; Bayha et al. 2017; Sørhus et al. 2017; Rodgers et al. 2018b, 2018a). These effects are thought to contribute to reduced survivorship potentially leading to population-level effects, and have been implicated in the collapse of the pink salmon (*Oncorhynchus gorbuscha*) fishery following the Exxon Valdez spill in 1989 (Rice et al.

2001). While multiple studies have characterized the phenotypic outcomes of oil exposure, many of the molecular events underlying these effects are poorly understood. Following the DWH spill, the use of transcriptomic studies have been widely employed to elucidate the mechanisms by which oil elicits observed effects. This approach is particularly relevant given the process by which oil contaminants are metabolized.

1.2 Metabolism of polycyclic aromatic hydrocarbons (PAHs)

Crude oil is composed of a hydrocarbon mixture including PAHs, alkanes and alkenes. Of these, PAHs are generally considered most toxic (Carls et al. 2008). PAH metabolism is mediated by the aryl hydrocarbon receptor (AHR), a ligand-binding transcription factor with a high affinity for PAH and polychlorinated biphenyl ligands (Billiard et al. 2002). Ligand binding activates the AHR, which then translocates to the nucleus and forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT) (Hankinson 2002) (Figure 1.1). This heterodimer binds to xenobiotic response elements (XREs) in the promoter of target genes to modulate their transcription (Nebert et al. 2000; Dalton et al. 2002; Hankinson 2002). Many genes in the AHR gene battery are those involved in xenobiotic clearance, such as cytochrome p450 1A (CYP1A) (Figure 1.1) (Nebert et al. 2000; Dalton et al. 2002). Induction of *CYP1A* transcription is such a ubiquitous action of AHR activation that its upregulation is considered a classic biomarker of contaminant exposure (Goksøyr 1995).

CYP1A belongs to a class of enzymes that commonly perform monooxygenase reactions, converting non-polar PAH substrates into more polar metabolites (Poland et al. 1974). This primes substrates for conjugation with other functional groups to allow their

secretion in bile or urine (Buhler and Williams 1988). While rendering PAHs more soluble for clearance, transformation via CYP1A can also result in the formation of highly reactive epoxide intermediates, which frequently bind to adenine or guanine residues in DNA to form DNA adducts (Figure 1.2) (Miller and Ramos 2001; Xue and Warshawsky 2005; Ewa and Danuta 2017). These adducts represent permanent mutations that disrupt replication and translation and are highly carcinogenic (Ewa and Danuta 2017). Therefore, biotransformation of PAH substrates by CYP1A is often said to activate their toxicity.

The metabolism of PAHs can also enhance their toxicity through the generation of reactive oxygen species (ROS) (Figure 1.2). This occurs when oxygen is incompletely reduced during biotransformation, resulting in superoxide (O_2^-) or peroxide molecules (e.g. H_2O_2) (Miller and Ramos 2001). These electron scavenging molecules cause oxidative stress, including genotoxicity in the form of base modifications and DNA strand breakage (Sharma et al. 2014). Additionally, oxidative stress initiates transcription of a battery of genes involved in redox-homeostasis via activation of anti-oxidant response elements (AREs), which are cis-acting DNA regulatory elements that function similarly to XREs (Nebert et al. 2000; Miller and Ramos 2001).

Because the process of PAH biotransformation and clearance activates transcriptional machinery and enhances genotoxicity, many studies have used transcriptomic profiling to examine the effects of oil exposure. For example, transcriptional studies have been used to elucidate oil-induced cardiotoxicity and immune dysfunction (Xu et al. 2016, 2017; Bayha et al. 2017; Rodgers et al. 2018b), as well as to investigate the interaction of oil with environmental stressors such as hypoxia or elevated

temperature (Serafin 2017; Simning 2017). Despite the breadth of transcriptional studies examining oil effects, many questions remain about how oil modulates gene expression in various contexts. In this study we used transcriptional profiling to address several questions regarding the molecular effects of oil contamination:

Chapter 2 primary question: *What are the transcriptional effects of environmentally relevant oil and dispersant concentrations in adult estuarine fish?*

1. Do these effects change over time?
2. Does the combination of oil and dispersant elicit different effects than either contaminant alone?

Chapter 3 primary question: *What are the transcriptional effects of oil contamination in developing estuarine fish?*

1. Do these effects changes across developmental stages?
2. Do these effects differ in ecologically similar species?

To address these questions we employed two techniques to measure global transcriptional patterns: microarrays and RNA sequencing (RNA seq).

1.3 Transcriptional profiling methods

Microarrays are a hybridization-based technique in which RNA is extracted from samples, converted to complementary DNA (cDNA), then labeled with a fluorescent dye (Figure 2.3a). Samples are then washed over a glass slide coated with single-strand DNA

(ssDNA) probes and allowed to hybridize. The intensity of fluorescence at a particular slide location is proportional to the number of RNA transcripts hybridizing to that ssDNA probe, thus allowing measurement of gene expression (Govindarajan et al. 2012). Microarrays are a well-established technology that until recently, were the most cost-effective option for measuring global transcriptional profiles (Mantione et al. 2014). Analysis of microarray data requires minimal computing resources, as data files are typically on the order of < 1GB, and software analytical tools are widely available (Mantione et al. 2014). However, microarrays require extensive *a priori* knowledge of genomic information to generate probe sets and therefore are of limited use in non-model systems (Wang et al. 2009b; Mantione et al. 2014). Additionally, measurement of fluorescent intensities provides lower resolution measurements than alternative methods, like RNA seq (Mantione et al. 2014). As costs for high-throughput sequencing continue to decrease, RNA seq is becoming a more widely-used method for transcriptional profiling (Pedro De Magalhães et al. 2010; Sboner et al. 2011).

Like microarrays, RNA seq involves isolation of mRNA from samples of interest. Samples are converted to cDNA, fragmented, ligated to adaptors, then sequenced on a high-throughput platform to generate short reads for one or both ends (Wang et al. 2009b) (Figure 3b). Data can be analyzed using a reference guided approach, in which reads are aligned to a previously sequenced genome or transcriptome, or a *de novo* approach, in which similar reads are binned together for analysis (Wang et al. 2009a). This method has several advantages over conventional microarrays. Read counts provide higher resolution measurements than intensity data, and direct sequencing allows detection of splice variants, rare transcripts, and novel sequences (Pedro De Magalhães et

al. 2010; Mantione et al. 2014). Additionally, there is no need for existing genomic information, rendering this technique useful for study of non-model organisms (Wang et al. 2009a). However, because library preparation involves fragmenting of mRNA, RNA seq data can be biased towards longer transcripts (Fang and Cui 2011; Mantione et al. 2014). Additionally, library preparation may require amplification prior to sequencing, which can introduce confounding PCR artifacts into data (Fang and Cui 2011). RNA seq produces an abundance of data, requiring substantial computing resources and bioinformatic knowledge for analysis (Sboner et al. 2011; Muir et al. 2016). However, despite these drawbacks, the advantages associated with RNA seq have rendered it the method of choice for transcriptional profiling.

1.4 DNA methylation and gene expression

As previously mentioned, oil exposure induces significant changes to gene expression in fish across life stages. These changes are often thought to represent transient responses that aid in the clearance of xenobiotic toxicants and enable a return to homeostasis. However, a growing body of literature suggests oil exposure can induce epigenetic modifications, which may alter gene expression patterns and could have long-term impacts on the health and survival of exposed animals (Corrales et al. 2014b; Mirbahai and Chipman 2014a; Vignet et al. 2015).

DNA methylation is the best studied of the epigenetic marks. It involves the addition of a methyl group to the 5' carbon of cytosine via the action of DNA methyltransferase (DNMT) enzymes, and most often occurs at cytosine/guanine (CpG) dinucleotide motifs (Labbé et al. 2015). In vertebrates, DNA methylation is common,

occurring in 5% of all mammalian and avian cytosine residues, and in 10% of the cytosine residues in fish and amphibians (Field et al. 2004). Typically methylation is evenly distributed throughout the genome, however one exception is in the methylation of CpG dinucleotides located in the promoter region of genes. These dinucleotides often occur in clusters, called CpG islands, and are usually unmethylated (Jones and Takai 2001, Vandegehuchte and Janssen 2011). Unmethylated CpG islands are targets for transcription factors and associated with active gene expression; conversely, methylation of CpG islands is known to be associated with gene silencing (Robertson and Jones, 2000, Jones and Takai, 2001).

Methylation patterns are mitotically heritable and therefore can constitute permanent changes in gene expression; as such, DNA methylation is important in modulating tissue differentiation and development (Dolinoy et al. 2007). Environmental perturbation and exposure to chemicals can induce changes in normal DNA methylation patterns, and abnormal methylation profiles are often correlated with disease (Head et al. 2012). In particular, both promoter hyper-methylation and global genomic hypo-methylation are associated with tumorigenesis in mammalian and fish models (Mirbahai et al. 2011; Mirbahai and Chipman 2014). Recent studies show that exposure to benzo[a]pyrene, a PAH, can alter DNA methylation patterns in fish, including methylation changes in the promoters of genes as well as global genomic hypo-methylation (Fang et al. 2013; Corrales et al. 2014a). However, there is little information regarding how a mixture of PAHs, such as that present in oil, might impact DNA methylation in fish.

In this study we address several questions related to the alteration of DNA methylation following oil exposure:

Chapter 4 primary question: *Does oil exposure impact transcription of dnmts in developing estuarine fish?*

1. Is *dnmt* transcription associated with promoter methylation of key AHR pathway genes?

2. Are promoter methylation patterns sustained into adulthood?

Chapter 5 primary question: *Does oil alter genome-wide methylation patterns in developing estuarine fish?*

1. Is altered methylation correlated to gene transcription?

To answer questions related to measurement of DNA methylation, we employed two methods: sodium bisulfite sequencing and methylated DNA immunoprecipitation followed by high throughput sequencing (MeDIP seq.).

1.5 Methods for quantifying DNA methylation

To measure methylation changes in promoter regions, we used bisulfite conversion followed by PCR. This method is based on the premise that treatment of DNA with sodium bisulfite results in deamination of unmethylated cytosine residues, converting them to uracil, while methylated cytosines remain unchanged (Figure 1.4a) (Fraga and Esteller 2002). Following treatment, PCR primers flanking regions of interest can be designed; PCR amplification will result in incorporation of thymine residues at converted loci and incorporation of cytosine residues at methylated loci. PCR products

can then be sequenced to identify single nucleotide polymorphism (SNPs) resulting from bisulfite conversion.

In the context of this study, we employed bisulfite conversion as described to identify methylation in the promoter of the aryl hydrocarbon receptor repressor (AHRR). However, this approach can also be used to evaluate genome wide methylation changes. In whole genome bisulfite sequencing (WGBS), DNA is bisulfite converted, fragmented, and sequenced using high throughput methods (Xu et al. 2018). While this method is considered the "gold standard" for examining genome wide methylation changes, it can be cost prohibitive and generates quantities of data requiring substantial computational resources. A second method, reduced representation bisulfite sequencing (RRBS), uses restriction enzymes recognizing CpG cut sites to fragment DNA; therefore regions with high CpG densities will result in smaller fragments (Fouse et al. 2010). DNA is then size selected, bisulfite converted, and sequenced. While lower in cost and computationally less intensive than WGBS, this method is biased towards CpG islands, with reduced CpG coverage genome wide (Xu et al. 2018). Additionally, analysis of bisulfite converted sequence necessitates a reference guided approach in which read data is aligned to a sequenced genome that has been "bisulfite converted" *in silico*. This reduces sequence complexity in both reference and sample data, resulting in poor mapping efficiencies, and may be of particular concern in non-model systems with poor genome annotation (Otto et al. 2012; Aluru et al. 2018).

Due to the aforementioned constraints, we opted to use methylated DNA immunoprecipitation coupled with high throughput sequencing (MeDIP seq) to examine genome-wide methylation changes following oil-exposure (Figure 1.4b). In this

approach, DNA is fragmented to a range between 200-500 bp, then immunoprecipitated using an antibody against 5-methyl cytosine (5meC) (Fouse et al. 2010; Xu et al. 2018). This results in a pool of DNA enriched for methylated regions which can then be sequenced. This approach has been shown to result in good genomic CpG coverage at a much lower cost than WGBS (Harris et al. 2010). Additionally, because MeDIP sequence data require no altering, efficiency of read mapping to the reference genome is not compromised, allowing enhanced identification of loci associated with methylation changes that can then be used in functional analyses.

1.6 Model organisms

The overarching goal of this research was to identify molecular responses to oil exposure in estuarine fish. To examine these effects, we used the sheepshead minnow (*Cyprinodon variegatus*) in laboratory exposures. *C. variegatus* is a resident, non-migratory species inhabiting northern Gulf of Mexico estuaries (Raimondo et al. 2015). These fish are well adapted to broad fluctuations in environmental parameters, rendering them easily reared in a laboratory environment (Nordlie 2006). For this reason, they have been widely used in toxicology testing, and there is an abundance of literature regarding their physiology and genomic sequences (Hansen et al. 1975; Karels et al. 2003; Pirooznia et al. 2010). In the laboratory they are continuous breeders, however, in estuarine environments spawning occurs May-September, coinciding with the oiling of estuaries during the DWH event (Talbot and Able 1984).

To examine molecular responses to oil exposure across two ecologically similar species, we compared transcriptional responses of *Fundulus grandis* with *C. variegatus*.

Like *C. variegatus*, *F. grandis* is a native of northern Gulf of Mexico estuaries that spawns during the spring and summer, and is easily reared in laboratory conditions (Nordlie 2006; Brown et al. 2011, 2012). While there is no sequenced genome available for *F. grandis*, there is a breadth of genomic and physiological data about its congener, *F. heteroclitus* (Garcia et al. 2012). Our use of resident species in laboratory bioassays renders this work translatable to effects that may be observed in estuaries following oil spill events.

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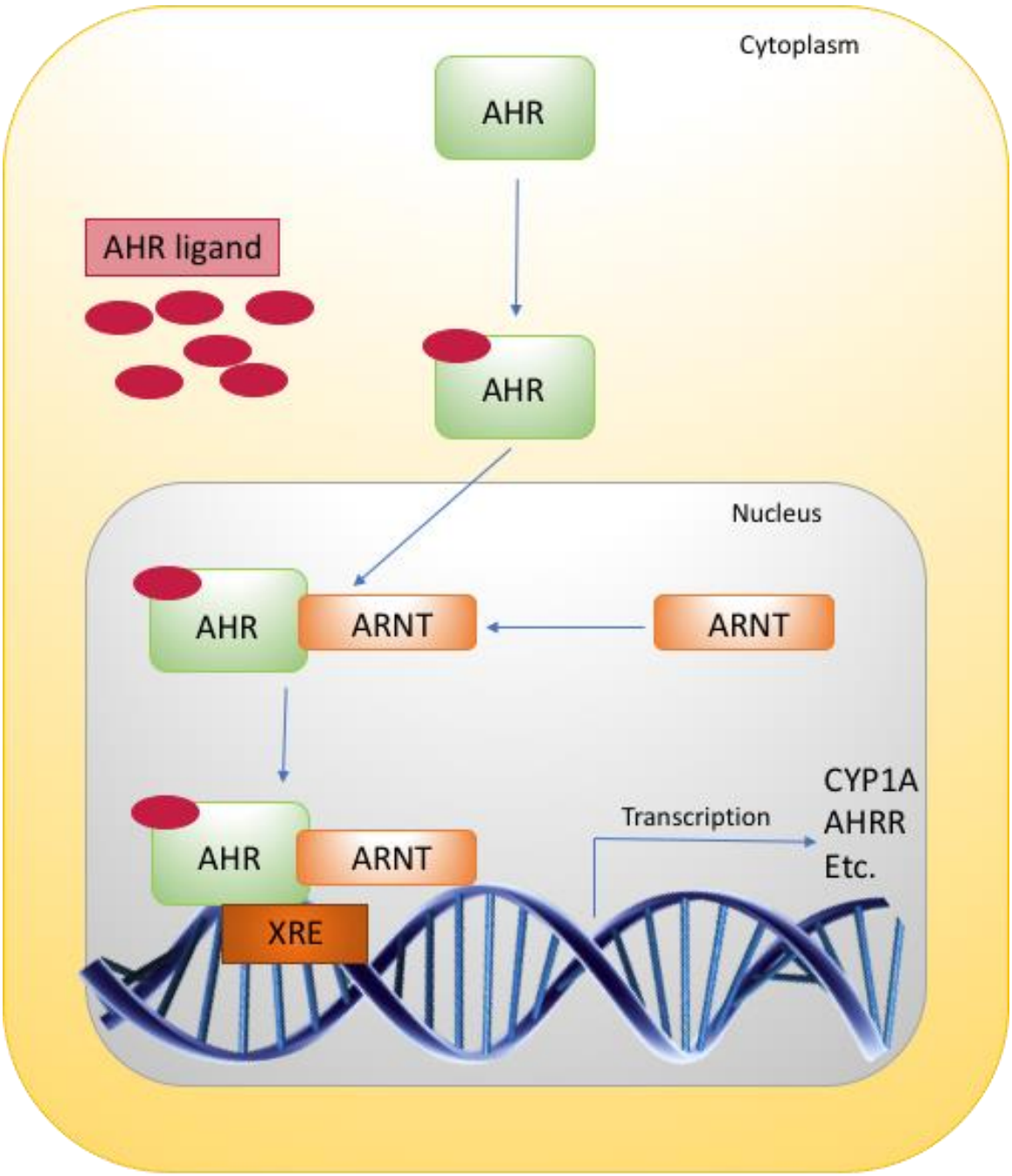

Figure 1.1 Mechanism of activation for aryl hydrocarbon receptor (AHR).

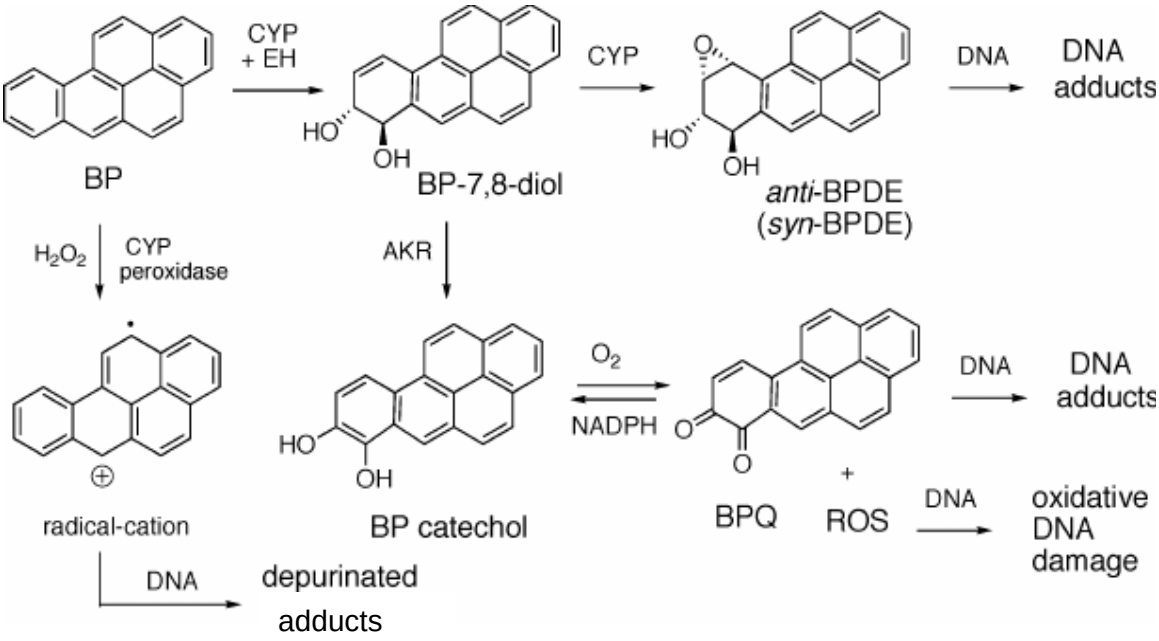

Figure 1.2 Biotransformation of benzo[a]pyrene, a representative polycyclic aromatic hydrocarbon (PAH).

From Harvey et al. (2005)

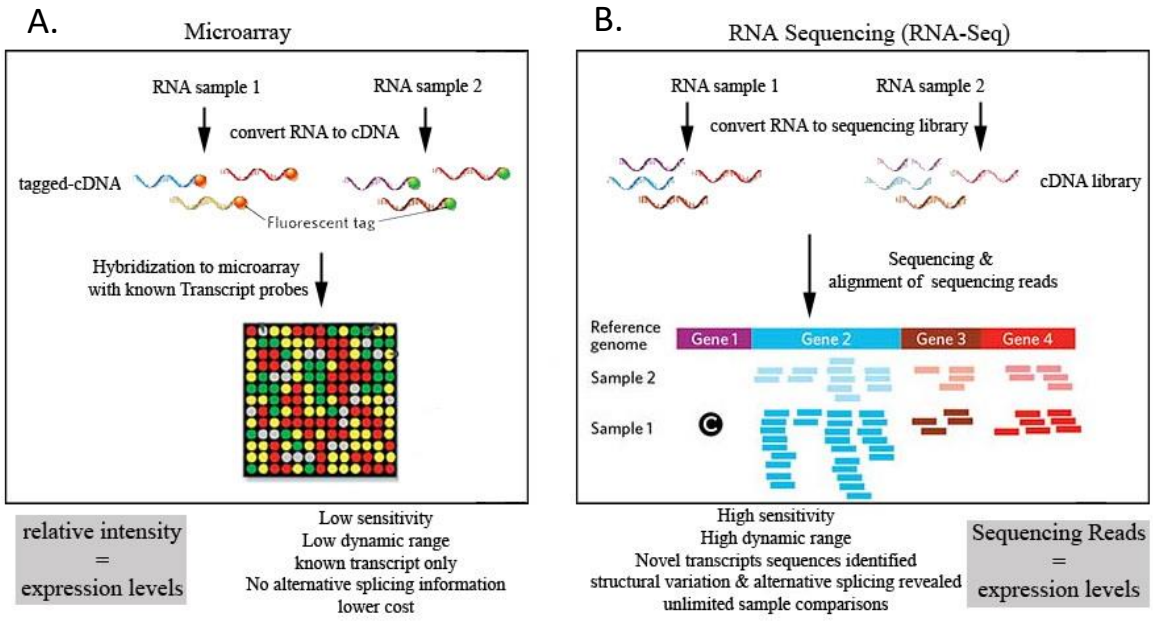

Figure 1.3 Diagram of microarray (A) and RNA seq (B) techniques.

https://www.otogenetics.com/rna-sequencing-vs-microarray/

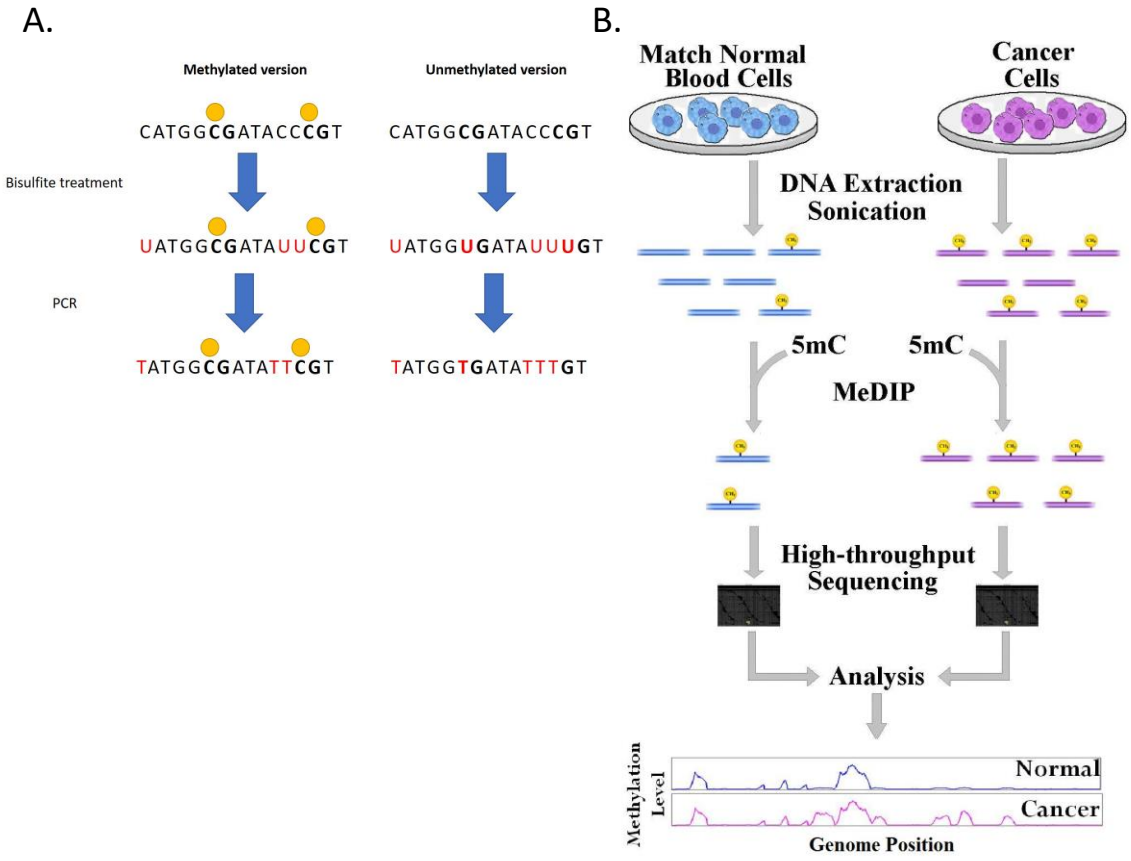

Figure 1.4 Methods for measuring DNA methylation.

A. Bisulfite conversion followed by PCR (https://toptipbio.com/bisulfite-pyrosequencing/). B. MeDIP seq (https://commons.wikimedia.org/wiki/File:Study-flow.jpg).

CHAPTER II EXPOSURE TO DEEPWATER HORIZON OIL AND COREXIT 9500 AT LOW CONCENTRATIONS INDUCES TRANSCRIPTIONAL CHANGES AND ALTERS IMMUNE TRANSCRIPTIONAL PATHWAYS IN SHEEPSHEAD MINNOWS

2.1 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. These results indicate that oil composition, concentration, and exposure duration all affect molecular responses in exposed fish, and suggest that low-concentration exposures may result in sub-lethal adverse effects.

2.2 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.

2.3 Methods

2.3.1 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.

2.3.2 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 sea water 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 sea water 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).

2.3.3 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 sea water (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.

2.3.4 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.

2.3.5 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.

