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The effects of natural and anthropogenic stressors on the stress response and tissue-level response in teleost fish

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THE EFFECTS OF NATURAL AND ANTHROPOGENIC STRESSORS ON THE STRESS RESPONSE AND TISSUE-LEVEL RESPONSE IN TELEOST FISH.

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological Sciences

By

Benjamin David Dubansky
B.S., Virginia Commonwealth University, 2006
May 2013
The following work is dedicated to Thomas (Tommy) Mayes, Travis Krause, and Jacob Martin. Benjamin Alexander, Layton Nidever, Terry Douglas, John Tomaske, Travis Swann, Valerie Lanzi, Kendal Oncale, Matthew Frontiero, Brogan, and those who still suffer.
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ABSTRACT

Analysis of integrative physiological responses is a valuable approach to studying the biological impacts of natural and anthropogenic stressors in biota. By measuring fluctuations in multiple physiological characteristics, we are able to predict the impact that exogenous stressors may have on organismal health. Laboratory studies are useful in studying the effects of a single or multiple stressor interactions in a controlled environment, although field-based studies allow for the understanding of how fish are affected in situ, where multiple natural and anthropogenic stressors exist. Using laboratory and field-based experimental design, physiological indices of fish health were measured in fish exposed to natural and anthropogenic stressors in two unique incidences. First, controlled laboratory-based studies were performed where fish were exposed to larval freshwater mussels, a natural stressor found in North American freshwater environments. Unionid freshwater mussel larvae, called glochidia, must attach to a