2.3.6 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/µg, with the exception of two 14-day control samples, which were excluded from further analysis due to low dye incorporation. Hybridization was performed according to Agilent's One-Color Microarray-Based Gene Expression protocol (Santa Clara, CA, USA), and arrays scanned using an Agilent G2505B scanner. Expression data were extracted from TIFF images using Agilent Feature Extraction Software (v10.7.3.1), and data was quality assessed via manual inspection of software generated quality control reports. Based upon inspection, all array data were determined to be of high quality and acceptable for inclusion in gene expression analysis. Raw expression data were imported into JMP Genomics v 5.1, $\log_2$ transformed and quantile normalized. Data were filtered to assign all data with intensities ≤4.5 a value of 4.5 (7-d samples) or ≤ 5.5 a value of 5.5

(14-d samples) as these values were determined to represent the limits of detection for microarrays analyses and were a conservative assessment (based on limits of detection for Agilent RNA spike-in and negative controls). After data filtering, differentially expressed genes (DEGs) were identified by one way analysis of variance (ANOVA) followed by a false discovery rate (FDR) of 5.0%.

To gain insight into biological pathways responsive to exposure to Corexit, CEWAF or HEWAF, enrichment analyses for all transcripts were performed using Pathways Studio 9.0 software (Elsevier Inc., Rockville, MD, USA). Prior to enrichment analyses, 14,000 of the sheepshead minnow probes were mapped to human homologs using official gene symbols + aliases. Gene set enrichment analysis (GSEA) was performed using 1000 permutations of the Kolmorgorov-Smirnov algorithm, and enrichment was set at alpha = 0.05. Gene sets used in the GSEA were based upon existing gene ontology knowledge as well as 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.

2.4 Results

2.4.1 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 (± SD) tank temperature was 26.8 (± 0.7) °C, mean pH 8.6 (± 0.1), and mean dissolved oxygen 5.8 (± 0.7) mg/L.

Stocks of HEWAF and CEWAF as well as all test solutions were analyzed for a suite of 50 PAH constituents (tPAH50). Stocks of HEWAF contained higher concentrations of PAHs than CEWAF stocks (Table 2.1); additionally tPAH50 concentrations were more stable in HEWAF stocks than CEWAF stocks (Table 2.1). For test solutions, analyses revealed low tPAH50 concentrations in both CEWAF and HEWAF treatments, with high treatments having concentrations of 5.98 μg/L for HEWAF solutions and 1.10 μg/L for CEWAF solutions, and low treatments having concentrations of 0.26 μg/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.

Dispersant concentrations for CEWAF and Corexit stocks, as well as all test solutions, were determined using measurement of dioctyl sodium sulfosuccinate (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 2.1). 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 these 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.

2.4.2 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.

2.4.3 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 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 2.2). 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 2.2). 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 2.1 A-D).

Pathways Studio 9 was used to identify gene sets and gene sub-networks significantly affected by treatment groups. 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 2.2, Figure 2.2 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 2.2, Figure 2.2). 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 2.2, Figure 2.3 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 2.2). 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 2.3). 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 2.3).

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 2.3). 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 (Figure 2.4). However, individuals from the 14-d treatment exhibited many up-regulated immune pathways, particularly in CEWAF and Corexit treatments. 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. 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.

Pathways related to blood and circulation were also impacted across all treatment groups. 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 2.4). These pathways included sub-networks associated with blood clotting, cardiac function, endothelial cell function, erythrocyte function and degradation, and hematopoiesis. 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.

2.5 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 µg/L tPAH50) and CEWAF (0.35 and 1.10 µg/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 current 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 exposure concentrations used herein 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 μg/L in water from an oiled site in Louisiana. The lowest concentrations measured in this study were tPAH50 0.26 μg/L for low HEWAF and tPAH50 0.35 ug/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 particularly interesting given that prior

studies document low to moderate Corexit toxicity in fish. Acute toxicity studies have reported LC_{50} 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). Similarly, our pathway analysis showed alteration of pathways related to circulation in treatment groups containing dispersant (Corexit and CEWAF treatments), however treatments without dispersant (HEWAF) also elicited similar responses (Table 2.2). 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

pathway analyses 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. 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 analyses 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. 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 these results suggest the possibility that adult fish may also be susceptible to the cardio-toxic and hemorrhagic effects of DWH oil exposure.

Overall, these 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 these 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.

2.6 Conclusion

This study reports 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. These 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 exposure concentrations used in this study suggest these 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.

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Table 2.1 Test solution concentrations.

Solution	tPAH50	DOSS
Control	0.04 ± 0.08	1.12 ± 0.63
Corexit	N/A	148600.00 ± 25620.00
HEWAF	2467.00 ± 199.90	N/A
CEWAF	535.80 ± 297.00	14560.00 ± 1236.00

tPAH50 and DOSS concentrations (± SD) for stock solutions prepared throughout the course of the experiment (n=9 samples for each stock).

Table 2.2 Summary of differentially expressed genes.

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

(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 2.3 Biological processes or themes categorizing altered sub-networks

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

Only sub-networks with mean changes greater or less than zero were included in analysis.

Table 2.4 Common immune sub-networks significantly affected in the liver of sheepshead minnow

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

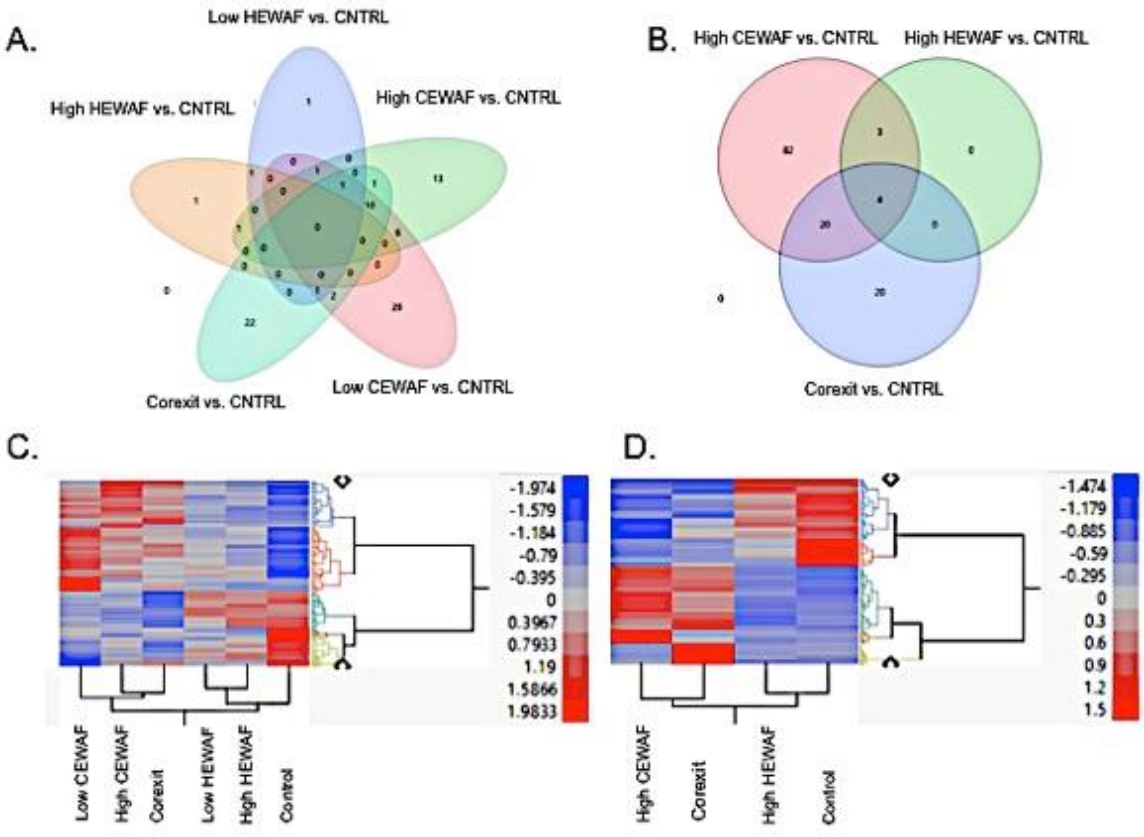

Figure 2.1 Effects of HEWAF, CEWAF, and COREXIT exposure on differential gene expression in sheepshead minnow liver

(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.

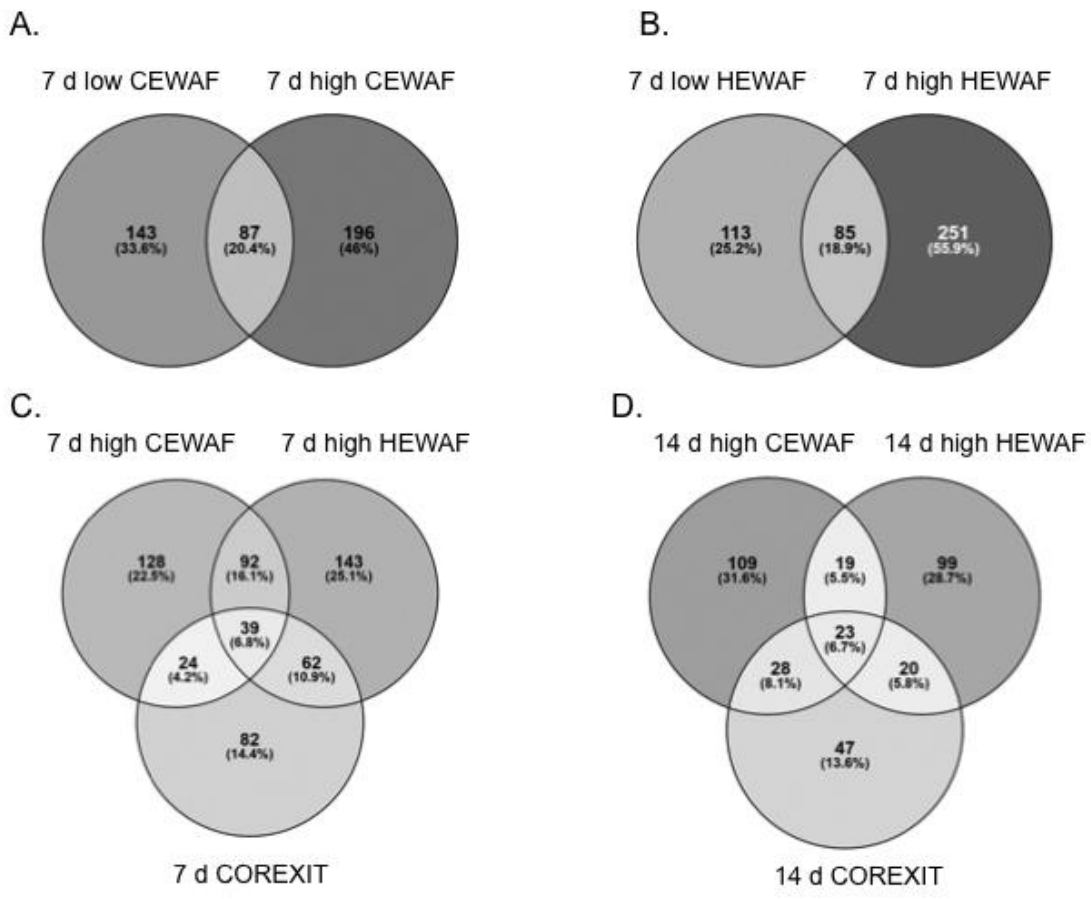

Figure 2.2 Effects of HEWAF, CEWAF, and COREXIT exposure on pathway sub-networks as determined by SNEA.

(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.

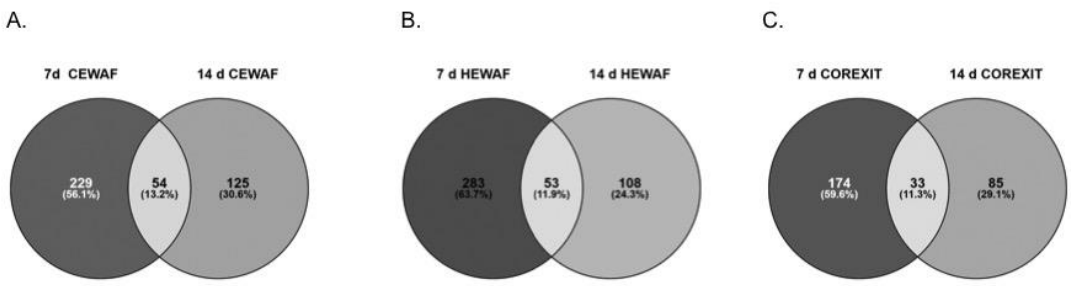

Figure 2.3 Effect of exposure time on sub-networks for high PAH doses and Corexit

(A) CEWAF, (B) HEWAF, and (C) COREXIT.

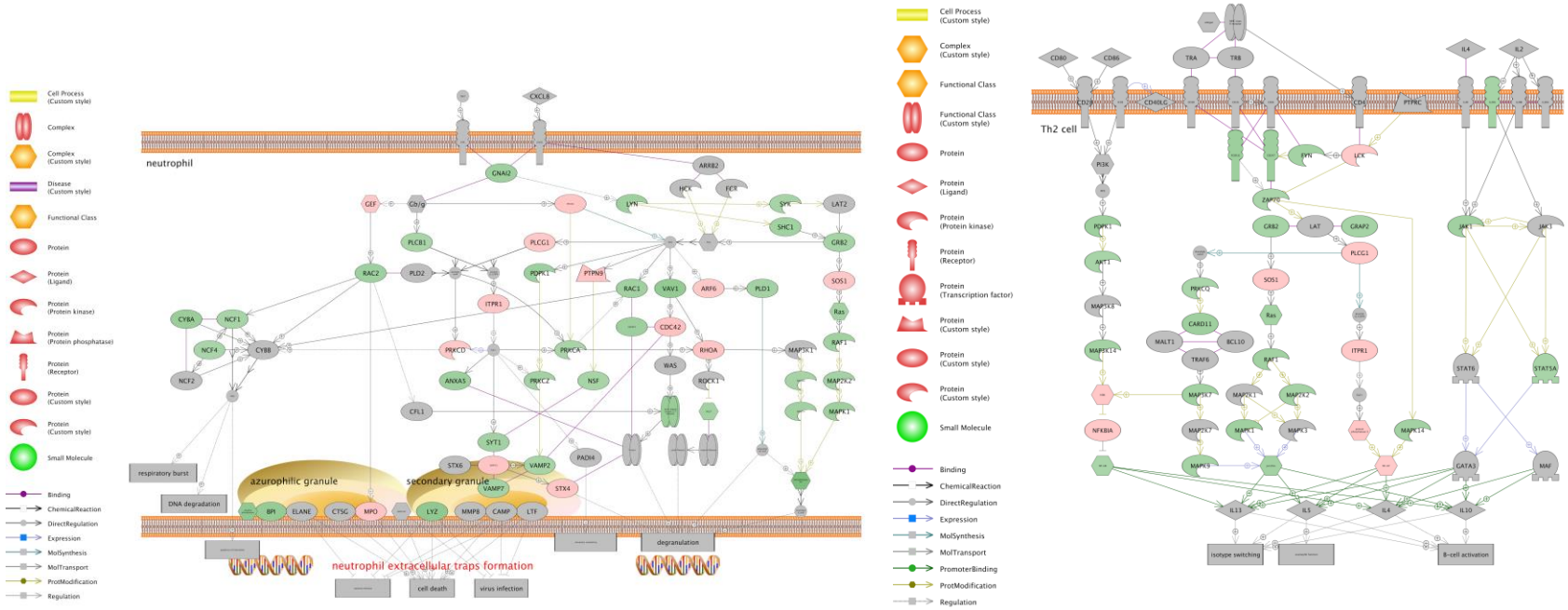

Figure 2.4 Representative immune pathways significantly affected by 7 d high dose HEWAF treatment

Green indicates down-regulation of genes and pathways; red indicates up-regulation. A: Innate immune pathway: Neutrophil degranulation via FPR 1 signaling in 7 d High HEWAF treatments. B: Adaptive immune pathway: Th2 cell differentiation in 7 d High HEWAF treatments.

CHAPTER III TRANSCRIPTOMIC RESPONSES TO OIL EXPOSURE IN ESTUARINE FISH ACROSS DEVELOPMENTAL STAGES AND TAXA

3.1 Abstract

Because oil spills frequently occur in coastal regions that serve as spawning habitat, characterizing the effects of oil in estuarine fish carries both economic and environmental importance. There is a breadth of research investigating the effects of crude oil on fish, however few studies have addressed how transcriptional responses to oil change throughout development or how these responses are conserved across taxa. To investigate these effects, we performed RNA seq and pathway analysis following oil exposure 1) in a single estuarine species (*Cyprinodon variegatus*) at three developmental time points (embryos, yolk-sack larvae, free-feeding larvae), and 2) in two similar species (*C. variegatus* and *Fundulus grandis*), immediately post-hatch (yolk-sack stage). Our results indicate that *C. variegatus* embryos mount a diminished transcriptional response to oil compared to later stages, and that few transcriptional responses are conserved throughout development. Pathway analysis of larval *C. variegatus* revealed dysregulation of similar biological processes at later larval stages, including alteration of cholesterol biosynthesis pathways, cardiac development processes, and immune functions. Our cross-species comparison showed that *F. grandis* exhibited a reduced transcriptional response compared to *C. variegatus*. Pathway analysis revealed that the two species shared similar immune and cardiac responses, however pathways related to cholesterol biosynthesis exhibited a divergent response as it was predicted activated in *C. variegatus* but inhibited in *F. grandis*. Our results suggest that examination of larval

stages may provide a more sensitive estimate of oil-impacts than examination of embryos, and challenge assumptions that ecologically comparable species respond to oil similarly.

3.2 Introduction

Oil spills in coastal regions are a recurring problem (Harfoot et al. 2018). Often these areas serve as fish spawning and nursery habitats; therefore, characterizing the effects of oil exposure in fish species bears economic and environmental importance. The timing of the 2010 Deepwater Horizon (DWH) oil spill coincided with the spawning period of many fish species in the northern Gulf of Mexico, and exposed early life stages of several commercially important species to oil contamination (Rooker et al. 2013; Dubansky et al. 2013). Concern regarding the ecosystem-level impacts of the spill prompted a surge of research on the effects of oil exposure in early life stage fish (see Beyer et al. 2016 for review), however most studies focused on particular stages of individual species; therefore questions remain about how these responses differ across development or across taxa. Characterizing transcriptional patterns conserved among developmental stages and taxa would allow identification of robust responses likely to be present in field-exposed animals, where the complex natural environment may alter contaminant-induced transcription and obscure more subtle responses.

Few studies have addressed how responses to oil change across developmental windows, when tissue and organ differentiation occur. Exposure to crude oil and its constituents is known to affect developing fish via increased mortality, delayed hatch, and developmental abnormalities dominated by cardiovascular and craniofacial defects

(Incardona et al. 2004, 2009; de Soysa et al. 2012b; Whitehead et al. 2012; Dubansky et al. 2013; Hedgpeth and Griffitt 2016). Much of this research has concentrated on exposure during the embryonic stage (Couillard 2002; Greer et al. 2012; Whitehead et al. 2012; Dubansky et al. 2013; Adeyemo et al. 2015; Incardona et al. 2015; Rodgers et al. 2018a), however, numerous studies have established that hatched larvae are generally more sensitive to toxicants than embryos, implying that examining responses in multiple age stages is important (Barry et al. 1995; Hutchinson et al. 1998; Humphrey and Klumpp 2003; Mhadhbi and Beiras 2012). Recent studies of transcriptomic responses to oil across developmental stages in mahi-mahi (*Coryphaena hippurus*) and red drum (*Scieanops ocellatus*) report substantial increases in numbers of dysregulated genes from embryonic to larval stages following oil exposure, suggesting that the timing of exposure may affect responses at the molecular level (Xu et al. 2016, 2017b, 2017a). However, these studies exposed fish continuously from embryonic stages through hatch, rendering it difficult to determine whether increased numbers of dysregulated genes result from ontogenetic differences or greater cumulative oil dose. Because tissue differentiation and development of organ systems occur during different developmental windows, it is likely that the timing of oil exposure determines the type and magnitude of response. There remains a need for studies which initiate exposure at discrete developmental windows to fully characterize the effects of oil on fish development; assuming that the effects of transient exposure across different developmental stages will be the same may not be valid.

It is also unclear whether responses to oil might vary substantially among similarly evolved taxa occupying comparable ecological niches. In particular, studies investigating the effects of oil contamination on resident marsh fish have frequently employed the Gulf killifish (*Fundulus grandis*) or sheepshead minnow (*Cyprinodon variegatus*) as model organisms to define oil responses in estuarine species (Beyer et al., 2016; Crowe et al., 2014; Dasgupta et al., 2016; Hedgpeth and Griffitt, 2016; Jones et al., 2017; Maria L. Rodgers et al., 2018; Whitehead et al., 2012). Both species are residents of northern Gulf of Mexico (nGOM) estuaries that spawn during spring and summer months when oil contamination from the DWH spill would have arrived along shorelines (Talbot and Able, 1984; Taylor et al., 1979), making them environmentally relevant models in which to study the effects of developmental oil exposure. The assumption that these two species serve as interchangeable examples for marsh species responses may be flawed, as they exhibit markedly different sensitivities to oil exposure, particularly during development (as defined by LC50 indices) (Rodgers et al., 2018, Simning 2017). Generally, the morphological effects of developmental oil exposure are assumed to be consistent across fish taxa, and transcriptomic studies repeatedly identify dysregulation of genes related to cardiac development, craniofacial development, and cholesterol biosynthesis across species (Sørhus et al. 2017; Carls et al. 2008; Xu et al. 2016, 2017a). However, the differences in oil-induced mortality between *F. grandis* and *C. variegatus* larvae suggest there may be nuanced differences in molecular responses contributing to differential sensitivity. To accurately identify these potential differences, it is important that experiments be carried out under similar conditions, however previous studies

examining oil effects in these two species have been conducted under varying experimental conditions rendering comparisons difficult.

To test whether early life stage fish, regardless of age or developmental state, respond similarly to oil, and whether two comparable estuarine species have similar responses to oil exposure, we compared transcriptional effects 1) across life stages of a single species (*C. variegatus*) and 2) across two similar species (*C. variegatus* and *F. grandis)* at the same developmental stage, immediately post-hatch (yolk-sack stage). Our results indicate substantial differences in transcriptional effects among developmental stages, and identify subtle disparities in molecular responses across species.

3.3 Materials and methods

3.3.1 Animal husbandry

C. variegatus brood stock were maintained at the Shoemaker Toxicology Laboratory at The University of Southern Mississippi Gulf Coast Research Laboratory (Ocean Springs, MS). Brood stock were kept in 300-L recirculating raceways containing 15 ppt artificial sea water at 26 - 27°C on a 12:12 light/dark cycle and fed commercial flake food twice daily. Spawning events occurred over 24-hour periods initiated by the placement of breeding nets in raceways. Collected eggs were examined to determine fertilization and, if not immediately used in experiments, embryos were placed into 4L holding tanks containing 30 ppt artificial sea water at 30°C. Embryos were then reared to yolk-sack (< 24 hours post hatch) or free-feeding (>4 days post hatch) larval stages. See Simning (2017) for additional detail.

F. grandis brood stock were collected near Biloxi, Mississippi, and transported to Purdue Aquaculture Research Laboratory (Purdue University, West Lafayette, IN.) Brood stock were maintained in 130-L recirculating tanks containing artificial sea water at 10 ppt at 25-26°C on a 16:8 light/dark cycle and fed frozen chironomids and *Artemia* nauplii twice daily. Spawning was initiated by the placement of Pentair coarse filter mats in breeding tanks. Up to 40 embryos were collected from each tank, placed in mesh cylinders within the breeding tanks, and checked daily for hatch. Yolk-sack larva were collected < 24 post hatch for use in experiments. See Serafin (2017) for additional detail.

3.3.2 Preparation of HEWAF test solutions

High energy water accommodated fraction (HEWAF) solutions for both *C. variegatus* and *F. grandis* experiments were prepared from DWH source oil using methods outlined in Forth et al. (2016), and concentrations for 29 parent PAHs, alkyl PAHs, and alkyl PAH homologs (tPAH29) were assessed via gas chromatography coupled with tandem mass spectrometry (GC/MS/MS) at the University of Connecticut Center for Environmental Sciences and Engineering (Storrs, Connecticut) for each HEWAF stock. Oil concentrations for HEWAF dilutions used in exposures were inferred using a fluorescence method detailed in Greer et al. (2012.). All exposures were conducted under normoxic (≥ 5 mg/L oxygen) conditions for 48 hours at 30°C and 30 ppt.

3.3.3 Experimental Design

To examine the effects of oil exposure throughout development and across taxa, we examined a total of 23 RNA seq libraries generated from *C. variegatus* and *F. grandis* (Figure 3.1; Table 3.1). *C. variegatus* libraries were constructed from three life stages (embryonic, yolk-sack, or free-feeding) subjected to oil exposure at a nominal concentration of 12.5% HEWAF (ranging from 69.79 ± 7.18 to 128.61 ± 6.94 μg/L tPAH29) ; *F. grandis* libraries were constructed from a single life stage (yolk-sack) exposed to a nominal oil concentration of 6.25% HEWAF (14 μg/L) (see Table 3.1 for measured concentrations). These doses were deemed equitoxic, as they produced similar mortality across species (Table 3.1) (Simning 2017, Serafin 2017).

3.3.4 RNA sequencing and bioinformatics

For RNA extraction and library generation, multiple individuals (10 per sample *for C. variegatus*, 3 per sample for *F. grandis*) were pooled to generate sufficient RNA mass for sequencing. Total RNA was extracted from three replicates for each treatment using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA). Extracted RNA was shipped on dry ice to Purdue University Genomics Core Facility for sequencing. Samples were prepared using Illumina TruSeq Stranded mRNA Sample Preparation Guide (Illumina, San Diego, USA) to construct polyA+ libraries. Libraries were sequenced on an Illumina HiSeq 2500 in high output mode to generate 2 x 100 bp reads at a minimum depth of 15M reads per sample for *F. grandis* libraries, and 30M reads per sample for *C. variegatus* libraries.

Sequencing data quality was checked by FastQC toolkit (Andrews, 2010), and adaptor sequences and low-quality reads were trimmed using Trimmomatic software (Bolger et al., 2014) for a mean Phred score of 30. Downstream data analysis was performed using CLC Genomics Workbench 10.1.1 (Qiagen, Hilden, Germany). Data were examined for residual low-quality reads and sequencing adaptors, and paired ends matched. For each sample, paired data was aligned to the sequenced genome available for the nearest taxonomic relative; *C. variegatus* samples were aligned to the *C. variegatus* genome (NCBI genome ID 13078) and *F. grandis* samples were aligned to the *F. heteroclitus* genome (NCBI genome ID 743). Expression values for transcripts were calculated as transcripts per million (TPM). Normalized expression values were used in hierarchical cluster analyses (Cluster 3.0, 2004) to examine transcriptional patterns among samples.

Differential gene expression was determined using the "against controls" option in CLC to compare exposed samples to controls for each treatment. The p-values for gene expression were corrected using false discovery rate (FDR) to adjust for type I statistical error. Significant genes (FDR ≤ 0.05) were matched to human orthologs for use in downstream pathway analysis using Ingenuity Pathways Analysis (IPA) software (Qiagen, Hilden, Germany). IPA is curated for human and mammalian gene-association findings, and therefore represents a rich database of information allowing detailed analysis of gene interactions and system level effects. This approach has been shown to improve functional analyses over approaches based on teleost information alone, by providing access to a wider breadth of data than that currently available for fish models (Xu et al. 2017). Identification of significantly altered canonical pathways, molecular

and cellular functions, and tox functions was made using the "Core Analysis" feature of IPA; pathways and gene networks were considered significantly altered at $p \leq 0.05$. Comparisons of altered pathways and functions among treatments were made using the "Comparison Analysis" function in IPA.

3.4 Results

3.4.1 Alignment and annotation of RNA seq reads

Sequencing of C. *variegatus* libraries yielded 224 M total reads for embryonic samples, 399 M total reads for yolk-sack samples, and 253 M total reads for free-feeding samples. Embryonic groups averaged 40 M reads/sample, yolk-sack 67 M reads/sample, and free-feeding 40 M reads/sample. Efficiency of mapping to the *C. variegatus* genome was high for all groups, ranging from 88% in embryonic samples to 94% in yolk-sack samples. C. *variegatus* reads were mapped to a total of 25,075 loci, 10,077 of which were annotated and matched to human orthologs for downstream functional analysis in IPA.

Sequencing of *F. grandis* libraries yielded a total of approximately 114 M total reads and averaged 23 M reads/sample. Despite use of a related genome, mapping efficiency remained quite high for all *F. grandis* samples, averaging 93%. To avoid confounding effects due to differential annotation of the two genomes used for alignments, comparisons between species were conducted using only genes that could be identified in both the *C. variegatus* and *F. heteroclitus* genomes. For cross species comparisons a total of 8196 genes were used in hierarchical cluster, differential expression, and functional analyses. Sequencing data from this study is publicly

available in NCBI's Gene Expression Omnibus (GEO) database under accession numbers GSE123469 for *C. variegatus data*, and GSE (accession number pending) for *F. grandis* data.

3.4.2 Transcriptional responses to oil exposure in *C. variegatus*

Global transcriptional patterns in *C. variegatus* were primarily influenced by life stage as a hierarchical cluster analysis grouped each of the life stages apart from each other (Figure 3.2A). Within each life stage, exposure to oil resulted in a clear treatment related separation of global transcriptional profiles, indicating that oil exposure elicited a unique transcriptional effect within each age stage. The number of differentially expressed genes (DEGs) was also influenced by age; oil exposure significantly affected expression of 686 genes in yolk-sack individuals and 614 in free-feeding groups, but only 48 in embryos (Figure 3.2B). There were relatively few genes that were significantly affected in multiple ages; the greatest point of overlap was between yolk-sack and free-feeding larvae, which shared 151 significantly affected genes.

We used Ingenuity Pathway Analysis (IPA) to place DEGs into known categories, including Canonical Pathways, Toxicology Lists, Diseases, and Molecular and Cellular Functions (Table 3.2). IPA uses activation z-scores to determine whether dysregulated pathways and functions are predicted to be activated or inhibited based on gene expression patterns; a z-score of >2 is considered activated, while a z-score <2 is considered inhibited. There were few canonical pathways identified as either activated or inhibited in oil-exposed embryonic *C. variegatus* (Figure 3.2C), although the Diseases analysis did identify processes related to liver disease, heart disease, and metabolism as

affected. The yolk-sack and free-feeding stages had many more canonical pathways affected by oil exposure than did the embryos. Many of the activated pathways in yolk-sack and free-feeding *C. variegatus* were related to immune function, including those linked to inflammation (e.g. IL-8 signaling, NF-kB signaling, HMGB1 signaling, Acute Phase Response signaling) and cell-mediated immunity (B-cell receptor signaling) (Figure 3.2C). While most canonical pathways were predicted to be activated by oil exposure, "PPAR Signaling" was predicted as inhibited in both life-stages; this prediction is likely an artifact of increased expression of *cox 2* (cyclooxygenase 2) in both groups, which is associated with oxidative stress response, but competes with PPAR for fatty acid substrates, thereby inhibiting PPAR signaling (Knopfová and Smarda, 2010).

For both yolk-sack and free-feeding *C. variegatus*, pathways related to cholesterol biosynthesis were predicted as highly activated, including "Superpathway of Cholesterol Biosynthesis", "Cholesterol Biosynthesis I", "Cholesterol Biosynthesis II", and "Cholesterol Biosynthesis III" with z-scores ranging from 2.47 to 4 (Figure 3.3A). Cholesterol biosynthesis is a subsidiary process nested within the Cellular and Molecular Functions categories of "Lipid Metabolism" and "Small Molecule Biochemistry"; therefore, combined evidence from altered Canonical Pathways and impacted Cellular and Molecular Functions analyses suggests that dysregulation of cholesterol biosynthesis is a major impact of oil exposure in yolk-sack and free-feeding larval *C. variegatus*. This activation was associated with increased expression of several key genes involved in cholesterol biosynthesis pathways including *lss* (lanosterol synthase), *sqle* (squalene monooxygenase), *nsdhl* (NADP steroid dehydrogenase-like) and *ebp* (3β-hydroxysteroid isomerase) (Figure 3.2B). There was also increased gene expression for upstream

regulators of cholesterol homeostasis, including sterol regulatory element binding transcription factors (*srebf1* and *srebf2*), as well as their chaperone, *scap* (SREBP cleavage activating protein) (Eberle et al. 2004) (Fig. 3B).

Cardiac processes were also among the top dysregulated processes in yolk-sack and free-feeding larvae (Table 3.2). Both the top Tox Lists and top Disease functions identified cardiac dysfunction as an effect of oil exposure in larval *C. variegatus*, including cardiac hypertrophy, cardiac fibrosis, and cardiac necrosis. There was little commonality in which cardiac disease terms were affected following oil exposure, although impacted disease processes generally trended towards inhibition (Figure 3.4A). Processes related to cardiac development were generally activated in both later age stages, including "Angiogenesis", "Development of Vasculature", "Vasculogenesis", and "Formation of Blood Vessels" (Figure 3.4A). We hypothesize that this is likely due to differences in the developing heart, particularly in the transition to the free-feeding larval stage, which necessitates more powerful movements. We also observed activation of the "BMP signaling" canonical pathway for both groups, which IPA defines as contributing to growth and differentiation of organ systems, including cardiovascular development. This upregulation was associated with increased expression of genes for *bmp 2* (bone morphogenic factor 2) and *bmp 4* (bone morphogenic factor 4); we hypothesize that upregulation of the "BMP Signaling" canonical pathway is linked to the observed general activation of cardiac development processes, given the role bone morphogenic proteins have in cardiac growth (Reifers et al. 2000) (Figure 3.4C).

Despite the differences observed across ages in *C. variegatus*, there were several pathways that were affected across all three, including "Glucose Degradation", "Folate

Transformation", and "Androgen Biosynthesis" (Table 3.3); it is possible that these processes are representative of a conserved response that is affected in developing fish regardless of the precise age of the individual.

3.4.3 Transcriptional responses across species in post-hatch *C. variegatus* and *F. grandis*

To assess whether transcriptional responses induced by exposure to an equitoxic dose of oil were conserved across species, we compared the responses of yolk-sack *C. variegatus* to those of yolk-sack *F. grandis*. We restricted our analysis of transcriptional patterns across species to only those genes annotated in both genomes used for transcript alignment; using this restricted list reduced the number of DEGs in post-hatch *C. variegatus* from 686 to 524 that passed ≤ 0.05 FDR cutoff. However, this diminished list of DEGs did not fundamentally alter the outcome of our species-specific functional analysis; in *C. variegatus* IPA still identified pathways related to cholesterol biosynthesis as the top altered Canonical Pathways; and the top impacted molecular and cellular functions still included "Lipid Metabolism" and "Small Molecule Biochemistry". "Cardiovascular Disease" also remained one of the top altered disease processes (Table 4).

Hierarchical cluster analysis of the genes present in both the *C. variegatus* and *F. grandis* datasets indicated that while both species had a transcriptional response to an equitoxic oil exposure, the two species responded differently. The hierarchical cluster analysis (Figure 3.5A) shows that the treatment-driven differences are much less than the differences between species. In *F. grandis*, oil exposure elicited differential expression

of 240 genes using an FDR cutoff of ≤ 0.05 (Figure 5B), as compared to 524 in *C. variegatus*. There was a surprisingly small overlap between the genes affected by oil in the two species; only 58 of the 240 genes were also affected in the *C. variegatus* dataset (Figure 5B). When placed into larger processes, IPA identified 106 significantly dysregulated Canonical Pathways in *F. grandis*, with the top most affected pathways related to oxidative stress or inflammatory processes (Table 3.4). Interestingly, the top impacted Molecular and Cellular Functions were very similar to those in *C. variegatus*, including "Lipid Metabolism" and "Small Molecule Biochemistry", and top Disease processes included "Cardiovascular Disease"; however, each of these processes were associated with fewer genes than the same processes in *C. variegatus* (Table 3.4).

Pathway activation analysis also shows that despite the relatively low number of genes that were differentially expressed in the two species, there is evidence for a consistent effect of oil exposure. In particular, immune related pathways were affected similarly ("IL-6 Signaling", "LPL/IL-1 Mediated Inhibition of RXR Function", "PI3K Signaling in B Lymphocytes", "Acute Phase Response Signaling", and "B Cell Receptor Signaling"), indicating that effects on the developing immune system may be shared across species (Figure 3.5C). There were also similar responses in cardiac related pathways. When cardiac processes were ranked by z-score, processes related specifically to cardiac development were impacted similarly and trended towards activation (Figure 3.6A). Activation of cardiac processes in both species was associated with upregulation of *bmp4*, which encodes a growth factor critical to cardiac development. (Figure 3.6B).

In contrast to the similarity in responses in immune and cardiac related pathways between species, there was a divergent effect of oil exposure on cholesterol biosynthesis. While dysregulation of processes related to cholesterol biosynthesis was a hallmark characteristic of oil exposure in larval *C. variegatus*, *F. grandis* exhibited fewer significantly impacted functions that were related to cholesterol biosynthesis, and although those pathways were predicted as activated in *C. variegatus*, they were predicted as inhibited in *F. grandis* (Figure 3.7A). These opposing directions of dysregulation were associated with decreased expression of cholesterol biosynthesis genes in *F. grandis* and increased expression in *C. variegatus;* in particular, expression of the cholesterol regulating gene *srebf1* was decreased in *F. grandis*, while *srebf2* (also a regulator of cholesterol biosynthesis) expression increased in *C. variegatus* (Figure 3.7B).

3.5 Discussion

In this study we sought to answer whether oil exposure elicits similar responses at different developmental stages in fish. Because oil contamination frequently impacts critical spawning and nursery habitat, many previous studies have emphasized the effects of oil exposure on embryonic stages as a means of predicting the population-level impacts (e. g. Carls and Thedinga 2010; de Soysa et al. 2012a; Dubansky et al. 2013; Incardona et al. 2015; Rodgers et al. 2018; Morris et al. 2018). The rationale for this emphasis is likely due to two factors; 1) in many cases, the embryos of estuarine fish are associated with sediment, where concentration of oil may be greatest, and 2) disruption of organogenesis would almost certainly cause delayed effects on survivorship and

reproduction, and therefore bear population-level consequences. However, while organ differentiation begins during embryonic development, most organ systems are only present in rudimentary form at hatch (O'Connell 1981); therefore exposure to oil during later larval stages may also impair development and have environmental repercussions.

Our results demonstrate that *C. variegatus* embryos mount a diminished transcriptional response following oil exposure compared to later developmental stages, despite being exposed to slightly higher oil concentrations. These data corroborate work from mahi-mahi and red drum which also found that newly hatched larvae exhibited greater transcriptional responses than embryonic groups (Xu et al. 2016, 2017b, 2017a). However, those studies employed continuous oil exposure throughout development, making it difficult to determine whether increased transcriptional responses were the result of cumulative dose or ontogenetic effects. An important feature of our work was the introduction of oil at each of three critical developmental windows, allowing assessment of how each ontogenetic stage mounts responses to oil. Decreased sensitivity to contaminants in fish embryos is common (see Hutchinson et al. 1998 for review), and may be due to the embryonic chorion providing a protective barrier against contaminant uptake (Mhadhbi and Beiras, 2012). Our data support the hypothesis that the embryonic chorion confers protection from oil exposure to fish embryos. Although there is evidence that PAHs from oil pass the through the chorion (Carls et al. 2008), it is likely that the chorion diminishes PAH dose and could account for our observed muted embryonic response. The similarity in responses we observed in yolk-sack and free-feeding larvae suggest that greater contact with contaminated water (via gills, integument, and drinking) may elicit greater transcriptional dysregulation due to a higher PAH dose.

Our larval groups shared a substantial number of similarly altered genes and pathways, and in particular, our data suggest that disruption of cholesterol biosynthesis is a primary effect of oil on larval *C. variegatus*. Cholesterol is an essential component of cell membranes, and a precursor to synthesis of steroids, vitamin D, and bile acids; therefore, dysregulation of cholesterol production has implications for both growth and endocrine function. Exposure to crude oil and other PAH mixtures in fish consistently elicits transcriptional changes in cholesterol-related genes, (Meland et al., 2011; Oleksiak, 2008; Olsvik et al., 2014), with upregulation of genes and pathways related to cholesterol biosynthesis noted in developing mahi-mahi and Atlantic haddock (Sørhus et al., 2017; Xu et al., 2017b, 2016). The mechanism underlying these effects is unclear. Sørhus et al. (2017) proposed that this upregulation represents a means to compensate for a lack of dietary cholesterol. In their study, they observed diminished yolk absorption in embryos and early larvae as well as empty stomachs in older larvae. This led them to hypothesize that oil exposure inhibits nutrient uptake and feeding behavior, and thus triggers an increase in endogenous biosynthesis of cholesterol. Whether this mechanism is the underlying cause of increased cholesterol synthesis developing *C. variegatus* is uncertain. We did not measure yolk consumption in yolk-sack groups, so it is unclear whether diminished nutrient uptake might have contributed to activation of cholesterol pathways. In free-feeding groups we observed more *Artemia* remaining in oiled containers than in control groups, suggesting larvae exposed to oil were not feeding at the rate control animals were. The possibility that our experimental animals experienced a lack of dietary cholesterol input is supported by our gene expression data. In both post-hatch and post-larval groups transcription of genes encoding SREBF1 and SREBF 2

(both regulators of cholesterol homeostasis) were upregulated. In the cell, activity of these proteins is triggered by low concentrations of cellular cholesterol (Eberlé et al., 2004); therefore increased transcription of these genes could be evidence for a lack of circulating cholesterol and may be the mechanism underlying activation of cholesterol biosynthetic pathways.

A second aim of this study was to examine oil responses conserved across taxa, with the intent to determine whether ecologically comparable species exhibit similar responses to oil. Inherent in the use of ecologically relevant models is the assumption that responses among species will be similar, thus allowing predictions of ecosystem effects to be made from examining a single species. To directly test this assumption, we selected two ecologically comparable species (*C. variegatus* and *F. grandis*) which occupy similar estuarine niches and are resident to areas impacted by oil spills like DWH. We found transcriptional patterns for each species to be largely distinct from one another, and in some cases, such as cholesterol biosynthesis, to respond in an opposing manner. Our cholesterol biosynthesis results corroborate previous work demonstrating that PAH induced modulation of cholesterol-related gene expression can vary depending on context. For example, studies examining the effects of the PAH benzo[a]pyrene (BaP) in human hepatocarcinoma cell lines consistently document transcriptional repression of cholesterol-related genes (Naem et al., 2012; Souza et al., 2016). In amphibians, BaP exposure elicited a biphasic effect on transcription, with repression of cholesterol-related genes early and later in exposure (6 and 24 hours) and upregulation of genes at intermediate time points (12 and 18 hours) (Regnault et al., 2014). In rat livers, a second PAH moiety, dibenz[*a,h*]anthracene (DB[a,h]A) elicited upregulation of cholesterol

biosynthesis pathways (Staal et al. 2007). These combined results suggest that the effects of PAH exposure on cholesterol biosynthesis may vary by both taxa and type of PAH. However, because both species in our study were subjected to HEWAF preparations with similar PAH composition, it is likely that the transcriptional patterns noted herein are an artifact of taxonomy. Results such as these suggest the possibility that variations in transcriptional patterns previously assumed to be due to differences in PAH constituents or oil dose may in fact be the result of taxonomic differences. The extent to which our observed transcriptional variation might manifest as divergent phenotypes is unclear; however our data caution against generalized assumptions based on superficial examination of dysregulated processes and highlight that ecologically similar species do not necessarily respond similarly to oil. While these two species were exposed to oil doses that produced comparable mortality at similar developmental stages, it remains possible that these effects are due to different concentrations used, or different absolute ages. *F. grandis* develop much slower than *C. variegatus*, therefore yolk-sack stages occur later due to longer time to hatch (~3 days for *C. variegatus* vs ~10 dph for *F. grandis*) (Rodgers et al. 2018, Simning 2017). However, given that the two exposures were done at similar effect concentrations and similar developmental stages, we assert that the comparison is valid.

Although we observed transcriptional differences across life stages and species, two areas where transcriptional responses to oil appear conserved are dysregulation of cardiac development and immune related processes. Cardiotoxicity is considered a hallmark characteristic of oil exposure in developing fish (Incardona et al. 2004, Incardona et al. 2009, Hicken et al. 2011). Similar to previous work, our data predicted

dysregulation of cardiac related processes in both *F. grandis* and *C. variegatus*, however we found differences in the nature of these responses. Processes related to cardiac disease showed variation among taxa and life stages with few processes conserved across groups, and disparity in activation or inhibition of disease related pathways. In contrast, processes associated with cardiac development were more consistently affected, and generally found to be activated. For all groups this activation was linked to expression of genes encoding bone morphogenic proteins (BMP 2 and 4 in *C. variegatus*, and BMP 4 in *F. grandis*). Bone morphogenic proteins are growth factors involved in development of organ and tissue architecture and are critical to cardiac development (Reifers et al. 2000). Increased expression of genes encoding these proteins, particularly BMP 4, is associated with cardiac hypertrophy (Sun et al., 2013), and has been noted in other fish taxa following PAH exposure, including mahi-mahi and zebrafish (*Danio rerio*) (Fang et al., 2015; Xu et al., 2017b). Interestingly, cardiac morphology consistent with ventricular hypertrophy has been noted following developmental oil exposure in zebrafish (Hicken et al., 2011). These results in combination with our data suggest one possible mechanism underlying oil-induced cardiotoxicity in early life stage fish could be increased expression of BMP genes resulting in tissue hypertrophy.

We also noted that pathways related to immunity and inflammation were consistently activated across species and life stages. Previous work has linked oil exposure in fish with impaired immunity resulting in health impacts including skin lesions, fin-erosion, increased incidence of infection, and mortality following bacterial exposure (Bayha et al., 2017; Hogan et al., 2018; Murawski et al., 2014; van den Heuvel et al., 2000). Oil exposure has also been associated with altered expression of immune

genes across a variety of taxa and life stages, and in particular, with changes in expression of genes related to the inflammatory response (Bayha et al., 2017; Jones et al., 2017; Maria L Rodgers et al., 2018). Our data documenting oil-induced modulation of immune genes and activation of pro-inflammatory processes corroborate these findings. Rodgers et al. (2018b) noted that in red snapper (*Lutjanus campechanus*), genes related to inflammation exhibited a time dependent expression pattern, with pro-inflammatory gene expression upregulated immediately after oil exposure but diminished after prolonged exposure. Those results suggest that the pattern of upregulation for immune genes and pathways in our data are likely due to the acute and recent oil exposure of our fish. How this activation of the immune system might impact later development and survival is unclear, but the similarities between our data and previous work suggests that dysregulation of immunity is a pervasive and conserved effect of PAH exposure across fish taxa.

3.6 Conclusion

This study describes responses to DWH oil in estuarine fish across development and taxa. We found that *C. variegatus* embryos are more resistant to transcriptional dysregulation from oil exposure than later developmental stages, suggesting that examination of larvae may provide a more sensitive assessment of oil spills than embryos. Additionally, our data reveal that yolk-sack and free-feeding larvae respond similarly to oil, as they share a substantial number of similarly altered genes and pathways, including those related to cholesterol biosynthesis, cardiac effects and immune dysfunction. Numerous studies have previously documented similar transcriptional

effects at various developmental stages and across multiple taxa (Bayha et al. 2016, Incardona et al. 2004, 2009, Jones et al. 2017, Rodgers et al. 2018, Sørhus et al., 2017; Xu et al., 2017b, 2016); thus our data contributes to an identification of conserved effects following oil exposure in teleosts. Our comparison between *C. variegatus* and *F. grandis* larvae found differences in oil-induced transcription, challenging assumptions that ecologically comparable species respond similarly to oil exposure. In particular, we found gene expression related to cholesterol biosynthesis was substantially upregulated in *C. variegatus* larvae, but downregulated in *F. grandis*, and suggest that future work focus on elucidating the phenotypic consequences of disrupted endogenous cholesterol production across teleost species.

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Table 3.1 Experimental parameters for each group

Species	Life stage	Age	# of libraries	# of ind./library	tPAH29 μg/L	Cumulative mortality (%)
C. variegatus	Embryonic	≤ 15 hpf	3 oil/ 3 control	10	128.61 ± 6.94	23
	Yolk-sack	≤ 24 hph	3 oil/ 3 control	10	69.79 ± 7.18	15
	Free-feeding	≤ 4 dph	3 oil/ 3 control	10	82.64 ± 4.85	51
F. grandis	Yolk-sack	≤ 24 hph	2 oil/ 3 control	3	14	30

Table 3.2 Top altered pathways and functions in developing *C. variegatus* with numbers of associated DEGs, as predicted by IPA software.

	Embryonic	# of genes	Yolk-sack	# of genes	Free-feeding	# of genes
Top Cannonical Pathways	Glucose and Glucose-1-phosphate Degradation	2/11	Osteoarthritis Pathway	23/212	Superpathway of Cholesterol Biosynthesis	16/28
	Maturity Onset Diabetes of Young (MODY) Signaling	2/21	Cholesterol Biosynthesis I	6/13	Cholesterol Biosynthesis I	9/13
	Estrogen Biosynthesis	2/41	Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	6/13	Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	9/13
	Intrinsic Prothrombin Activation Pathway	2/42	Cholesterol Biosynthesis III (via Desmosterol)	6/13	Cholesterol Biosynthesis III (via Desmosterol)	9/13
	Type II Diabetes Mellitus Signaling	3/154	LPS/IL-1 Mediated Inhibition of RXR Function	21/222	Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	7/17
Top Tox Lists	Genes upregulated in response to chronic renal failure	1/5	Liver proliferation	29/240	Cholesterol Biosynthesis	13/16
	Glutathione depletion - Hepatocellular Hypertrophy	1/8	Cardiac Hypertrophy	28/352	Cardiac Hypertrophy	34/352
	Liver Necrosis/Cell Death	3/310	Cardia Fibrosis	20/215	Liver Necrosis/Cell Death	26/310
	FXR/RXR Activation	2/126	LPS/IL-1 Mediated Inhibition of RXR Function	22/254	Cardiac Necrosis/ Cell Death	23/294
	Cholesterol Biosynthesis	1/16	PXR/RXR Activation	10/65	Cardiac Fibrosis	18/25
Top Diseases	Connective Tissue Disorders	14	Cancer	641	Cancer	579

Table 3.2 (continued)

	Hereditary Disorder	25	Gastrointestinal Disease	615	Organismal Injury and Abnormalities	585
	Organismal Injury and Abnormalities	47	Organismal Injury and Abnormalities	650	Gastrointestinal Disease	539
	Cardiovascular Disease	10	Cardiovascular Disease	160	Dermatological Diseases and Conditions	353
	Hematological Disease	18	Inflammatory Response	172	Cardiovascular Disease	148
Top Molecular and Cellular Functions	Carbohydrate Metabolism	10	Lipid Metabolism	161	Amino Acid Metabolism	37
	Lipid Metabolism	11	Molecular Transport	184	Small Molecule Biochemistry	144
	Molecular Transport	12	Small Molecule Biochemistry	185	Lipid Metabolism	110
	Small Molecule Biochemistry	20	Vitamin and Mineral Metabolism	55	Vitamin and Mineral Metabolism	48
	Cell-To-Cell Signaling and Interaction	7	Cell Death and Survival	252	Cell Morphology	79

"Canonical Pathways" and "Tox Functions" consist of predefined lists of genes; numbers represent the total number of DEGs in each group out of the total number possible. Gene numbers for "Diseases" and "Molecular and Cellular Functions" represent the total number of significant DEGs associated with each category.

Table 3.3 Pathways conserved across developmental stages following oil exposure in *C. variegatus*

Canonical Pathway	-log P value		
	E	YS	FF
Glucose and Glucose-1-phosphate Degradation	3.58	1.36	1.44
Folate Transformations I	1.7	2.66	2.79
UDP-N-acetyl-D-galactosamine Biosynthesis II	1.62	1.36	1.44
Androgen Biosynthesis	1.51	3.14	3.32
γ-glutamyl Cycle	1.51	2.08	2.2

(E = embryo, YS = yolk-sack, FF=free-feeding). Significance is reported as -log p-values such that higher numeric value indicates greater statistical significance.

Table 3.4 Top altered pathways and functions in yolk-sack *C. variegatus* and *F. grandis*

	Yolk-sack *C. variegatus*	# of genes	Yolk-sack *F. grandis*	# of genes
Top Canonical Pathways	Cholesterol Biosynthesis I	5/13	NRF2-mediated Oxidative Stress response	11/199
	Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	5/13	Acute Phase Response Signaling	10/176
	Cholesterol Biosynthesis III (via Desmosterol)	5/13	TNFR1 Signaling	5/50
	Osteoarthritis Pathway	16/212	TNFR2 signaling	4/30
	LPS/IL-1 Mediated Inhibition of RXR Function	16/222	Death Receptor Signaling	6/63
Top Tox Lists	Liver proliferation	22/240	Cardiac Hypertrophy	15/352
	Cardiac Hypertrophy	25/352	Liver Proliferation	11/240
	LXR/RXR Activation	11/123	NRF2-mediated Oxidative Stress Response	11/251
	Cardiac Firbrosis	15/215	Liver Necrosis/Cell Death	12/310
	FXR/RXR Activation	11/126	Hepatic Cholestasis	8/163
Top Diseases	Cancer	498	Cancer	227
	Organismal Injury and Abnormalities	503	Organismal Injury and Abnormalities	231
	Gastrointestinal Disease	476	Gastrointestinal Disease	218
	Cardiovascular Disease	126	Caradiovascular Disease	57
	Hepatic System Disease	352	Skeletal and Muscular Disorders	58
Top Molecular and Cellular Functions	Lipid Metabolism	126	Lipid Metabolism	48
	Molecular Transport	148	Small Molecule Biochemistry	70
	Small Molecule Biochemistry	147	Vitamin and Mineral Metabolism	21
	Vitamin and Mineral Metabolism	41	Amino Acid Metabolism	10

Table 3.4 (continued)

	Cell Death and Survival	192	Cell Morphology		56

Values from analyses considering only genes annotated in both genomes used for transcript alignments. In IPA, "Canonical Pathways" and "Tox Functions" consist of predefined lists of genes; numbers represent the total number of DEGs in each group, out of the total number possible. Gene numbers for "Diseases" and "Molecular and Cellular Functions" represent the total number of significant DEGs associated with each category.

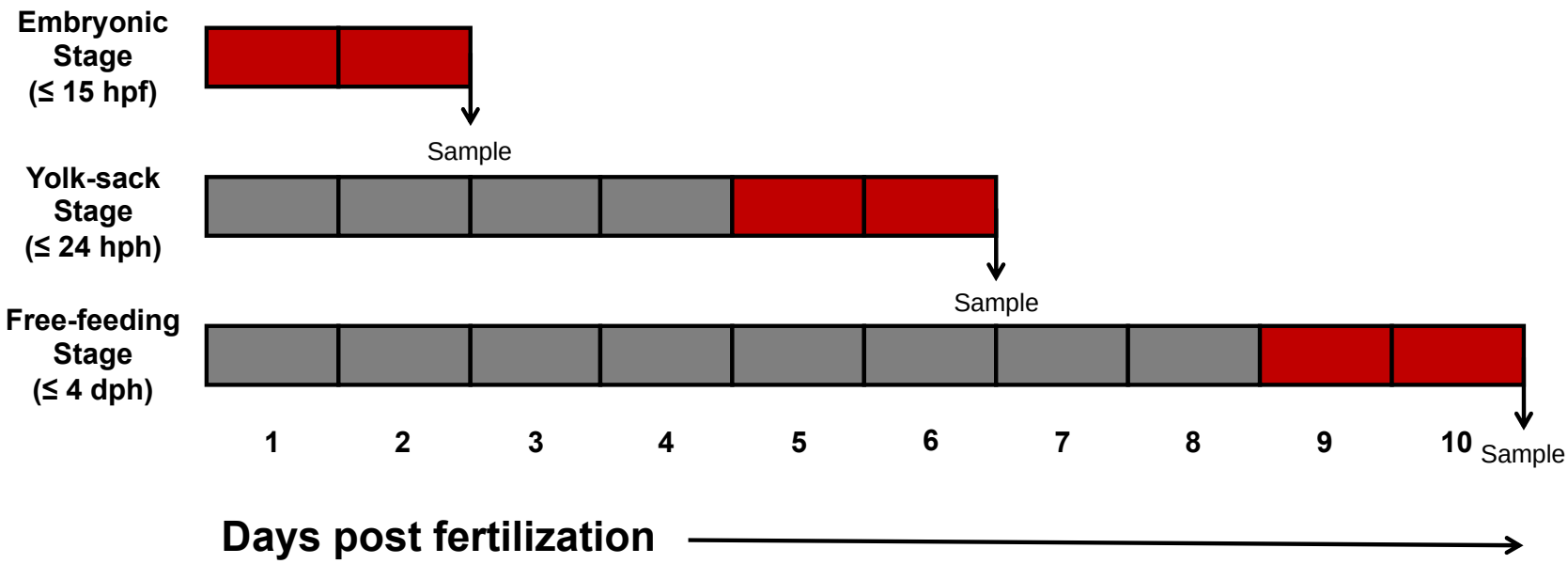

Figure 3.1 Exposure design across *C. variegatus* developmental stages

Red boxes indicate exposure windows.

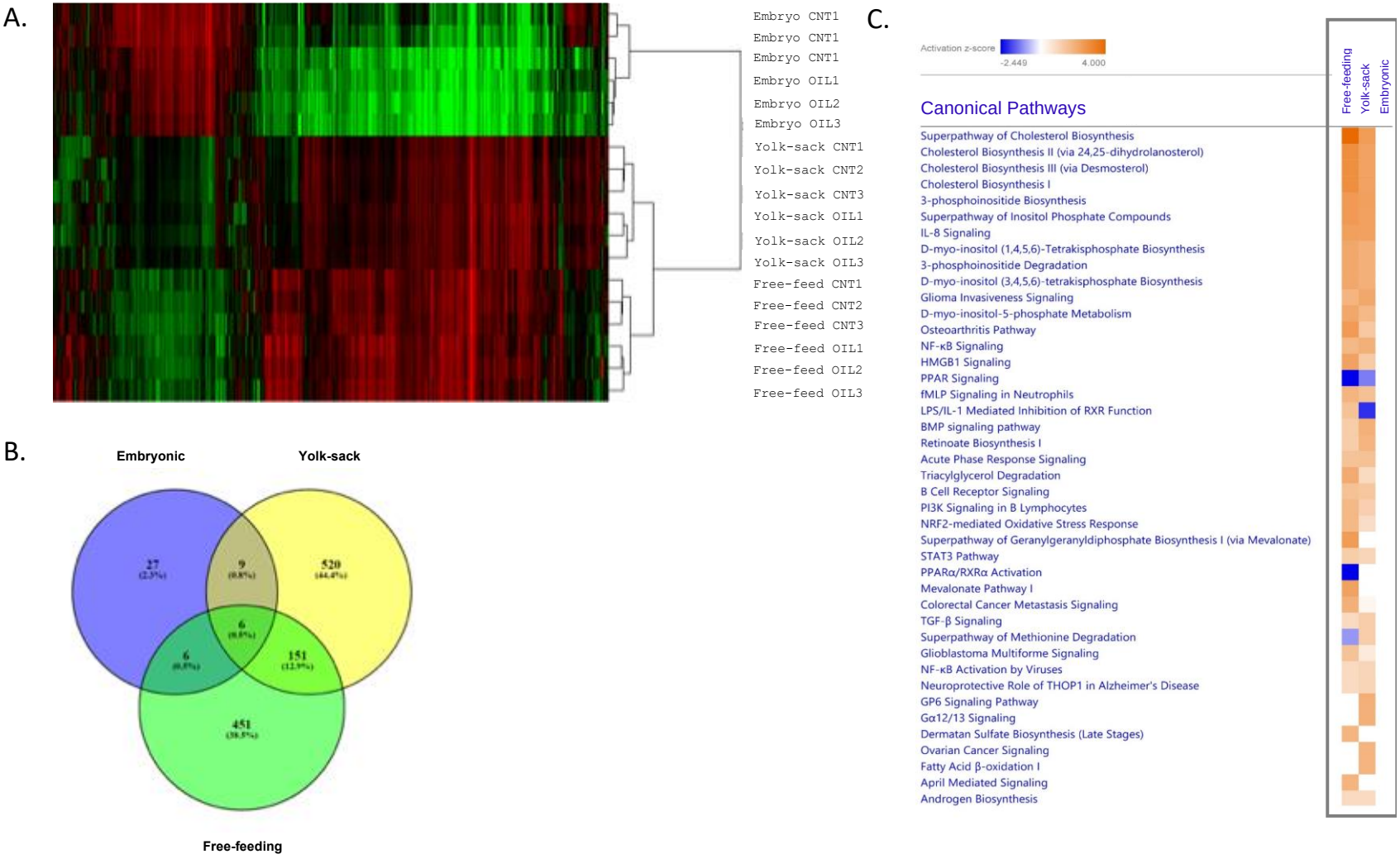

Figure 3.2 Transcriptional patterns across life stages in *C. variegatus*.

Figure 3.2 (continued)

A) Hierarchical cluster analysis of expression (TPM) using all measured genes. Note that embryonic transcriptional patterns differ substantially from yolk-sack and free-feeding patterns, which were similar to each other. B) Venn diagram illustrating the unique and shared DEGs following oil treatment across life stages. Note that oil exposure elicited differential expression of few genes in embryos, but resulted in a substantial number of differentially expressed genes in yolk-sack and free-feeding groups. C) Altered canonical pathways following oil exposure. Pathways and functions are ranked by z-score, a measure predicting activation or inhibition of processes based on fold change data of significant DEGs (FDR $\leq$0.05). Functions in orange are predicted to be activated; those in blue are predicted inhibited. Blank squares indicate insufficient information to make a prediction. Note that for embryos there were few genes that passed FDR cutoff, therefore few processes had sufficient information to calculate z-scores.

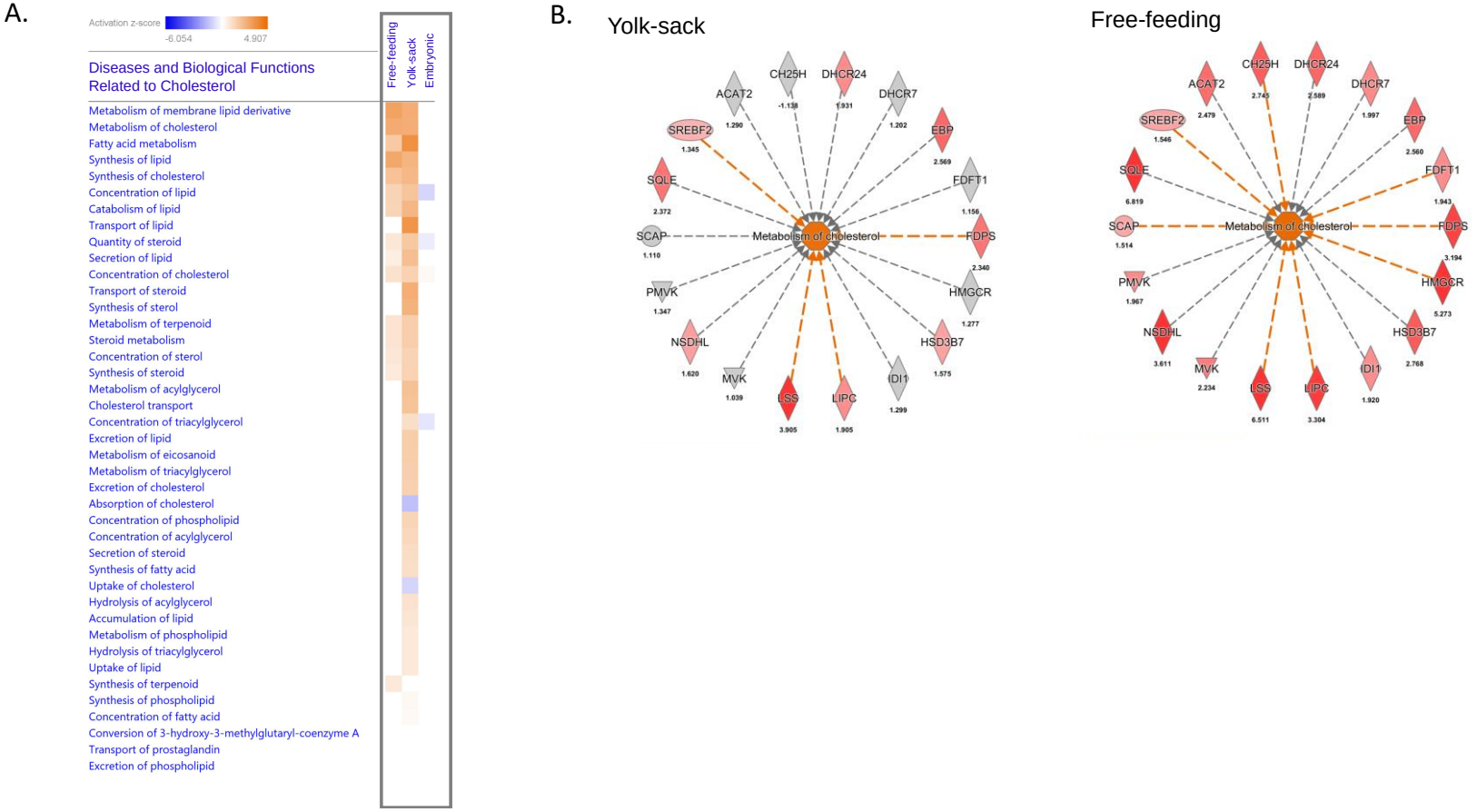

Figure 3.3 Altered cholesterol-related processes following oil exposure in larval *C. variegatus*.

A)Altered functions ranked by z-score. Note functions are predicted as activated in both larval life stages. B) Gene networks related to cholesterol metabolism. Upregulated genes are depicted in red; downregulated genes in blue. Genes in gray did not pass statistical cutoff (FDR ≤0.05). Numbers represent log2 fold change from control. Note upregulation of genes encoding cholesterol biosynthesis enzymes and cholesterol homeostasis regulators.

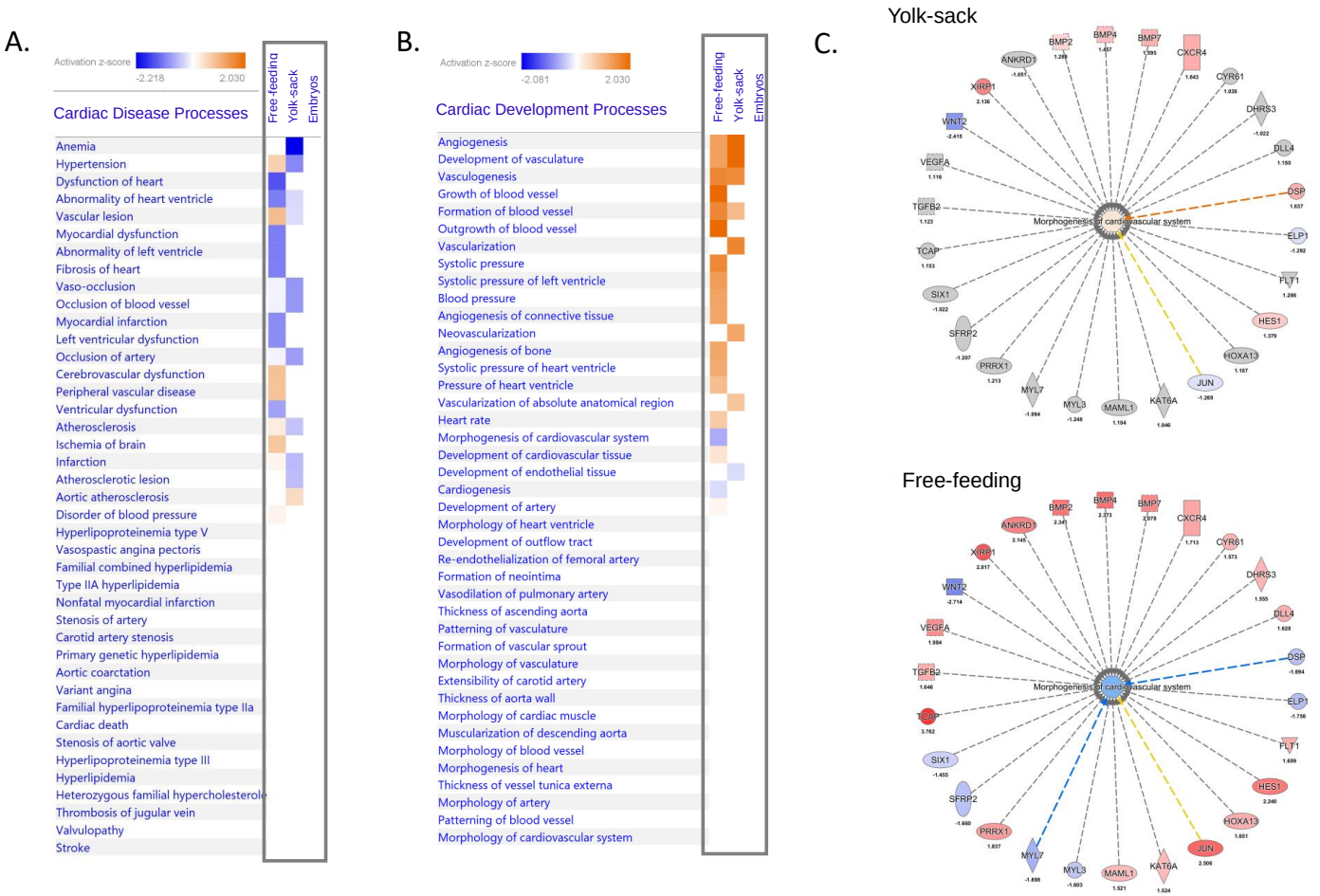

Figure 3.4 Altered processes related to cardiac function and development following oil exposure in larval *C. variegatus*

A) Altered cardiac disease processes ranked by z-score. B) Altered cardiac development processes ranked by z-score. Note that while few terms are shared among life stages, developmental processes were generally upregulated in larval stages. C) Gene networks related to morphogenesis of cardiovascular system in larval stages. Note upregulation of *bmp4* in both groups.

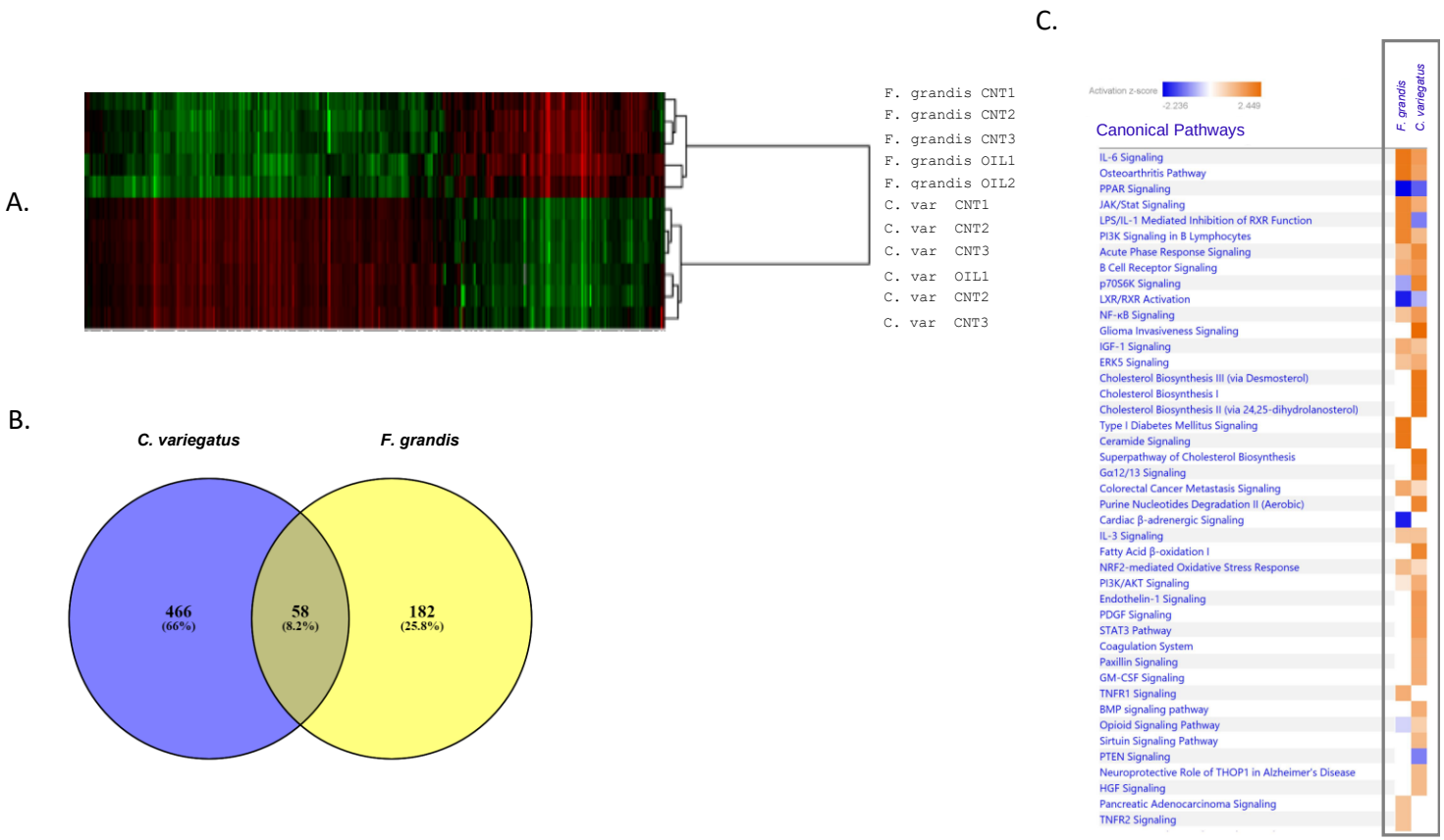

Figure 3.5 Transcriptional patterns in post-hatch *F. grandis* and *C. variegatus*

A)Hierarchical cluster analysis of expression (TPM) using all genes measured in both data sets. Note that transcriptional patterns differ between species. B) Venn diagram illustrating the unique and shared DEGs following oil treatment between species. Note that oil exposure elicited differential expression of fewer genes in *F. grandis* than in *C. variegatus*. C) Altered canonical pathways following oil exposure, ranked by z-score.

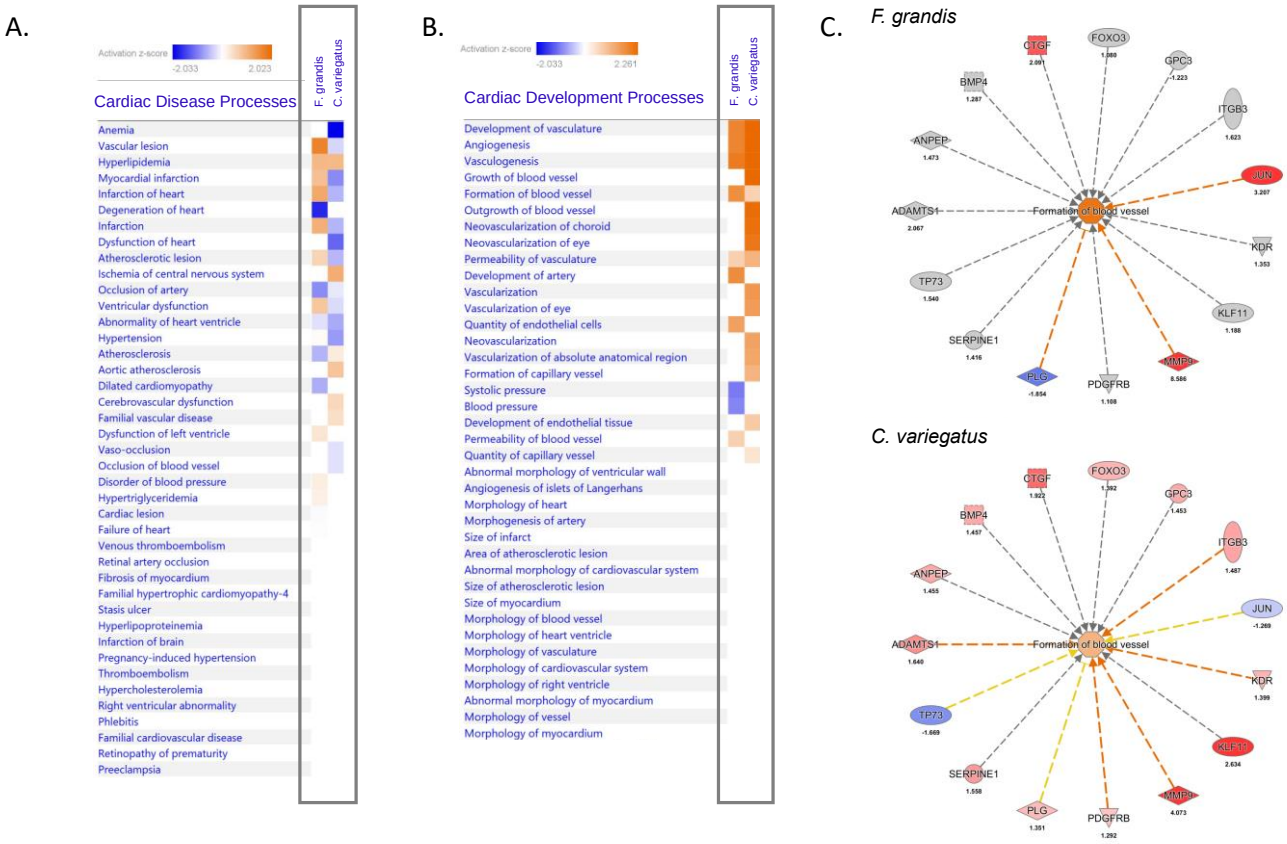

Figure 3.6 Altered processes related to cardiac function and development following oil exposure in yolk-sack *F. grandis* and *C. variegatus*.

A) Altered cardiac disease processes ranked by z-score. B) Altered cardiac development processes ranked by z-score. Note that while few terms are shared among life stages, developmental processes were generally upregulated in both species. C) Gene networks related to morphogenesis of cardiovascular system in larval stages. Note upregulation of BMP4 in both groups (BMP4 did not pass FDR cutoff in F. grandis).

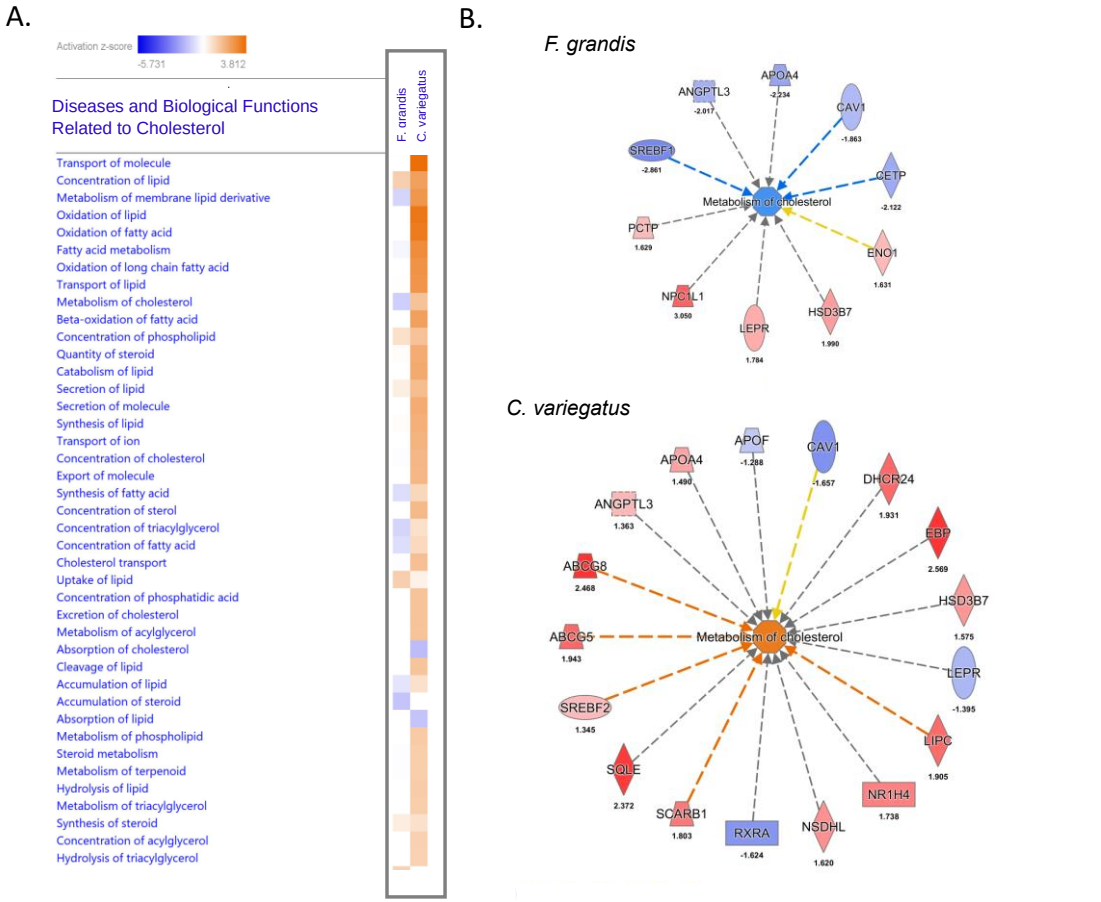

Figure 3.7 Altered processes related to cholesterol following oil exposure in *C. variegatus* and *F. grandis*

A) Impacted cholesterol functions. B) Gene networks predicting activation or inhibition of cholesterol metabolism. Blue symbols represent measured downregulation of genes; red symbols represent upregulation. Note opposing expression patterns for *srebf1* and *2*, which are master regulators of cholesterol biosynthesis

CHAPTER IV – TRANSIENT OIL EXPOSURE ALTERS DNA METHYLTRANSFERASE (DNMT) EXPRESSION IN LARVAL CYPRINODON VARIEGATUS

4.1 Abstract

DNA methylation in the promoter of genes can silence transcription, therefore there is typically an inverse relationship between promoter methylation and gene expression. Studies examining DNA methylation in fish exposed to PAH contaminants have documented altered methylation of gene promoters and general genomic hypomethylation following exposure. We hypothesized that oil, a PAH mixture, affects establishment of DNA methylation patterns in developing fish by disrupting transcription of DNA methyltransferase enzymes (*dnmts*). To test this hypothesis, we exposed developing *Cyprinodon variegatus* to high energy water accommodated fraction (HEWAF) oil solutions for 48h and measured *dnmt* transcription and methylation of the aryl-hydrocarbon receptor repressor (*ahrr*) promoter. To determine whether larval methylation patterns were sustained long-term, we reared exposed larvae to adulthood and measured *ahrr* methylation in adult liver tissue. Finally, to examine relationships between promoter methylation and gene expression we re-exposed adults previously exposed to oil and measured induction of *ahrr* transcription. Our results indicate that transient exposure to oil during development alters *dnmt* expression but does not impact establishment of *ahrr* methylation. Additionally, we found no evidence of an inverse relationship between *ahrr* promoter methylation and gene expression in adult *C. variegatus*. These data suggest a mechanism underlying PAH-induced hypomethylation

noted in previous studies, and contribute to a growing body of literature revealing a complex relationship between promoter methylation and gene expression.

4.2 Introduction

DNA methylation is one of the most extensively studied epigenetic marks. In vertebrates, it involves the addition of a methyl group to cytosine/guanine (CpG) dinucleotide motifs. Genomic regions with a high occurrence of CpG sites, referred to as CpG islands, frequently occur in the promoter of genes and generally lack methylation (Deaton and Bird 2011). Unmethylated CpG islands are targets for transcription factors and associated with active gene expression; conversely, methylation of CpG islands is known to be associated with gene silencing (Robertson and Jones 2000; Jones and Takai 2001). Therefore, there is generally an inverse relationship between promoter methylation and gene expression. The majority of DNA methylation is established early in development and modulates gene expression during tissue differentiation and organogenesis (Reik et al. 2001; Kamstra et al. 2015). However, exposure to environmental contaminants can disrupt normal methylation patterns (Head et al. 2012; Head 2014; Vandegehuchte and Janssen 2014). DNA methylation is mitotically heritable, therefore aberrant methylation patterns may persist throughout an organism's lifetime and have long-term effects on gene expression, possibly leading to disease states in adulthood (Dolinoy et al. 2007).

One class of contaminants known to alter DNA methylation in developing fish is polycyclic aromatic hydrocarbons (PAHs) (Herbstman et al. 2012; Breton CV 2014; Yan et al. 2014; Brandenburg and Head 2018). Studies in zebrafish (*Danio rerio*) document

the ability of individual PAH moieties to alter methylation patterns, as embryonic exposure to the PAH benzo(a)pyrene (BaP) elicits a reduction in global genome methylation and alters methylation of promoters in several disease-related genes (Fang et al. 2013; Corrales et al. 2014). However, in these studies, altered methylation was not broadly correlated with gene expression, prompting questions about the relationship between PAH-induced methylation changes and transcriptional profiles (Corrales et al. 2014). Additionally, while these studies have described the ability of individual PAH moieties to alter global and gene specific methylation, there is little information regarding how oil, a PAH mixture, impacts methylation in fish models.

One possible mechanism governing methylation changes following PAH exposure includes dysregulation of genes encoding DNA methyltransferase (DNMT) enzymes. In mammals, DNA methylation is accomplished via the action of three DNMTs; DNMT3a and 3b, which establish de novo methylation patterns, and DNMT1 which maintains methylation during mitosis (Okano et al. 1999; Bestor 2000; Reik et al. 2001; Goll and Bestor 2005). Studies from mammalian cell lines demonstrate that PAH exposure elicits upregulation of *DNMT* expression and is associated with increased methylation of gene promoters (Liu et al. 2013). How PAH exposure impacts *dnmt* expression and methylation patterns in fish models is less clear. One study in Japanese flounder (*Paralichthyes olivaceous*) noted down-regulation of *dnmt* transcription in adult gill tissue following oil exposure, although methylation levels were not measured in that study (Zhu et al. 2016). In developing zebrafish, while BaP elicited effects on both global and gene specific methylation in developing zebrafish, it did not cause associated changes in *dnmt* expression or activity (Corrales et al. 2014). Interestingly, exposure to

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin), which also causes global and gene-specific methylation changes, has been linked to altered *dnmt* expression in developing zebrafish (Aluru et al. 2015). PAHs and dioxins share similar modes of action, eliciting effects via activation of the aryl hydrocarbon receptor (AHR), a ligand binding transcription factor which attaches to xenobiotic response elements (XREs) in the promoters of target genes to modulate transcription (Billiard et al. 2002). Promoters in the zebrafish *dnmt3* family of genes all contain a XRE, and transactivation assays confirm the ability of zebrafish AHR to bind to *dnmt3* promoters (Aluru et al. 2015). These results suggest that the mechanism underlying methylation changes following PAH exposure could be AHR mediated disruption of *dnmt* gene expression.

Genes in the AHR pathway are particularly susceptible to methylation changes (Novakovic et al. 2014; Aluru et al. 2015; Bojesen et al. 2017; Brandenburg and Head 2018). One well studied example is methylation in the AHR repressor (AHRR). The AHRR inhibits the activity of the AHR, thereby diminishing transcriptional induction of AHR activated genes (Brauze et al. 2006). Repressor inhibition of the AHR pathway confers protection against toxic intermediates generated during xenobiotic transformation; in particular, the activity of cytochrome p4501A1 (CYP1A1) often results in epoxide intermediates, which are highly reactive and can form DNA adducts leading to oncomutations (Androutsopoulos et al. 2009; Becker et al. 2015). Transcription of the AHRR is controlled by the AHR, therefore ligand activation of the AHR initiates a negative feedback loop which down-regulates expression of genes in the AHR pathway (Tsuchiya et al. 2003; Haarmann-Stemmann and Bothe 2007). Exposure to AHR ligands is known to alter DNA methylation patterns in the AHRR gene. In

humans, epidemiological studies document hypo-methylation of the AHRR in smokers, who are chronically exposed to PAH contaminants in cigarette smoke (Monick et al. 2012; Bojesen et al. 2017). There is a dearth of information regarding these effects in fish, however Aluru et al. (2105) found that dioxin exposure resulted in hypermethylation of the *ahrr* promoter in embryonic zebrafish. This hypermethylation was not correlated to *ahrr* mRNA expression, therefore the effects of altered methylation on *ahrr* negative feedback are unclear. There remains a need for data regarding whether PAHs alter methylation of the *ahrr* promoter in fish and whether differential methylation modulates negative feedback of the AHR pathway.

In this study we hypothesize that oil, a PAH mixture, affects establishment of DNA methylation patterns in developing fish by altering transcription of *dnmt*s. To test this hypothesis, we measured *dnmt* expression and methylation of the *ahrr* promoter following oil exposure in developing sheepshead minnows (*Cyprinodon variegatus)*. To determine whether developmental effects were sustained into adulthood, we reared exposed individuals to reproductive maturity and measured *ahrr* methylation. We also measured induction of AHR pathway genes in adults previously exposed to oil to examine relationships between promoter methylation and gene expression. Our results indicate that transient exposure to oil during development substantially alters *dnmt* expression but does not impact establishment of *ahrr* methylation in larvae. Additionally, we found no evidence of an inverse relationship between *ahrr* promoter methylation and gene expression in adult *C. variegatus*.

4.3 Materials and Methods

4.3.1 Animal husbandry

C. variegatus broodstock were maintained at the University of Southern Mississippi's Toxicology Lab in Ocean Springs, MS. Fish were kept in 300L circulating raceways containing 15 ppt artificial sea water at 25 C 15 ppt on a 16:8 light/dark cycle and fed commercial flake food daily. Breeding was initiated by the placement of breeding nets in raceways. After 12 hours embryos were collected and examined for fertilization. Embryos were either immediately used in exposures or reared to yolk-sac (24 hph) or free-feeding (4 dph) larval stages. See Simning (2007) for additional detail.

4.3.2 Preparation and analysis of High Energy Water Accommodated Fractions (HEWAF)

All HEWAF stocks were prepared using Deepwater Horizon source oil according to methods described by Forth et al. (2016). Concentrations for 29 parent PAHs, alkyl PAHs, and alkyl PAH homologs (tPAH29) were assessed via gas chromatography coupled with tandem mass spectrometry (GC/MS/MS) for each HEWAF stock. Stocks from *dnmt* transcription experiments were analyzed at the University of Connecticut Center for Environmental Sciences and Engineering (Storrs, Connecticut); stocks from *ahrr* promoter methylation experiments were analyzed at Mote Marine Laboratory (Sarasota, FL). Measured PAH concentrations for test solutions were inferred using a fluorescence method described by Greer et al. (2012) (Table 1).

4.3.3 Measurement of DNMT expression

To examine the effects of oil exposure on *dnmt* expression, *C. variegatus* at three developmental stages (embryonic, yolk-sac, or free-feeding) were exposed to HEWAF for 48 h (Fig. 1A). HEWAF stocks were serially diluted to create test solutions at 100%, 50%, 12.5%, 6.25% and 0% (control) of stock concentration (Table 1). Exposures were conducted under normoxic conditions (≥ 5 mg/L O2) at 30C and 10 ppt. After 48h, organisms were sampled and stored in RNA later at -80C until further analysis. See Simning et al. (2018) for additional detail.

To obtain sufficient material for gene expression analyses, total RNA was extracted from 10 pooled animals per replicate (n=4 replicates per treatment) using RNEasy Mini kit (Qiagen, Hilden, Germany). RNA was quantified spectrophotometrically (Nanodrop 2000, Thermo Scientific, Waltham, MA) and reverse transcribed using RevertAid RT kit (Thermo Sceintific, Waltham, MA). Quantitative PCR (qPCR) assays were performed to measure expression of *cyp1a1* (as a biomarker of PAH exposure), *dnmt1, dnmt3a, dnmt3b,* and *β-actin* (housekeeping gene) (primers listed in Table 4.2). Samples were assayed in triplicate using Fast SYBR Green Master Mix on an ABI 7500 Fast Real Time PCR System (Thermo Scientific, Waltham, MA.) Relative expression was calculated as ($2^{-\Delta\Delta CT}$) where $\Delta\Delta CT = \Delta CT(experimental) - \Delta CT(controls)$ (Livak and Schmittgen 2001).

4.3.4 Identification of AHRR promoter and CpG islands

A reference genome for *C. variegatus* is publicly available (NCBI Genome ID: 13078), however it lacks annotation of promoter regions. To locate the *C. variegatus ahrr* promoter, we compared sequence 3500 bp upstream of the *ahrr* transcription start site to published sequence for the *Fundulus heteroclitus ahrr* promoter (Karchner et al. 2002). Putative *C. variegatus* promoter sequence was analyzed using MethPrimer software to identify CpG islands (Li and Dahiya 2002).

4.3.5 Measurement of AHRR methylation

For experiments investigating the effects of oil exposure on *ahrr* methylation, freshly hatched *C. variegatus* were purchased from Aquatic Biosystems (Fort Collins, CO) and reared to 4 days post-hatch when yolk-sacks were absorbed and larvae were free feeding. Animals were reared in 15 ppt artificial sea water, under normoxic conditions (≥ 5 mg/L O2) at 30°C and a 12:12 light/dark cycle. Upon initiation of feeding, animals were fed *Artemia* nauplii daily; feeding continued throughout exposures.

We exposed larvae to 25% HEWAF (tPAH29 437.51 ± 7.38 μg/L) or artificial sea water at 30°C and 15 ppt for 48h. Sixty larvae were randomly placed into 250 ml beakers containing 100 ml of test solution. After 48h, 10 larvae from each replicate (n=4 replicates per treatment) were collected for qPCR analysis; remaining larvae were transferred to clean artificial sea water (15 ppt) and depurated for 48h to allow stabilization of oil-induced methylation effects. Following depuration, 10 additional larvae were sampled for promoter methylation analysis; all remaining larvae were reared to adulthood under standard animal husbandry conditions (Figure 4.1B).

Gene expression was measured for *cyp1a1* and *ahrr* following 48h oil exposure to examine induction of the AHR pathway and initiation of AHRR negative feedback loop. RNA was extracted and reverse transcribed from a pool of 5 larvae per replicate, and assayed via qPCR as described above (see Table 4.2 for primer sequences).

DNA from depurated free-feeding larvae (n=4 pools per treatment, 5 larvae/pool) and adult male and female livers (n=3 per treatment, no pooling) was extracted and RNAse treated using Qiagen's DNEasy Blood and Tissue kit (Qiagen, Hilden, Germany). Extracted DNA was bisulfite treated using EZ DNA Methylation Gold kit (Zymo Research, Irvine, CA) and used as template in PCR reactions to amplify the identified CpG island region of the AHRR promoter. Bisulfite specific primers (Table 2) were designed using MethPrimer software (Li and Dahiya, 2002), and amplification reactions performed using Platinum Taq High Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA). PCR product was ligated into p-GEM T Easy Vector System (Promega, Madison, WI) and transformed into JM-109 Competent *E. coli* Cells (Promega, Madison, WI). Transformed bacteria were plated onto LB ampicillin agar plates containing 40 μL each of X gal (20 mg/mL), IPTG (100mM). Following overnight incubation at 37°C, ten white colonies from each sample were selected and plasmid DNA isolated using Zyppy Plasmid Miniprep kit (Zymo Research, Irvine, CA). Purified plasmid DNA was sent to Eurofins (New Orleans, LA) for sequencing and sequence data analyzed using CLC Genomic Workbench 12 (Qiagen, Hilden, Germany). Efficiency of DNA bisulfite conversion was calculated as (# of converted non-CpG cytosines/number of non-CpG cytosines in reference sequence); only samples with efficiencies ≥ 98% were

included in methylation analysis. Average methylation per sample was calculated as (∑ methylated CpG residues per sample)/(number of colonies sequenced) ± SD.

4.3.6 Induction of AHR pathway in previously exposed animals

To determine whether developmental exposure to oil alters transcriptional induction of AHR pathway genes, adults reared from free-feeding larval experiments were re-exposed to 25% HEWAF (tPAH29 407.23 μg/L) or clean artificial sea water for 24h (Figure 4.1C). RNA was extracted and reverse transcribed as previously described, and qPCR performed to measure expression of *cyp1a1* and *ahrr*.

4.3.7 Statistical analyses

All statistical analysis were conducted using StatPlus (6.1.7) software (AnalystSoft, Inc., Walnut CA). A one-way ANOVA followed by a Fisher LSD post-hoc test was performed to examine the effect of oil exposure on *dnmt, cyp1a1*, and *ahrr* expression, and promoter methylation. To investigate the effects of sex, previous exposure, and PAH dose on *cyp1a* and *ahrr* expression in adults, a three-way ANOVA was performed. All results were considered significant at $p \leq 0.05$.

4.4 Results

4.4.1 *dnmt* expression in developing *C. variegatus*

Significant oil-induced upregulation of *cyp1a1* was observed across all HEWAF doses in all three life stages, indicating PAH uptake and activation of the *ahr* pathway (Simning 2007). Mortality for all groups was low across all life stages and treatments,

and therefore was not considered a confounding effect on gene expression. See Simning (2007) for additional detail.

We detected transcriptional dysregulation for all three *dnmt* genes following oil exposure (Figure 4.2). Oil induced a significant, dose-dependent downregulation of *dnmt3a* across all three life stages. Transcription of *dnmt3b* was upregulated in embryos, but was not significantly affected in yolk-sack or free-feeding larvae. Dysregulation of *dnmt1* expression following oil-exposure was bi- directional, with slight upregulation in embryos and substantial down regulation in later larval stages.

4.4.2 Methylation of the *C. variegatus ahrr* promoter

Our previous work demonstrates that oil exposure elicits substantial transcriptional dysregulation in free-feeding *C. variegatus* compared to other developmental stages (Ch. 3); therefore, we used this stage to investigate oil-induced effects on *ahrr* promoter methylation. We measured transcription of *cyp1a1* and *ahrr* immediately following 48 hr oil exposure to ensure that our *ahrr* promoter results were associated with uptake and metabolism of PAHs. Both were significantly upregulated, indicating activation of the AHR pathway and initiation of the AHRR negative feedback mechanism (Figure 4.3).

Comparison of the putative *C. variegatus* promoter with sequence from *F. heteroclitus* revealed conservation of characteristic features including three xenobiotic response elements and a GC box (Figure 4.3). Submission of putative *C. variegatus* sequence to MethPrimer returned results for one CpG island 350 bp in length consisting

of 33 CpG residues. Bisulfite primers were designed using MethPrimer to amplify a 311 bp region of sequence spanning 28 CpG residues (primer sequences listed in Table 2).

Following 48h of exposure we depurated remaining larvae for 48h to allow stabilization of any oil-induced effects to methylation. We detected low overall methylation of the *ahrr* promoter in depurated free-feeding larvae, with 0.9 ± 1.2 methylated CpG sites in control promoters, and 0.7 ± 0.86 methylated CpGs in exposed promoters. Our analysis revealed no statistical differences between control and exposed groups, and no consistently methylated CpG residues in either treatment (Figure 4.5A). After larval sampling, we allowed remaining larvae to grow to adulthood for seven months. Analysis of *ahrr* promoter in adult livers also revealed low methylation levels. Adult females exhibited 0.6 ± 0.76 methylated CpGs in control promoters and 0.4 ± 0.68 in exposed; adult males exhibit similarly methylation levels, with 0.7 ± 0.82 methylated CpGs in control promoters and 0.7 ± 0.80 in exposed. As with larvae, we found no statistical differences among treatments or genders, and no consistently methylated CpG residues in any group (Figure 4.5B-C).

4.4.3 Re-exposure of previously exposed animals

We performed re-exposure experiments to determine whether prior exposure to oil impacts subsequent activation of AHR-mediated transcription, as this might suggest epigenetic changes aside from *ahrr* promoter methylation. After 24h of re-exposure, all adults exhibited significant upregulation of *cyp1a1* expression, indicating activation of the AHR pathway (Figure 4.6A). The magnitude of this induction was similar to that observed in larval samples exposed to similar oil concentrations; however there were no

differences between previously exposed and previously unexposed fish or gender specific differences. While expression of *cyp1a1* indicated activation of AHR-mediated transcription, expression of *ahrr* was not significantly altered in adults following 24 of re-exposure to oil. These results indicate no transcriptional activation of the AHRR negative feedback mechanism, despite activation of the AHR pathway and low methylation levels in the *ahrr* promoter.

4.5 Discussion

In this study we sought to examine the effect of transient oil exposure on expression of *dnmt* transcripts to better understand how PAHs modify the establishment of DNA methylation in developing fish. We observed oil-induced transcriptional effects for all three *dnmt* genes examined, with the majority of dysregulation related to decreased expression patterns. In particular, the *de novo* DNA methyltransferase *dnmt3a* exhibited significant downregulation across all stages, and the maintenance methyltransferase *dnmt1* was significantly downregulated in later larval stages. These results suggest a possible mechanism underlying genome-wide hypomethylation following PAH exposure in zebrafish embryos (Fang et al. 2013).

In zebrafish, the expression of *dnmt3a* orthologs gradually increases throughout development, during critical windows in which genome-wide and gene-specific methylation patterns are established (Smith et al. 2011). Therefore, diminished expression of *dnmt3a* could alter the establishment of normal methylation patterns and result in global hypomethylation such as that noted in embryonic zebrafish. Previous studies have also linked downregulation of *dnmt1* transcription to hypomethylation in

fish following exposure to other classes of contaminants (Xing et al. 2014; Laing et al. 2016) . This hypomethylation is likely linked to passive demethylation of cytosines which occurs when methylation is not maintained by *dnmt1* during mitotic cell division (Wu and Zhang 2010).

We observed downregulation of *dnmt1* in yolk-sack and free-feeding larvae which suggests that in addition to embryos, PAHs may also disrupt methylation patterns at later developmental stages via a "dilution" effect. Data regarding methylation following larval exposure to contaminants is lacking, as it has been assumed that the greatest disruptions to methylation would occur during embryonic development, when methylation patterns related to tissue differentiation and organogenesis are established (Kamstra et al. 2015). However, recent work suggests that larvae may be susceptible to substantial alterations to the DNA methylome following environmental perturbations (Anastasiadi et al. 2017). Whether the transcriptional effects noted herein impact genome-wide methylation changes is unclear, however our data suggests that future studies should seek to examine the effects of oil on global methylation patterns across developmental stages.

A second aim of this study was to investigate whether developmental exposure to PAHs elicits changes in methylation of the *ahrr* promoter. Our results indicate that there is minimal methylation in the proximal promoter of the *ahrr* in *C. variegatus* and that, despite dysregulation of *dnmt* transcription, oil exposure does not alter its methylation state. This limited promoter methylation should render *ahrr* transcriptionally labile, such that PAH exposure readily stimulates its transcription. This was true in our larval data, as 48h of oil exposure significantly upregulated *ahrr* transcription. However, data from adults revealed similarly low levels of methylation in the *ahrr* promoter, yet oil exposure

failed to induce a significant increase in *ahrr* transcription. It is possible this lack of induction is an artifact of exposure duration, as our larvae were exposed to oil for 48h while adults were exposed for 24h. However, both groups were exposed to similar oil concentrations (tPAH29 437 and 407 μg/L, respectively) and exhibited comparable induction of *cyp1a1*, therefore it can be assumed the AHR pathway was equivalently activated in both circumstances. Normally, the ability of the AHRR to repress expression of transcription of AHR pathway genes, such as *cyp1a1*, is considered a protective mechanism against the creation of toxic PAH intermediates during xenobiotic clearance (Becker et al., 2015). Therefore, the lack of *ahrr* induction in adults is surprising, given the low methylation we observed in the promoter.

This disconnect between *ahrr* promoter methylation and gene expression has also been documented by Aluru et al (2015) who found hypermethylation of the *ahrr* promoter in dioxin exposed zebrafish, but no associated changes in mRNA expression. These results in combination with our data contribute to a growing body of literature examining the relationship between promoter methylation and gene expression. For example, Corrales et al. (2014) observed altered methylation in promoters of 10 of genes in zebrafish following benzo[a]pyrene exposure, but found only two instances of a correlation between methylation state and gene expression. While the majority of studies correlating methylation with gene expression have focused on specific loci, a few studies have taken advantage of high throughput methods to investigate the relationship between methylation and transcription, with mixed results. While one study from human placentas confirmed the paradigm of an inverse relationship between promoter methylation and gene expression (Lim et al. 2017), several have found little correlation

between differentially expressed genes and differentially methylated promoters (Roforth et al. 2015; Day et al. 2016; Schachtschneider et al. 2016; Aluru et al. 2018). For example, a comparison of methylation in bone marrow between young and elderly women found that of 1528 differentially methylated genes, only 758 had mRNA expression in the opposite direction of methylation (Roforth et al. 2015). Another study investigating the effects of 3, 3', 4, 4', 5-pentachlorobiphenyl (PCB 126) on adult zebrafish brains found that of 1715 differentially expressed genes, only 33 were associated with altered methylation, and only 12 expressed in the opposite direction of methylation (Aluru et al. 2018). These results suggest DNA methylation plays a complex role in the modulation of gene expression, and highlight the need for additional studies to more accurately characterize interaction between methylation and transcriptional activation.

4.6 Conclusion

To our knowledge, this study documents the first evidence of oil-induced alterations to *dnmt* transcription in developing fish. While this transcriptional disruption was not associated with altered methylation of the *ahrr* promoter, our results suggest that oil may modify methylation throughout the genome, and imply a mechanism governing PAH-induced methylation changes observed in previous studies. Our results revealed substantial dysregulation of *dnmt* transcription in larval developmental stages, which suggests that in addition to embryos, the methylation altering effects of contaminants on larval stages should be examined. One unique feature of this study is the duration of exposure. We observed transcriptional effects to methylation machinery following 48h

of oil exposure; a much shorter duration than the majority of studies examining methylation effects (Vandegehuchte and Janssen 2011). This suggests the possibility that even short term exposure to contaminants during development can lead to epigenetic effects that might be sustained into later life. Finally, this work contributes to growing body of literature revealing a complex relationship between promoter methylation and gene expression.

4.7 References

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Table 4.1 tPAH29 HEWAF concentration for test solutions.

Test solution tPAH29, *dnmt* expression exposures				
Life stage	6.25%	12.5%	50%	100%
Embryonic	57.81 ± 0.56	90.94 ± 3.50	190.90 ± 9.64	268.38 ± 4.57
Yolk-sack	110.22 ± 5.10	172.03 ±10.43	311.17 ±3.37	368.55 ± 4.23
Free-feeding	68.76 ± 3.63	106.35 ± 13.17	162.35 ± 15.18	198.56 ±11.21

All concentrations inferred using fluorescence method described by Greer et al. (2012). All measurements µg/L ± SD.

Table 4.2 Primers used in PCR and qPCR

Gene	Direction	Sequence
ahrr promoter (Bisulfite)	Forward	TAGTATAAGTTTTTGTTTTGTGTGTGTG
	Reverse	TAACTCCAAAATCACATCCAATTTA
ahrr	Forward	ATGCAGTTTCAGGGTCGGTT
	Reverse	CCGCTTTTGTTCTTTCCCCG
cyp1a	Forward	GCAGATTAACCACGACCCAGAG
	Reverse	GCATCGCCTCCTTCCTAAGC
18s	Forward	GCTGAACGCCACTTGTCC
	Reverse	ATTCCGATAACGAACGAGACTC
dnmt3b	Forward	TGCTCAGGTAGGAGTGTGGT
	Reverse	CGACTAGAAGCCAAGCGTCA
dnmt3a	Forward	AAAGCCTCGCAAAAGCACAG
	Reverse	GGGTGCTCCAGGCTTACATT
dnmt1	Forward	GCGTTCCGCCTTAACAATCC
	Reverse	GCATTTCCACATCGCCCTTC

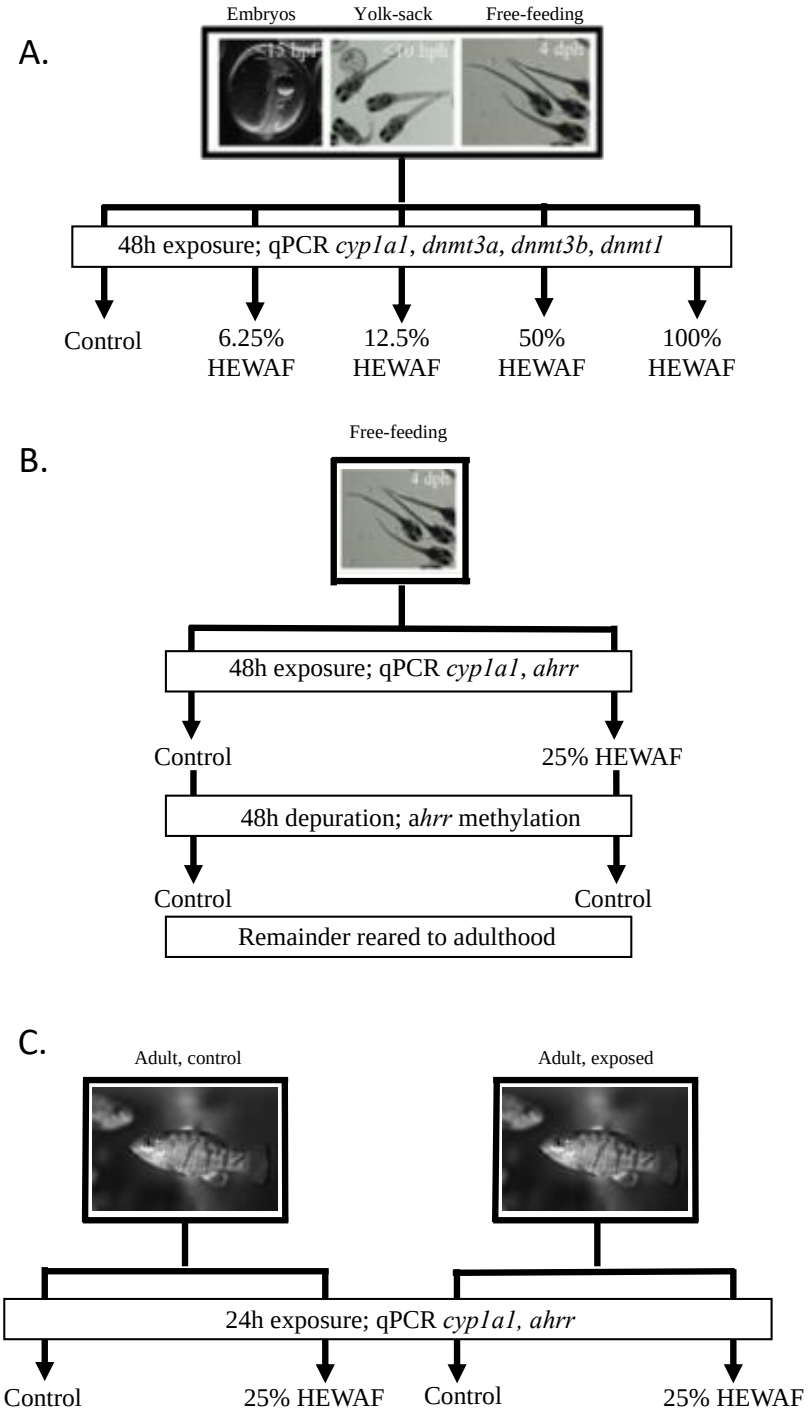

Figure 4.1 Experimental designs

A) exposures to measure dnmt expression, B) exposures to measure ahrr methylation, and C) re-exposures to measure effects of developmental exposure to oil on AHR pathway induction.

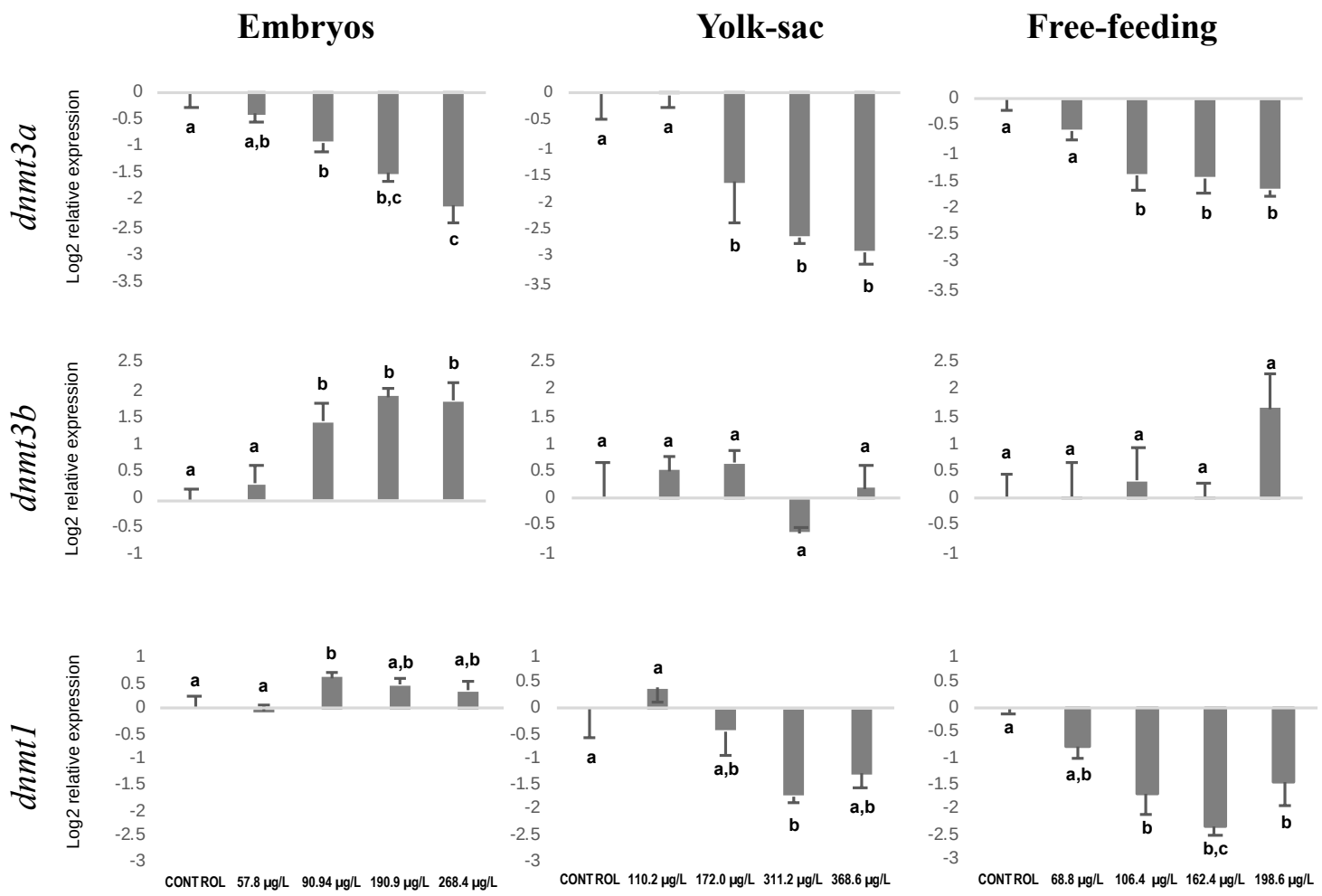

Figure 4.2 Relative expression (± SEM) of *dnmt* transcripts across developmental stages following 48 oil exposure

Concentrations are tPAH29 values derived from fluorescence assays. Letters denote statistical differences among groups.

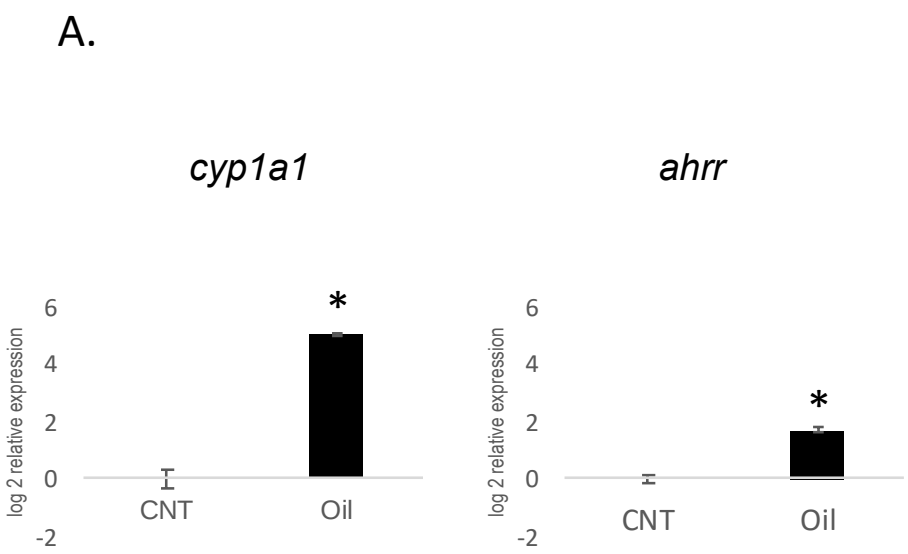

Figure 4.3 Expression of *cyp1a1* and *ahrr* following 48 h exposure in free-feeding larvae.

Asterisks indicate statistical differences from control

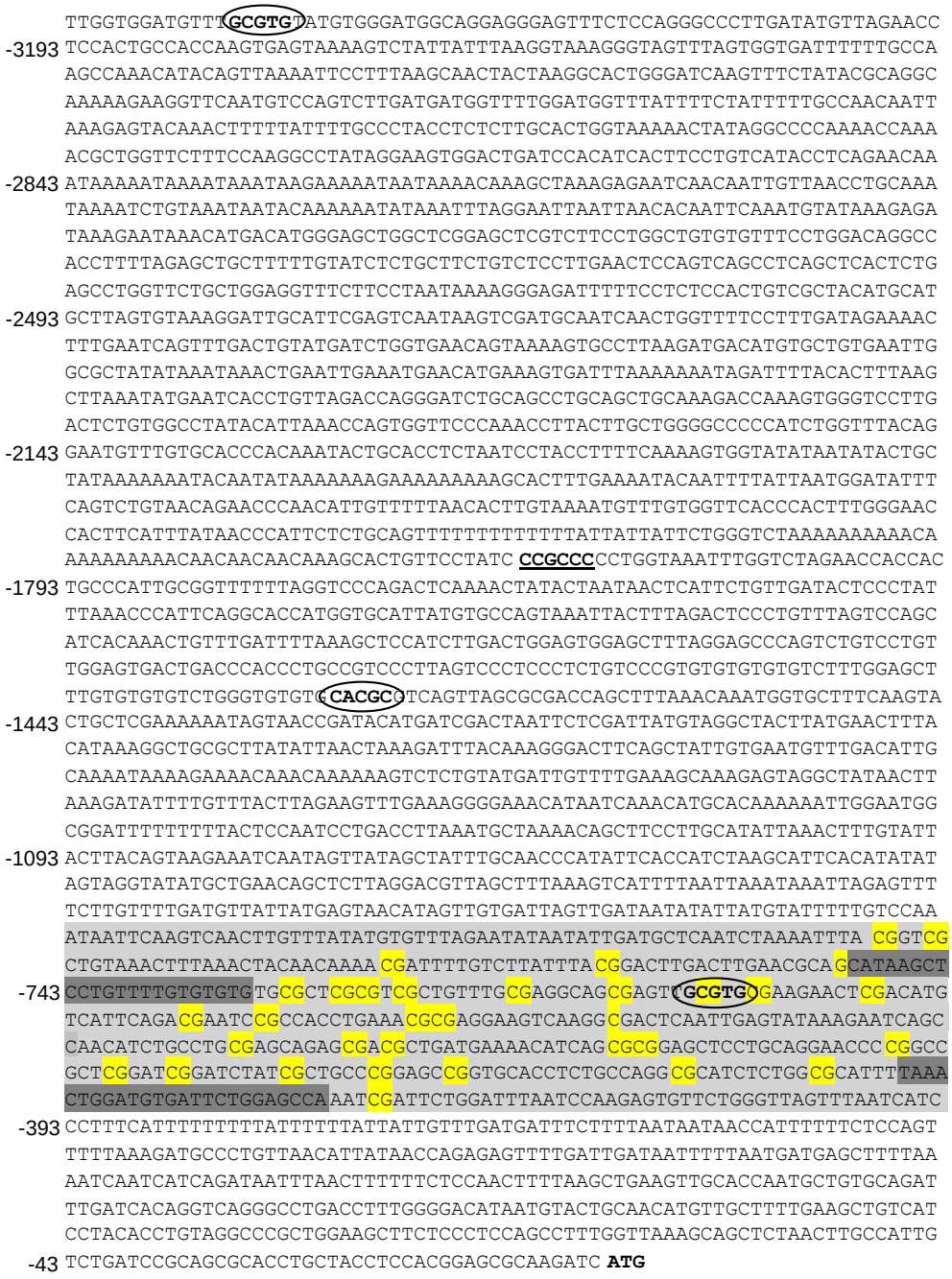

Figure 4.4 Putative *ahrr* promoter in *C. variegatus*

Sequence determined by comparison to the *F. heteroclitus* promoter. Numbers indicate distance upstream from transcription start site (ATG, bolded.) Three xenobiotic response elements (XREs) are circled, and one GC box underlined. Light grey highlight indicates location of CpG island, with CpG residues highlighted in yellow. Dark grey highlight indicates location of primers used in bisulfite PCR.

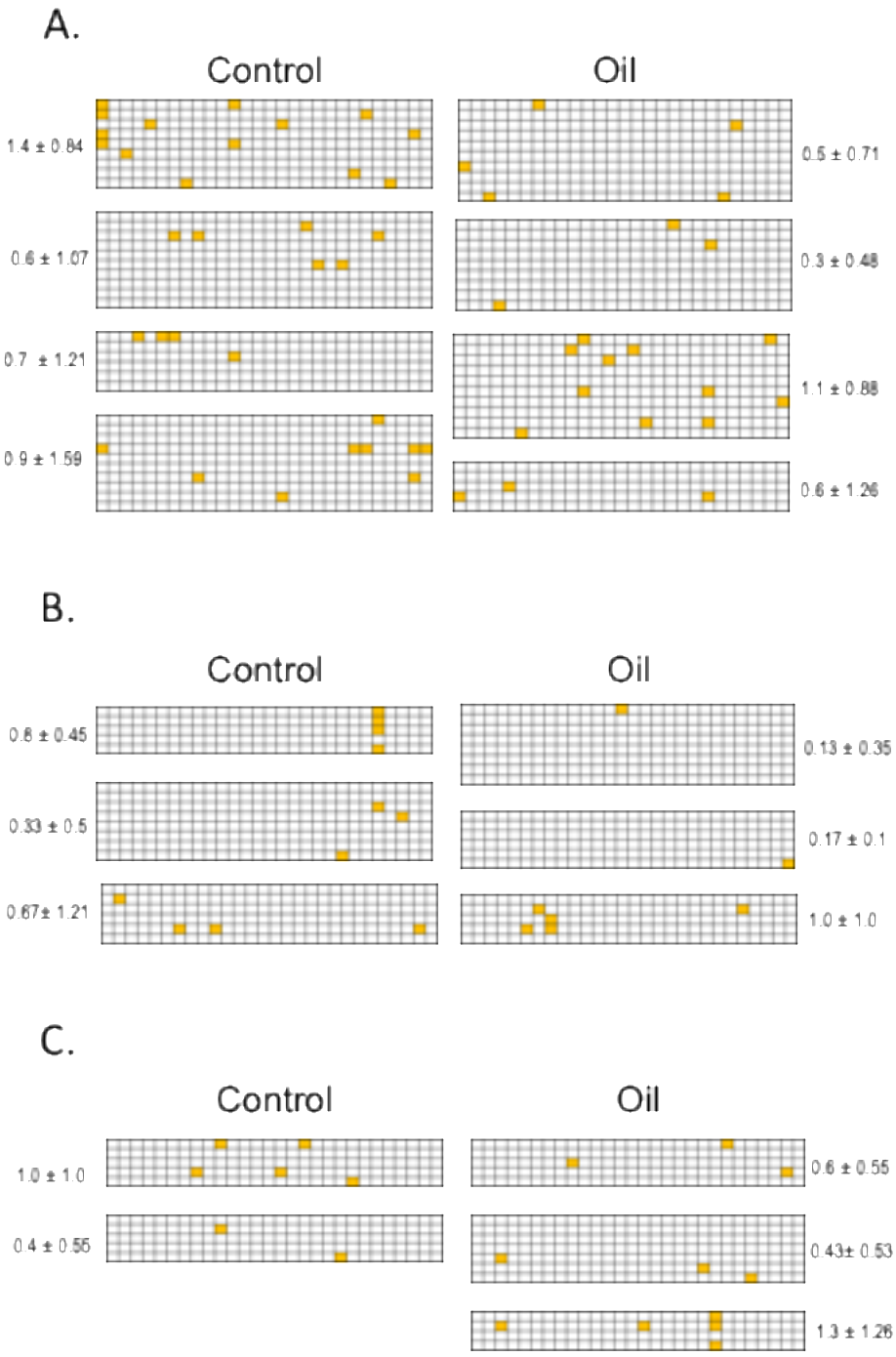

Figure 4.5 Oil induced effects on the *ahrr* promoter.

A) Methylation of *ahrr* promoter in larvae. Each rectangle contains data from a sample pool (n=5 larvae/pool). Rows represent individual sequences, columns represent CpG loci. Methylated CpG residues highlighted in yellow. Numbers are average methylated loci per sample, ± SD. B) Methylation of *ahrr* promoter in adult females reared from larval exposures. Rectangles represent sequences from liver tissue of individual females. C) Methylation of *ahrr* promoters in adult males reared from larval exposures.

Figure 4.5 (continued)

Rectangles represent sequences from liver tissue of individual males. Differences in number of sequences per group due to exclusion of low quality sequences from analysis.

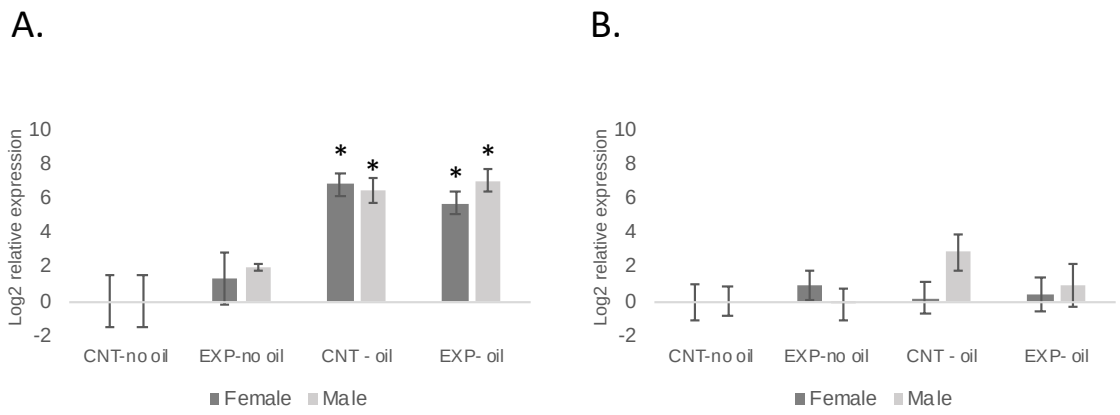

Figure 4.6 Expression of *cyp1a1* and *ahrr* following exposure to HEWAF (tPAH 407 μg/L) in adults reared from larval exposures.

A) *cyp1a1* expression; B) *ahrr* expression. Animals designated as CNT were not exposed as larvae; those designated as EXP were exposed to HEWAF (tPAH 437μg/L) as larvae. "No oil" and "oil" labels refer to re-exposure treatments. Asterisks indicate statistical difference from controls.

CHAPTER V – OIL AND HYPOXIA INDUCED ALTERATIONS TO DNA METHYLATION PROFILES IN LARVAL CYPRINODON VARIEGATUS

5.1 Abstract

DNA methylation is an important epigenetic mark involved in modulating transcription. While multiple studies document the ability of environmental stressors to alter methylation patterns, there is little information regarding the effects of oil and hypoxia on the methylome. Oil and hypoxia stress are particular threats in coastal ecosystems, which act as nursery habitats for developing fish. To explore the methylation altering effects of oil and hypoxia on developing fish, we exposed larval *Cyprinodon variegatus* to oil, hypoxia, or both for 48 h and used immunoprecipitation coupled with high-throughput sequencing (MeDIP seq) to evaluate genome-wide methylation changes. We also associated differential methylation with altered transcription by comparing MeDIP seq to RNA seq data from exposures carried out under similar experimental conditions. Our results indicate that all three treatments elicited significant effects to methylation, with hypoxia having a subtle effect, and oil a more pronounced effect. The combination of oil and hypoxia resulted in far greater impacts to methylation than either stressor individually, suggesting the two act synergistically to exert methylation changes. Functional analyses of oil and oil plus hypoxia groups revealed enrichment of processes related to DNA damage and cellular stress. In the oil plus hypoxia treatment, there was a significant association between methylation and transcriptional data, and overlap in pathways and functions enriched in

both data sets. These data demonstrate that oil and hypoxia impact the methylome of developing fish and strongly suggest that methylation plays a role in modulating transcriptional responses mitigating the effects of these stressors.

5.2 Introduction

One of the most studied epigenetic responses to environmental stressors is altered DNA methylation. Methylation is an important mechanism regulating transcription, and because methylation patterns are mitotically heritable, they have the potential to permanently alter gene expression (Jones and Takai 2001; Dolinoy et al. 2007). While numerous studies document the ability of environmental stressors to alter methylation patterns (Bernal and Jirtle 2010; Mirbahai et al. 2011; Skinner 2014; Wang et al. 2016; Beck et al. 2017; Aluru et al. 2018) there remains a gap in the literature concerning the methylation-modifying effects of oil and hypoxia, two stressors relevant to coastal ecosystems.

Coastal ecosystems are increasingly threatened by the risks of polycyclic aromatic hydrocarbon (PAH) contamination from oil spills and expansion of hypoxic "dead zones" from agricultural run-off (Rabalais et al. 2002; Harfoot et al. 2018). One example of these threats is the Deepwater Horizon (DWH) oil spill, which contaminated approximately 2100 km of shoreline from Florida to Texas with oil (Michel et al. 2013; Nixon et al. 2016). That same area is also characterized by broad fluctuations in dissolved oxygen content, as up to 29% of estuaries in the region experience seasonal hypoxia (Engle et al. 1999). The timing of the DWH spill in the spring and summer of 2010 coincided with the spawning periods of many estuarine residents, including fish

such as *Cyprinodon variegatus* (Talbot and Able 1984). Therefore, vulnerable early-life stages of coastal species were simultaneously exposed to oil contamination and annual summer periods of hypoxia, prompting questions regarding how both stressors interact to exert toxicity in estuarine fish species.

Both oil and hypoxia are well known to modify gene expression in developing fish. The transcriptional effects of oil exposure include altered expression of genes related to cardiac development, immunity, DNA damage repair, and cholesterol biosynthesis (Whitehead et al. 2012; Dubansky et al. 2013; Pilcher et al. 2014b; Xu et al. 2016; Jones et al. 2017; Sørhus et al. 2017; Rodgers et al. 2018). Developmental hypoxia exposure can also affect gene expression via suppression of growth related genes and induction of genes related to oxygen delivery (Ton et al. 2003; Hala et al. 2014). However, simultaneous exposure to both stressors is of particular concern due to interactions between pathways mediating PAH detoxification and pathways involved in hypoxia response. Oil exposure activates the aryl hydrocarbon receptor (AHR); a ligand binding transcription factor that initiates expression of genes related to PAH metabolism (Hankinson 2002). Similarly, hypoxia exposure activates hypoxia inducible factor 1 (HIF1); a transcription factor that initiates expression of genes to optimize oxygen uptake (Wu 2002). Both transcription factors share a dimerization partner, the aryl hydrocarbon receptor nuclear translocator (ARNT) (Gradin et al. 1996). Competition for this partner can result in inhibition of transcription related to each pathway, thereby having a greater effect on gene expression than either stressor alone (Fleming et al. 2009; Fleming and Di Giulio 2011; Dasgupta et al. 2015, 2016; Simning 2017).

The extent to which oil or hypoxia induced transcriptional dysregulation is associated with altered DNA methylation is unclear. While there is little information regarding the combined effects of oil and hypoxia on DNA methylation, a few studies have investigated the effects of each stressor individually. In humans, exposure to airborne PAHs is correlated with altered methylation of gene promoters and genomic hypomethylation; in fish, aquatic exposure to individual PAH moieties in zebrafish (*Danio rerio*) has a similar effect (Pavanello et al. 2009; Herbstman et al. 2012; Fang et al. 2013; Novakovic et al. 2014; Corrales et al. 2014b). For hypoxia, much of the information about methylation effects is derived from studies of tumor tissues, and demonstrates that hypoxic exposure results in global genomic hypomethylation coupled with hypermethylation of specific promoter loci (Li et al. 2003; Wilson et al. 2006; Shahrzad et al. 2007; Thienpont et al. 2016). In fish, the methylation-altering effects of hypoxia are less well characterized; however one study determined that chronic hypoxia exposure in zebrafish resulted in global hypermethylation of sperm cells; an effect opposite that noted in human cancer lines (Wang et al. 2016). Whether these opposing effects are the result of taxonomically differing responses to hypoxia exposure, or simply an artifact of differing tissue types is unclear. Although studies have begun to characterize the effects of PAHs and hypoxia on methylation patterns, their scope has largely been restricted to evaluation of a few loci of interest. Additionally, there remain questions regarding the relationship between DNA methylation and gene expression. However, the advent of next generation sequencing technology makes it possible to evaluate genome-wide methylation changes in conjunction with differential transcription to better elucidate the epigenetic effects of stressors.

In this study, we used immunoprecipitation coupled with high-throughput sequencing (MeDIP seq) to evaluate genome-wide methylation changes following exposure to oil, hypoxia, or both in free-feeding larval *C. variegatus*. We also sought to associate differential methylation with altered gene expression by comparing our data to transcriptional data from exposures carried out under similar experimental conditions. Our results indicate that all three stressors elicit effects to methylation, and our functional analyses suggest that differentially methylated regions and differentially expressed genes are associated with similar biological processes, suggesting that methylation and transcription work together to modulate molecular responses to oil and hypoxia.

5.3 Materials and Methods

5.3.1 Experimental animals

C. variegatus brood stock were maintained at the University of Southern Mississippi's Toxicology Lab in Ocean Springs, MS. Fish were kept in 300 L circulating raceways containing 15 ppt artificial sea water at 25°C and 15 ppt salinity on a 16:8 light/dark cycle and fed commercial flake food daily. Breeding was initiated by the placement of breeding nets in raceways. After 12 hours embryos were collected and examined for fertilization. Embryos were rolled on mesh mats to remove external villi, and incubated in 15 ppt sea water at 30°C with agitation to prevent clumping. After hatching, embryos were reared until yolk-sacks were absorbed and larvae began free-feeding (4 days post-hatch). Upon initiation of feeding, larvae were fed *Artemia* nauplii once daily. Feeding continued throughout experiments.

5.3.2 Exposure conditions

Larvae were exposed to one of four conditions: control, hypoxia, oil, or oil plus hypoxia. Larvae (n =20) were placed in mesh cups, with three replicate cups per treatment. All larvae were exposed for 48 h and depurated in clean, normoxic water for an additional 48 h to allow stabilization of any treatment induced effects to methylation patterns. Exposures were conducted in flow through systems delivering test solutions at a rate of 2L/h. Hypoxic conditions were achieved via bubbling of nitrogen gas through test solutions until dissolved oxygen reached $\leq$ 2 mg/L O2. Environmental conditions were held at 15 ppt and 30°C across treatments and tanks were monitored daily for temperature and salinity. Dissolved oxygen was monitored every six hours to ensure maintenance of oxic regimes. Following 48h of depuration, larvae were sampled and stored in RNA later at -80°C until further analysis.

5.3.3 Preparation of HEWAF for oil exposures

High energy water accommodated fraction (HEWAF) solutions for both normoxic and hypoxic experiments were prepared using methods outlined in Forth et al. (2016). Briefly, Deepwater Horizon source oil (1g/mL) was added to artificial sea water (15 ppt) and mixed in a stainless steel blender (Waring Commercial) for 30s on low speed. The resulting mix was decanted into a separatory funnel, covered with foil to protect from UV light, and allowed to settle for 1 h. Following the settling period, HEWAF was diluted to 25% for use in exposures. One liter of each 25% HEWAF test solution was collected in 1L amber glass bottle and shipped on ice to the Mote Marine Laboratory (Sarasota, FL). Concentrations for 50 parent PAHs, alkyl PAHs, and alkyl PAH homologs (tPAH50)

were measured via gas chromatography coupled with tandem mass spectrometry (GC/MS/MS).

5.3.4 DNA extraction and immunoprecipitation

DNA was extracted and RNAse treated using DNeasy Blood and Tissue kit according the manufacturer's protocol (Qiagen, Hilden Germany). To ensure sufficient DNA mass for immunoprecipitation, DNA from 5 larvae was pooled for each replicate, resulting in n=3 samples per treatment, 5 larvae/sample. Extracted DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA).

To fragment DNA for immunoprecipitation, 8μg of DNA per sample was digested using MseI and DraI restriction enzymes according to recommended reaction parameters (New England BioLabs, Ipswitch, MA). Resulting fragments were end repaired using the large (Klenow) fragment of DNA polymerase (New England BioLabs, Ipswitch, MA). To check for complete digestion of genomic DNA, 5μL of each digestion reaction was run on a 1% agarose gel; samples were considered fully digested when all fragments were between 500-200 bp in size. To avoid interactions between restriction enzymes and immunoprecipitation antibodies, DNA was purified from digestion reactions using phenol-chloroform-isoamyl alcohol (PCI) (25:24:1) according to published protocol (Green and Sambrook 2017).

Immunoprecipitation reactions were performed using modifications to protocols published by Lizardi et al. (2017) and Beck et al. (2017). Purified, fragmented DNA (3 μg) was diluted in 450 μL of ultra-pure water, and heat denatured at 95°C for 10 minutes. Samples were then immediately placed on ice to prevent re-annealing of fragments.

Following cooling, 50 μL of 10x immunoprecipitation (IP) buffer and 4μL of 5-methyl cytosine (5meC) antibody (monoclonal mouse anti 5-methyl cytidine; Diagenode #C15200006) were added. Samples were then incubated overnight with rotation at 4°C.

For each sample, 50 μL of MagnaBind Goat Anti-Mouse IgG magnetic beads (Thermo Scientific, Waltham, MA) were washed three times with 1× PBS containing 0.1% BSA, then washed once with 1x IP buffer. Beads were collected in a magnetic rack, then added to each sample and incubated with rotation for 2 h at room temperature. Following incubation, beads were collected using a magnetic rack, and supernatent discarded. Beads were then washed three times in 1X IP buffer, and resuspended in in 500μL of proteinase K digestion buffer containing 5μL/mL proteinase K solution (stock concentration 20 mg/mL). Proteinase K reactions were incubated with rotation for 2 h at 50°C. After incubation, beads were collected using a magnetic rack and the supernatant collected. To purify immunoprecipitated DNA, the supernatant from each reaction was PCI purified according to published protocols (Green and Sambrook 2017).

5.3.5 Sequencing and bioinformatics

Immunoprecipitated DNA was quantified on a Qubit 4 fluorometer using a ssDNA assay kit (Thermo Scientific, Waltham MA.) Approximately 2ng of DNA per sample was shipped to the University of Colorodo Anschutz Medical Campus Microarray and Genomics Core (Denver, CO) for library preparation and sequencing. Libraries were prepared using ACCEL-NGS® 1S Plus & Methyl-SEQ kit (Swift Biosciences, Ann Arbor, MI.) and sequenced on an Illumina NovaSeq to generate 2 x 150 bp reads.

Initial processing of data was performed using CLC Genomics Workbench 12 (Qiagen, Hilden, Germany). Adaptor sequences and low quality reads were trimmed, paired ends matched, and reads aligned to the *C. variegatus* genome (NCBI genome ID 13078). After alignment, data were processed using the CLC "ChIP Seq" peak calling tool to identify methylated regions of the genome. Peaks were annotated with information regarding nearest upstream and downstream genes using the "Epigenomics Analysis Annotation" tool.

Differentially methylated regions were identified using the MEDIPS R package (v 1.34). Data quality was assessed for each sample including CpG coverage analyses and saturation analyses to ensure adequate sampling of the methylome, and genome-wide correlation analyses to determine similarity among sample libraries. To calculate differential methylation, the *C. variegatus* genome was divided into consecutive 100 bp windows, and read depth for each window compared between control and treatment samples. The edgeR Bonferroni adjusted p-value was used to identify differential read coverage between groups; regions were considered differentially methylated at edgeR p adj. of ≤ 0.1. Adjacent differentially methylated 100 bp windows were then merged to identify genomic regions particularly targeted for methylation changes following exposure.

Differentially methylated regions (DMRs) were annotated with gene information by comparing genomic location data generated in MEDIPS with annotated peaks identified by CLC. Genes associated with DMRs were matched to mammalian orthologs and input into Ingenuity Pathway Analysis (IPA) software for functional analysis. Identification of significantly altered canonical pathways, diseases, and molecular and

cellular functions were made using the "Core Analysis" tool of IPA. All biological processes were considered significantly altered at $p \leq 0.05$.

5.3.6 Association between DNA methylation and gene expression

To determine the relationship between methylation and gene expression, we compared DMRs identified in this study with differentially expressed genes (DEGs; FDR adj. p. value > 0.1) from exposures previously conducted under similar experimental conditions. Briefly, free-feeding *C. variegatus* larvae were exposed to oil, hypoxia, or both for 48 h, and RNA-seq performed to examine altered transcription following exposure. Differential gene expression was calculated using CLC Genomics Workbench 12 and functional analysis performed in IPA. See Simning (2017) for additional detail.

5.4 Results

5.4.1 Experimental results

Environmental conditions were relatively stable throughout exposures (Table 5.1). Analytical chemistry revealed similar concentrations and PAH constituents present in both normoxic and hypoxic HEWAF solutions. Mortality was low (<11%) in all treatments, however there was a slight treatment effect, with the greatest mortality observed in oil and oil plus hypoxia groups.

5.4.2 Sequence data quality

Samples were sequenced at a minimum depth of 14M reads/sample, and aligned to the *C. variegatus* genome with a mapping efficiency >84% (Table 5.2). To check for

non-specific binding of the 5meC antibody, we performed CpG coverage analyses; for all samples, fewer than 1.1% of reads mapped to regions devoid of CpG motifs, suggesting that immunoprecipitation was highly specific. Analysis of sequencing depth indicated that all samples were sequenced to saturation, and genome-wide Pearson correlation analyses revealed strong similarity among sample libraries (with correlation coefficients > 0.8); therefore all data were deemed appropriate for comparison.

5.4.3 Differential methylation

Because all samples were sequenced to saturation, we used CpG coverage as a proxy measurement for total genomic methylation. Coverage analyses showed that ≥48% of CpG residues were represented by at least 1x coverage in all samples. Genomic methylation patterns were similar in all treatments except the oil plus hypoxia group, which exhibited a shift toward increased total genomic methylation (Figure 5.1).

Differential methylation analysis demonstrated that all three treatments elicited methylation effects at specific genomic loci. Effects were much greater in the oil plus hypoxia treatment than in either stressor alone, suggesting that oil and hypoxia act synergistically to exert changes in DNA methylation profiles (Figure 5.2A-C). We used enrichment of DMRs as a measure of methylation status; DMRs with fold changes greater than 0 were considered hypermethylated relative to controls; conversely, those with fold changes less than 0 were considered hypomethylated. The majority of DMRs were hypomethylated across in all three groups, however, each treatment also exhibited a substantial number of hypermethylated regions, indicating that all treatments had a mixed effect on the methylome (Figure 5.2A-C).

After merging adjacent significantly methylated windows, the size distribution of DMRs ranged from 100 bp to 1300 bp. Overall, the largest DMRs were in the oil plus hypoxia group, however, the majority of differential methylation occurred in discrete 100 bp windows across all treatments (Figure 5.3A-C). To determine the genomic region of these methylation changes, DMR genomic locations were divided into four categories: gene body, promoter (<5000 bp upstream of nearest gene), distal promoter (<10,000 bp upstream of nearest gene) or intergenic (> 10,000 bp upstream of nearest gene). In the two larger data sets (oil and oil plus hypoxia groups) the majority of methylation changes were associated with gene body regions, followed by intergenic regions (Figure 5.4A-B).

None of the DMRs in the hypoxia group occurred in the other two treatments, however the oil and oil plus hypoxia groups shared several DMRs (Table 5.3). The majority of shared DMRs occurred in the gene body region and were hypomethylated. In both groups, one of the largest DMRs (900 bp in the oil plus hypoxia group, 600 bp in the oil group) was associated with the gene body of *acot8*, which encodes an enzyme involved in fatty acid oxidation (Hung et al. 2014). The only DMR to exhibit opposing methylation in oil vs oil plus hypoxia treatments was *arhgap32*, which encodes a GTP-ase activating protein (Mezzavilla et al. 2015).

5.4.4 Functional analysis of DMRs

To predict the biological outcomes associated with differential methylation, we matched DMRs with mammalian orthologs for use in functional analyses using IPA software. We excluded DMRs associated with intergenic regions from our analyses, as intergenic methylation is thought to be primarily associated with genomic stability, not

gene expression (Weber et al. 2007). We also excluded hypoxia data from functional analyses, as only one DMR from the hypoxic treatment matched with a mammalian ortholog. This DMR was located in the gene body for *pepd*, which encodes prolidase, an enzyme associated with increased activity of hypoxia-inducible factor 1 (Yang et al. 2017).

In the oil plus hypoxia group, we matched 230 DMRs to mammalian orthologs, which provided a robust dataset for functional analyses. IPA predicted dysregulation of 19 canonical pathways following oil plus hypoxia exposure, including those involved in transcription (tRNA Charging), neurotransmitter signaling (Agrin Interactions at Neuromuscular Junctions), DNA damage (Role of BRCA1 in DNA damage response), and cellular stress (BAG2 Signaling Pathway) (Figure 5.5A). Additional functional analyses in IPA predicted impacts to molecular and cellular functions related to cell death and cell to cell signaling, and disease effects including cancer, gastrointestinal disease, and hepatic disease (Table 5.4). Fewer pathways and functions were predicted as dysregulated in oil groups, as only 15 of the 35 DMRs suitable for analysis could be matched to human orthologs. Given this reduced set of analysis-ready DMRs, IPA predicted impacts to 4 canonical pathways (Figure 5.5B) and additional impacts to molecular and cellular functions including changes to cellular functions and DNA repair, as well as disease impacts including neurological disease and cancer (Table 5.4).

5.4.5 Association of DMRs with gene expression

To investigate how altered methylation influences gene expression, we compared methylation profiles with RNA seq data from previous exposures. All experiments

examining transcription were conducted using free-feeding larval *C .variegatus*, exposed to similar levels of hypoxia (≤ 2 mg/L 02) and similar oil concentrations (tPAH29 82.14 μg/L for oil groups; tPAH29 97.48 μg/L for oil plus hypoxia groups); see Simning (2017) for additional detail.

In the oil plus hypoxia group, we found evidence of a significant association of DMRs with differentially expressed genes; out of 507 DMRs occurring in gene body, promoter, or distal promoter regions, 98 were associated with differentially expressed genes (p. value of overlap > 0.031). However, of those only 50 were linked to DEGs with expression inversely proportional to methylation status. Data from the oil treatment showed no significant correlation between methylation and expression; out of 35 DMRs occurring in regions influencing expression, only 4 matched to DEGs, and none of the DEGs exhibited expression inversely proportion to differential methylation (Figure 5.5B).

To determine the extent to which methylation might influence biological function, we compared our IPA analysis of methylation data with an IPA analysis using transcriptomic data. We restricted this inquiry to data from the oil plus hypoxia group, as this represented a more robust data set containing a greater number of DMRs. We found substantial functional overlap in the two analyses. At the canonical pathway level, the data sets shared three dysregulated pathways: Role of BRCA1 in DNA Damage Response, Unfolded Protein Response, and Sonic Hedgehog Signaling. Additionally, the two data sets shared four out of five top altered disease processes, and two out of five top altered molecular and cellular functions (Table 5.4). These results suggest that

methylation and gene expression work together to modulate pathways and functions following oil plus hypoxia exposure.

5.5 Discussion

To our knowledge this is the first study to characterize genome-wide methylation changes following oil and hypoxia exposure in developing fish. We found that both stressors elicit significant effects on methylation in larval *C. variegatus*, with oil eliciting greater effects than hypoxia. However, the most marked effect we observed was in the oil plus hypoxia group, which exhibited far more DMRs than either stressor alone, suggesting that oil and hypoxia interact synergistically to elicit changes in the methylome.

The mechanisms underlying these changes are unclear. Previously, we reported that oil exposure inhibits transcription of DNA methyltransferases (DNMTs), the enzymes responsible for establishing and maintaining methylation patterns. Oil-induced down-regulation of DNMT transcripts suggests a mechanism driving the genomic hypomethylation observed in developing zebrafish following exposure to individual PAHs (Fang et al. 2013). Studies from mammalian models demonstrate that hypoxia also has an inhibitory effect on DNMT transcription (Zhang et al. 2014; Liu et al. 2016); therefore the combined effects of oil and hypoxia on DNMT expression may underlie the synergistic effects observed herein. Additionally, both oil and hypoxia are known to produce reactive oxygen species (ROS) which can elicit DNA damage in the form of strand breakage (Franco et al. n.d.; Xue and Warshawsky n.d.; Chandel et al. 2000). Studies examining simultaneous exposure to both oil and hypoxia stressors in fish have

noted increases in DNA strand breakage (Negreiros et al. 2011; Dasgupta et al. 2016), which can interfere with the ability of DNA to act as a substrate for DNMT enzymes, and result in genomic hypomethylation (Franco et al. 2008).

Across all treatments in this study, the majority of DMRs were hypomethylated, which is consistent with both mechanisms proposed above. However, each treatment also exhibited a substantial number of hypermethylated DMRs, and in the oil plus hypoxia group, CpG coverage data suggested a general shift toward genomic hypermethylation. Preliminary data from our lab (unpublished) suggests that simultaneous exposure to oil and hypoxia can increase DNMT transcription, which could account for the hypermethylation effects noted in the oil plus hypoxia group, but is insufficient to explain hypermethylation following exposure to each stressor individually. Clearly, additional work is needed to fully characterize how oil and hypoxia act to elicit altered methylation.

One of the primary rationales for investigating altered methylation patterns is elucidation of how epigenetic modifications relate to transcriptional effects. Our data revealed significant overlap of DMRs with DEGs following combined oil and hypoxia exposure, likely due to the prevalence of altered methylation in transcriptionally active gene body regions. Methylation studies often focus on promoters upstream from transcriptional start sites, as hypermethylation in these areas is known to be associated with transcriptional silencing (Robertson and A.Jones 2000; Jones and Takai 2001). Unlike promoters, methylation of gene bodies does not inhibit transcription (Yang et al. 2014a, 2014b). The purpose of gene body methylation is unclear, but it is hypothesized to play a role in silencing of internal promoters, retrotransposons, and other functional

elements to maintain efficient transcriptional patterns (Maunakea et al. 2010; Kulis et al. 2012; Yang et al. 2014b). Early investigations of gene body methylation at specific loci suggested intra-genic methylation is often positively correlated with gene expression, however, a few genome-wide studies have found both positive and negative correlation of gene expression with gene bodies (Maunakea et al. 2010; Kulis et al. 2012). Our data corroborate the latter, as expression associated with gene body DMRs in our study was both positively and negatively correlated with methylation status.

The size of the DMRs in our data may also be related to transcriptional activity. Our analyses revealed that the majority of methylation changes occurred in discrete 100 bp windows, suggesting that few genomic regions were broadly targeted for altered methylation. Typically, transcriptional modulation is associated with methylation of regions containing high CpG density (CpG islands), which can be located in promoters upstream from genes, in alternative promoters within gene bodies, and in intergenic regions (Maunakea et al. 2010). By definition CpG islands are greater than 200 bp in size (Gardiner-Garden and Frommer 1987); therefore, the size distribution of DMRs in our dataset indicate that most methylation changes did not occur across entire island regions, but across partial CpG islands or outside of island regions. However, recent evidence suggests that small scale methylation changes can modulate transcription, as studies have linked gene expression with methylation of specific CpG residues within island regions, or methylation of low-density CpG island "shores" adjacent to island regions (Fouse et al. 2010; Kulis et al. 2012; Monick et al. 2012). The correlation between gene expression and altered methylation in our dataset further support the idea that transcriptional activity may be modulated by smaller regions of methylation than previously thought.

At the pathway level, the association between methylation and transcriptional data suggests that responses to oil and hypoxia exposure are influenced by methylation of specific loci. For example, multiple studies document the genotoxic effects of oil and hypoxia in fish (Vanzella et al. 2007; Pilcher et al. 2014a; Le Bihanic et al. 2014b, 2014a; Dasgupta et al. 2016); our data exhibited enrichment of pathways and functions related to DNA damage responses using both DMR and DEG data sets, suggesting that transcriptional remediation of DNA damage may be governed by methylation. Similarly, studies examining the transcriptional effects of oil in developing fish consistently identify dysregulation of lipid metabolism as a hallmark effect of oil exposure (Meador et al. 2006; Xu et al. 2016, 2017; Sørhus et al. 2017; Loughery et al. 2018; Aluru et al. 2018, Jones, Ch. 3). Our data revealed differential methylation in the gene body of *acot8* in both oil and oil plus hypoxia groups. This gene encodes the protein acyl-coA thioesterase 8; an enzyme catalyzing the breakdown of fatty acyl-CoA into free fatty-acids and coenzyme A (CoA), and thought to play an important role in regulating lipid metabolism (Hung et al. 2014). In our hypoxia data, we observed differential methylation in the gene body of *pepd*, which encodes prolidase, an enzyme associated with increased expression of hypoxia-inducible factor 1α (HIF1α), indicating it may be important in responding to hypoxic stress (Surazynski et al. 2008; Yang et al. 2017). Although we did not find an association between these two DMRs and their mRNA expression, their association with well-established oil and hypoxia effects suggests that the methylation alterations in this study are not random, but occur in targeted regions of the genome related to mitigation of these stressors.

Our data strongly suggest that methylation plays a role in modulating transcriptional responses to oil and hypoxia, prompting questions regarding the longevity of these responses. DNA methylation is mitotically heritable, therefore transcriptional patterns resulting from disruption to methylation patterns could endure throughout an organism's lifetime (Dolinoy et al. 2007). One interesting feature of this study is that we measured methylation after 48h exposure followed by 48h depuration, while transcriptional samples were collected immediately after 48h exposures. This means that the association between methylation and transcriptional patterns we observed are from methylation changes persisting after exposure ended. DNA methylation is a dynamic process, and while methylation patterns can persist long-term, they can also be removed through passive and active demethylation processes (Wu and Zhang 2010). Whether the altered methylation in this study represents transient changes not yet removed or more permanent alterations to the methylome is unclear. Additional work in which methylation is measured at multiple time points following exposure would more fully characterize the long-term impacts of oil and hypoxia on fish.

5.6 Conclusion

In this work we describe genome-wide methylation alterations following oil and hypoxia in developing fish. Our results indicate that simultaneous exposure to both stressors has a substantial effect on methylation which primarily affects gene body regions. We also found a significant overlap between methylated regions and differentially expressed genes, suggesting that methylation influences transcriptional responses to oil and hypoxia. It should be noted that the samples used in methylation and

transcriptional analyses originated from two independent exposures conducted under similar experimental conditions; future studies including MeDIP seq and RNA seq data from the same exposure could uncover an even closer association between differential methylation and gene expression. One interesting feature of this work is the persistence of exposure induced methylation effects 48h after exposure. Additional work measuring methylation from exposure through maturation would elucidate the persistence of oil and hypoxia induced changes to the methylome.

5.7 References

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Table 5.1 Exposure conditions for MeDIP seq experiments.

	Normoxic exposure		**Hypoxic Exposure**		
	Control	*Oil*	*Normoxic Control*	*Hypoxia*	*Oil plus Hypoxia*
Salinity (ppt)	15.8 ± 0.5	15.8 ± 0.5	17.0 ± 2.0	15.5 ± 1.0	15.3 ± 1.0
Temperature (°C)	28.43 ± 0.28	28.45 ± 0.30	28.32 ± 0.35	28.66 ± 0.20	28.78 ± 0.43
Dissolved oxygen (mg/L)	8.84 ± 0.12	5.88 ± 1.72	5.94 ± 0.49	2.04 ± 0.40	1.73 ± 0.49
tPAH29 (μg/L)	0.3	102.5	0.3	0.3	93.19
Mortality (%)	0	10	0	1.2	11

Table 5.2 Sequencing information for MeDIP seq samples.

Treatment	**Total number of reads**	**% Mapping efficiency**	**% of non-CpG reads**
Normoxic exposure			
Control	124,049,566	87 ± 3.16	0.78 ± 0.08
Oil	176,412,146	90 ± 1.52	0.75 ± 0.14
Hypoxic Exposure			
Control (normoxic)	59,481,126	93 ± 0.76	0.58 ± 0.03
Hypoxia	95,642,746	94 ± 0.28	0.77 ± 0.27
Oil plus Hypoxia	272,061,386	93 ± 0.10	0.68 ± 0.02

Table 5.3 Differentially methylated regions (DMRs) present in oil and oil plus hypoxia treatments

DMR	**Location**		**Fold change (log_2)**		**Size of DMR (bp)**	
	Oil	*Oil plus Hypoxia*	*Oil*	*Oil plus Hypoxia*	*Oil*	*Oil plus Hypoxia*
LOC107100474	promoter	promoter	-0.48	-0.98	500	600
eif4e3	distal promoter	distal promoter	-0.45	-1.01	300	600
asxl2	distal promoter	distal promoter	-0.44	-0.9	200	400
ppef1	distal promoter	distal promoter	-0.41	-0.76	200	900
LOC107095832	gene body	gene body	-0.44	-0.9	200	400
slc1a2	promoter	gene body	-0.46	-0.61	400	300
abcg4	gene body	gene body	-0.42	-0.69	100	400
enpp7	gene body	gene body	-0.42	-0.93	300	600
LOC107094903	gene body	gene body	-0.37	-0.58	100	100
LOC107103315	gene body	gene body	-0.46	-0.98	100	600
LOC107102156	gene body	gene body	-0.41	-0.76	200	900
rcor3	gene body	gene body	-0.41	-0.9	100	600
LOC107083172	gene body	gene body	-0.46	-0.76	100	500
acot8	gene body	gene body	-0.47	-1.14	600	900
LOC107103061	gene body	gene body	-0.47	-1.01	200	400
arhgap32	gene body	gene body	-1.07	1.94	200	200
gltscr1l	gene body	gene body	-0.38	-0.54	100	400
LOC107096110	gene body	gene body	-0.45	-0.87	400	700
LOC107096832	gene body	gene body	-0.46	-0.61	100	500
LOC107096471	promoter	promoter	-0.39	-0.81	100	400
LOC107098674	promoter	promoter	-0.48	-0.78	100	600

Loci labels beginning with “LOC” are genes for which transcripts have been identified, but cannot be matched to known vertebrate orthologs.

Table 5.4 Top enriched diseases and molecular and cellular functions identified by IPA for methylation and transcription data.

Oil DMRs	**Oil + Hypoxia DMRs**	**Oil + Hypoxia DEGs**
	Diseases	
Developmental Disorder	Cancer	Cancer
Hereditary Disorder	Gastrointestinal Disease	Organismal Injury and Abnormalities
Neurological Disease	Hepatic System Disease	Endocrine System Disorders
Organismal Injury and Abnormalities	Organismal Injury and Abnormalities	Gastrointestinal Disease
Cancer	Endocrine System Disorders	Connective Tissue Disorders
	Molecular and Cellular Functions	
Cell Morphology	Cell Death and Survival	DNA Replication, Recombination, and Repair
Cellular Assembly and Organization	Cell-To-Cell Signaling and Interaction	Cell Cycle
Cellular Function and Maintenance	Cellular Assembly and Organization	Cellular Assembly and Organization
DNA Replication, Recombination, and Repair	Cellular Function and Maintenance	Cell Death and Survival
Amino Acid Metabolism	Cellular Development	Gene Expression

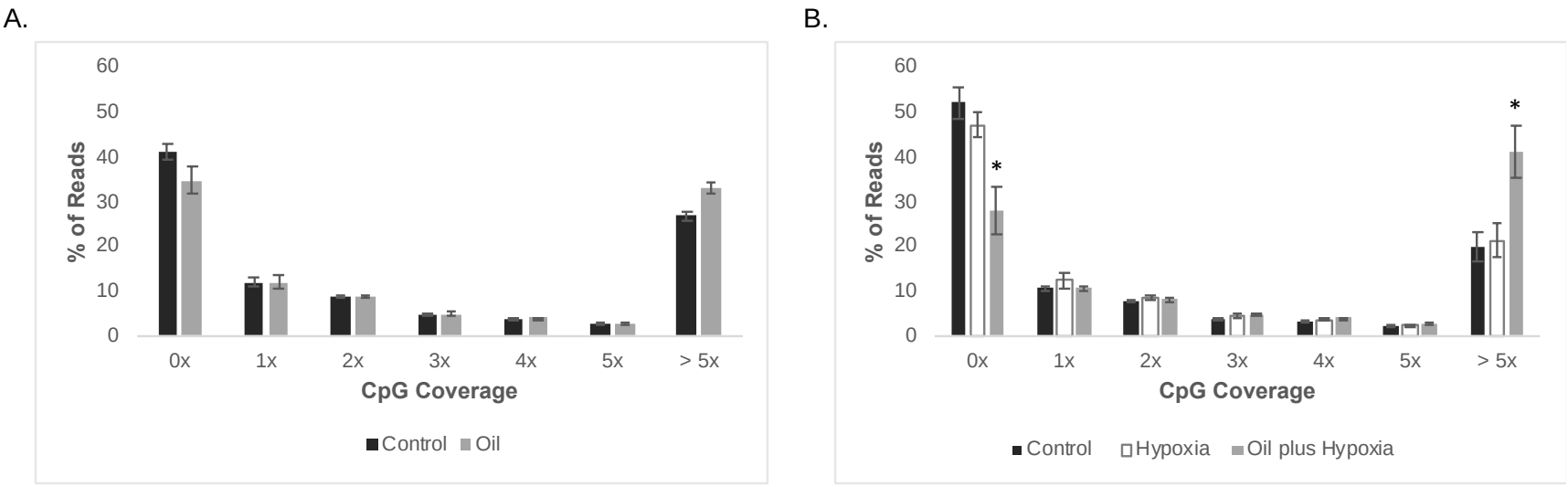


Figure 5.1 CpG coverage across genome for normoxic (A) and hypoxic (B) exposures

Asterisks denote statistical difference from controls (ANOVA; $p \leq 0.05$).

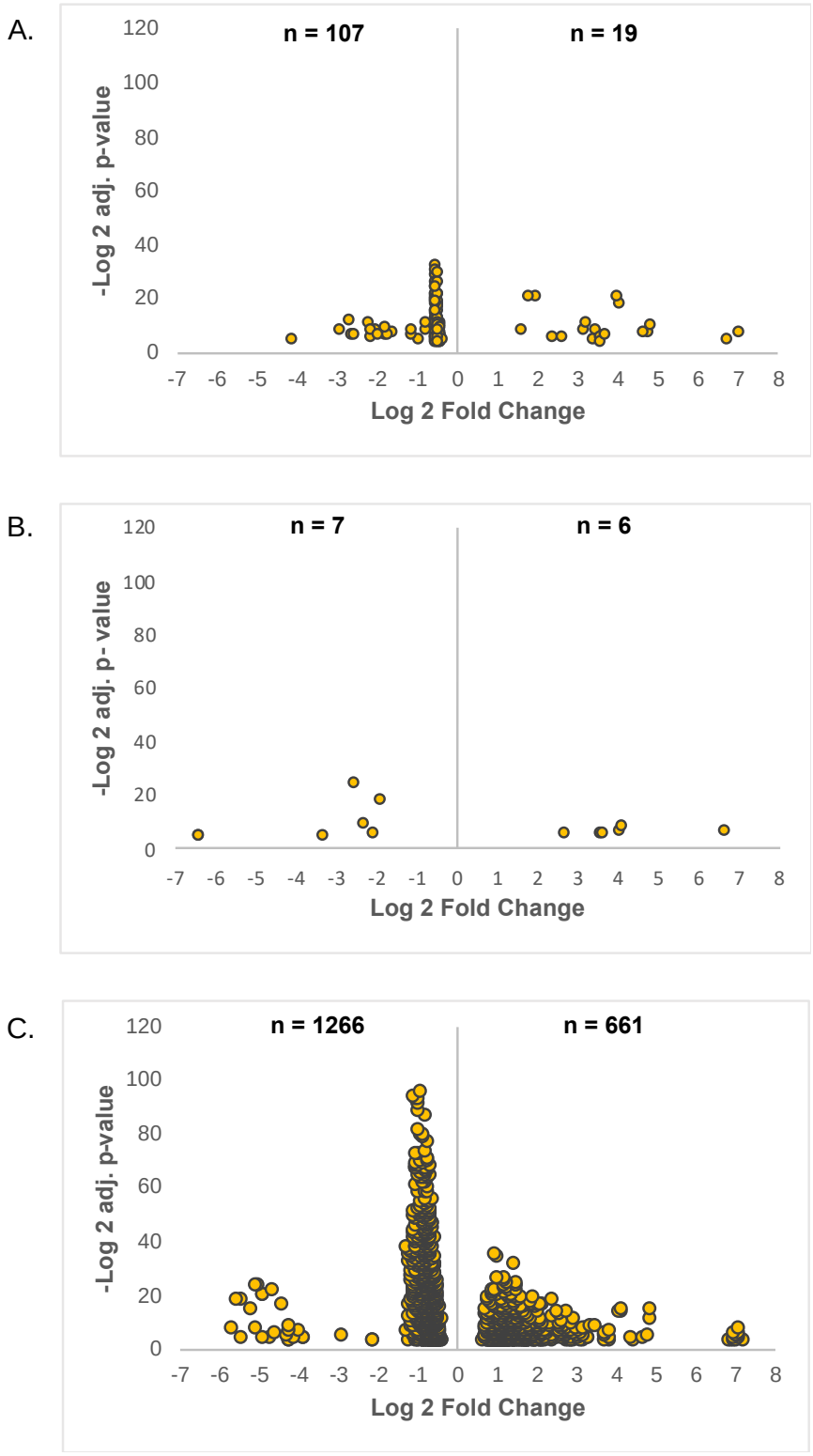
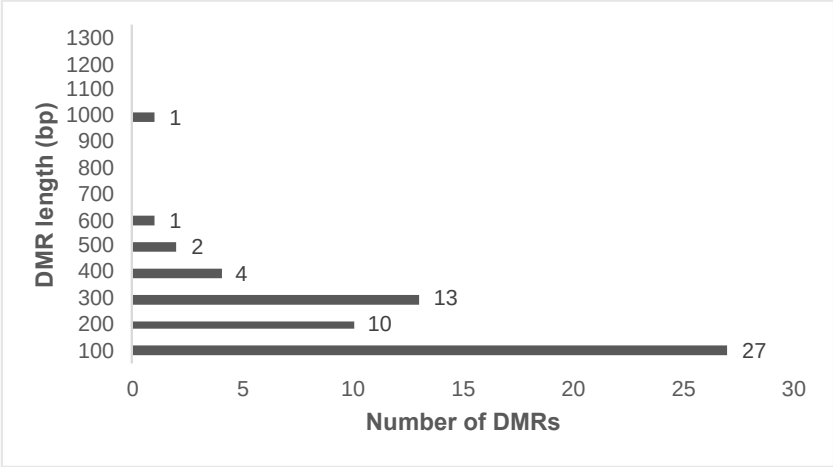


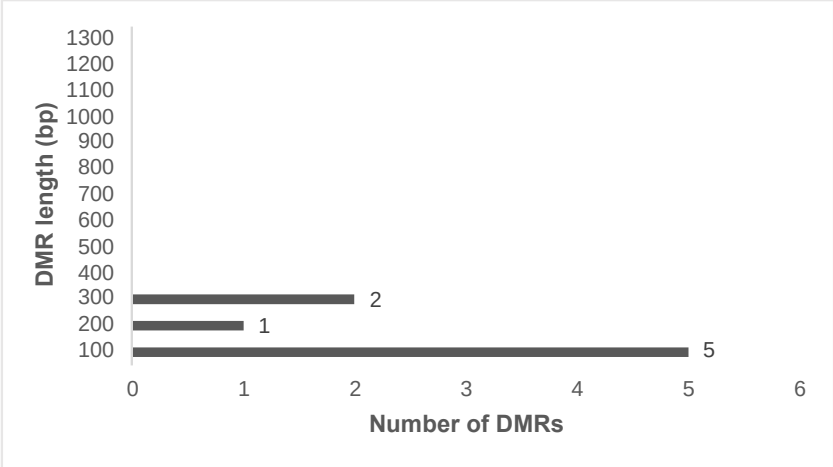
Figure 5.2 Fold change vs. p value for significant DMRs

All DMRs considered significant at adj. p value ≤ 0.1. A: Oil treatment, B: hypoxia treatment, C; Oil plus hypoxia treatment.

A.



B.



C.

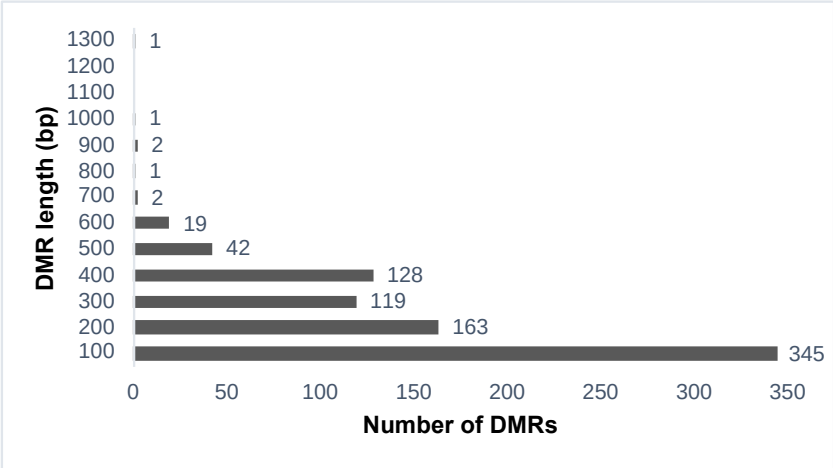
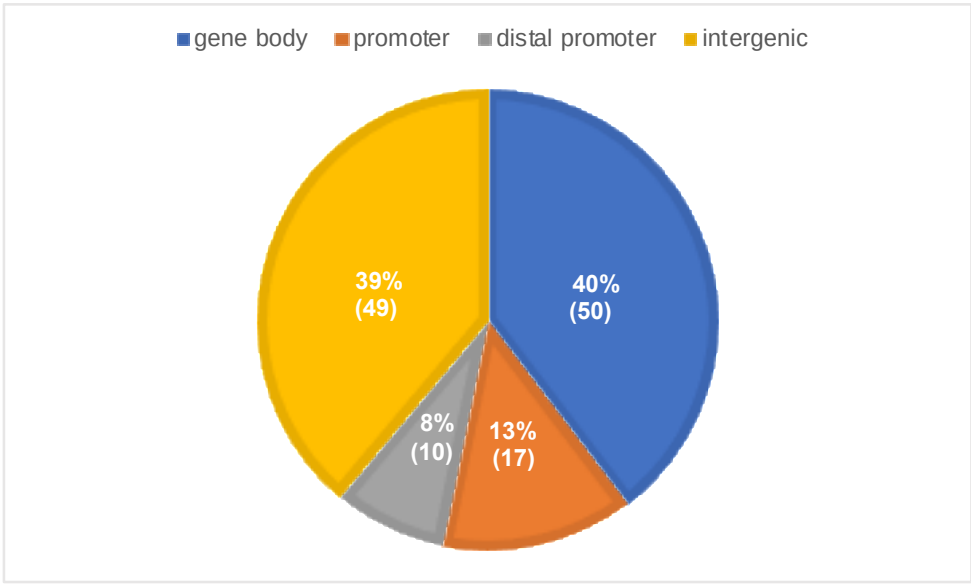


Figure 5.3 Size distribution of DMRs

Numbers indicate DMRs in each size category. A: oil treatment, B: hypoxia treatment, C: oil plus hypoxia treatment.

A.



B.

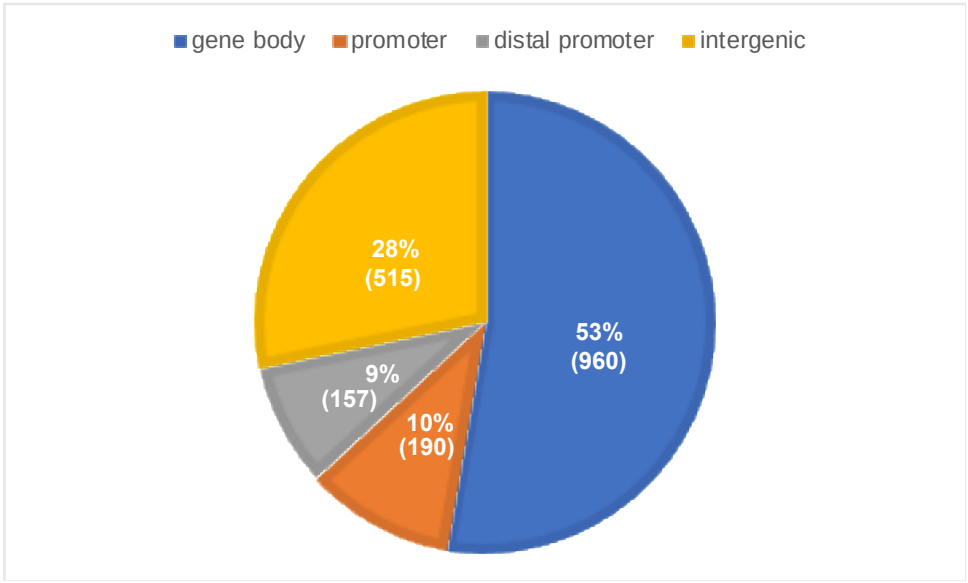


Figure 5.4 Genomic location of differentially methylated regions

Numbers represent total DMRs in each location. A: oil treatment, B: oil plus hypoxia treatment. Hypoxia only group was excluded due to low number of DMRs.

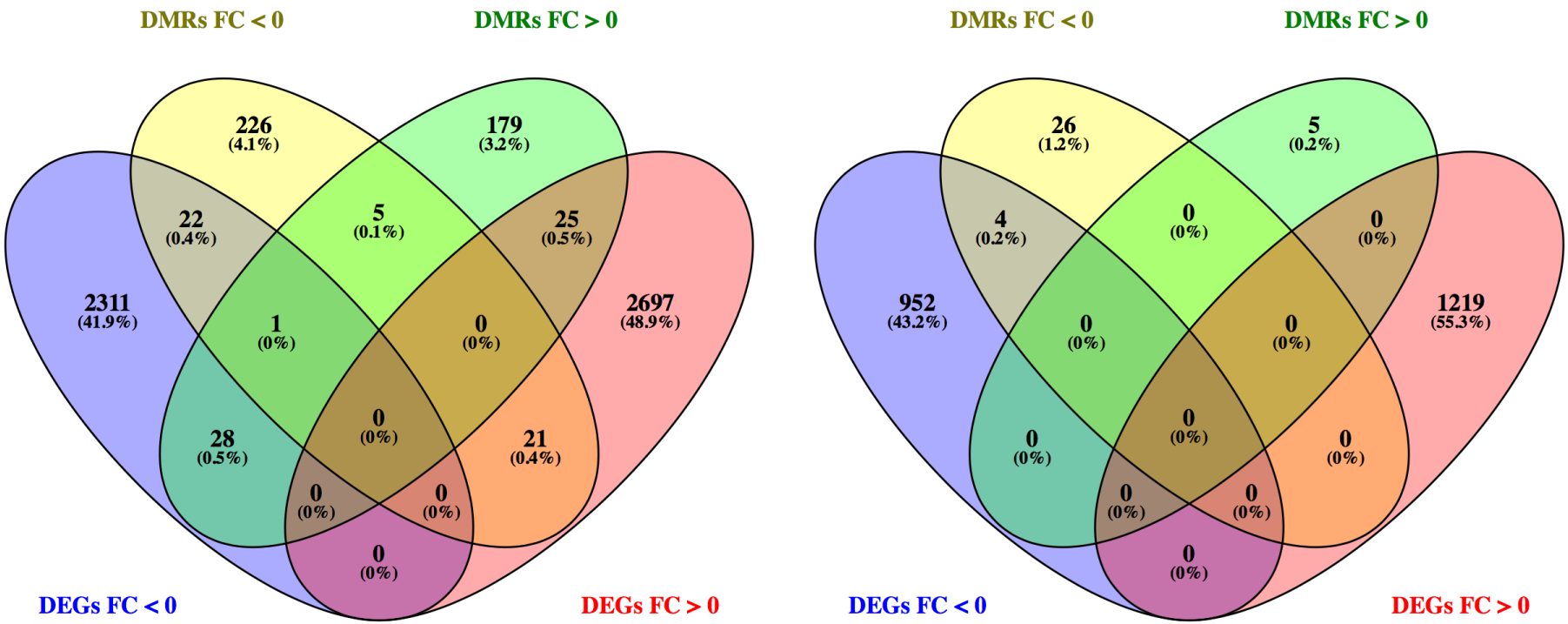
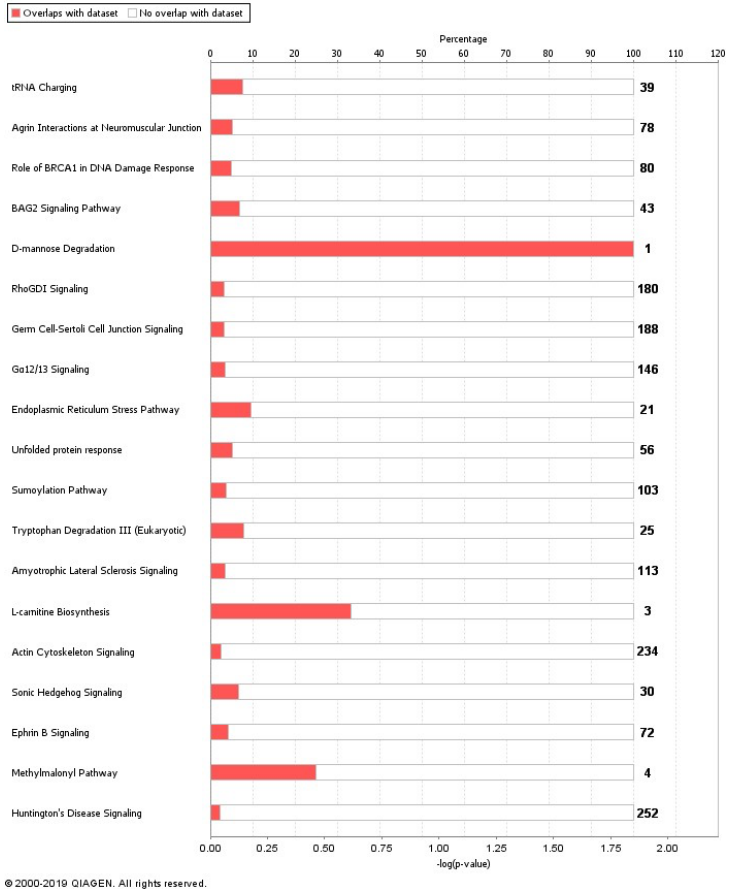


Figure 5.5 Overlap of DMRs with DEGs

A: oil plus hypoxia treatment, B: oil treatment

A.



B.

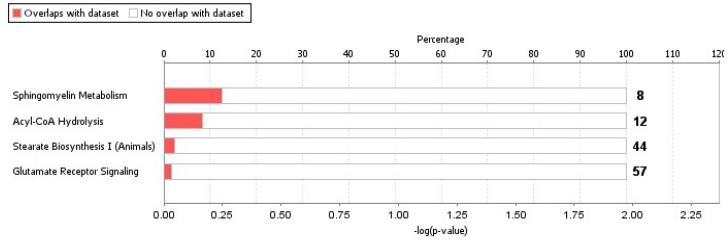


Figure 5.6 Enriched canonical pathways identified in IPA

Numbers indicate total number of genes in a pathway. Red indicates percent of genes represented in our data. A: oil plus hypoxia treatment, B: oil treatment

CHAPTER VI – SIGNIFICANCE OF RESEARCH

6.1 Concluding remarks

In this work we sought to identify molecular responses to oil contamination in estuarine fish. Oil exploration and extraction is predicted to increase along US coasts, particularly in the Gulf of Mexico (Harfoot et al. 2018). Therefore, in the future it is likely that estuarine organisms will encounter conditions similar to those of the Deepwater Horizon spill. This work elucidates how oil spills impact estuarine fish in a variety of contexts, such as across age stages, across taxa, and in conjunction with other environmental stressors. Additionally, our results suggest DNA methylation influences these responses, provoking questions about the duration of oil-induced effects.

One unique feature of this work is that it examines effects in a single species from fertilization through adulthood. Often, oil toxicity studies have focused on embryonic life stages due to concern regarding developmental disruption during organogenesis (Couillard 2002; Greer et al. 2012; Whitehead et al. 2012; Dubansky et al. 2013; Adeyemo et al. 2015; Incardona et al. 2015; Rodgers et al. 2018). However, our work highlights substantial effects across life stages. In adult *C. variegatus*, we found transcriptional effects following exposure to low, environmentally relevant concentrations of oil and dispersant. Adult life stages are often considered the most resistant to contaminant effects (Hutchinson et al. 1998), yet our work describes contaminant-induced alterations to immune and circulatory functions, suggesting adverse health outcomes resulting from oil exposure in adults.

Our comparison of transcriptional effects among early life-stage *C. variegatus* found that larval stages mount a significant transcriptional response to oil exposure, while embryonic responses were muted. Additionally, we found that, in larvae, transcriptional dysregulation was primarily associated with cholesterol synthesis and cardiac development, two functions critical to organismal growth. These combined results suggest that oil exposure during later life stages may have greater impacts to fish populations than embryonic toxicity.

We also examined whether transcriptional effects of oil exposure are conserved across taxa, with the intent to determine whether ecologically comparable species exhibit similar responses to oil. Although most studies investigating oil effects in estuarine fish have focused on single species (Whitehead et al. 2012; Crowe et al. 2014; Hedgpeth and Griffitt 2016; Dasgupta et al. 2016; Beyer et al. 2016; Jones et al. 2017; Rodgers et al. 2018), our comparison between larval *C. variegatus* and *F. grandis* illustrates that two taxonomically and environmentally similar species can mount distinct transcriptional responses to oil. This result was exemplified by opposing effects on transcription related to cholesterol biosynthesis. How these effects manifest in phenotypic differences is unclear, however our results suggest that nuanced differences in molecular effects exist among fish species, and should be considered when predicting the ecosystem-level effects oil contamination.

Perhaps the most novel feature of this work is the identification of oil-induced effects to DNA methylation. These effects are of particular interest as methylation can modulate gene expression and, because methylation marks are mitotically heritable, may therefore elicit permanent changes to transcriptional patterns (Dolinoy et al. 2007). Little

information exists regarding oil-induced alteration of the methylome in fish. We determined that oil exposure down-regulates DNA methyltransferase (*dnmt)* transcription in developing *C. variegatus*. Because *dnmts* are responsible for establishment of DNA methylation marks (Labbé et al. 2015), this downregulation suggests developmental oil exposure disrupts methylation patterns. Additionally, our results reveal substantial dysregulation of *dnmt* transcription in larval stages compared to embryos, further emphasizing that the molecular effects of oil exposure may be greater in later in development and suggesting that examination of larvae may provide a more sensitive assessment of oil spill effects than examination of embryos.

Our genome wide methylation analysis found that oil exposure alters methylation and that oil and hypoxia act synergistically to exert changes on the methylome in larval *C. variegatus*. These results are especially relevant given that contaminants from the DWH spill reached shorelines during annual periods of summer hypoxia (Engle et al. 1999; Michel et al. 2013; Nixon et al. 2016), and corroborate previous work documenting heightened toxicity following simultaneous exposure to both stressors (Dasgupta et al. 2015; Hedgpeth and Griffitt 2016; Rodgers et al. 2018). Following simultaneous oil and hypoxia exposure, we found genome-wide hypermethylation as well as altered methylation of specific loci. We also found a correlation between differentially methylated loci and gene expression in animals exposed to both stressors, suggesting that methylation influences transcriptional patterns. This relationship between methylation and transcription is of particular importance considering that methylation marks can be permanent, therefore, these results prompt questions regarding the long-term effects of oil exposure on gene expression. However, DNA methylation is a dynamic process, and

while methylation patterns can persist long-term, they can also be removed through passive and active demethylation processes (Wu and Zhang 2010). It is unclear whether the altered methylation in this study represents transient or permeant effects, and is an area for future study.

Finally, many of the molecular effects identified herein highlight biological processes consistently dysregulated following oil exposure in fish. In this work we found transcriptional disruption of processes related to immunity, cardiac and circulatory function, and cholesterol biosynthesis, as well as transcriptional and methylation effects related to DNA replication and repair. Dysregulation of these processes has been noted in other teleosts across multiple taxa, life stages, and habitats, suggesting these are pervasive and conserved effects of oil exposure (Bayha et al. 2016, Incardona et al. 2004, 2009, Jones et al. 2017, Rodgers et al. 2018, Sørhus et al., 2017; Xu et al., 2017b, 2016). Thus, our data contribute to identification of a conserved set of effects following oil exposure in fish models. Overall, our results suggest that the sub-lethal impacts of oil spill contaminants constitute a substantial insult to cellular and molecular functions in estuarine fish, and imply that effects to fish health following oil spills could be more widespread and persistent than previously thought.

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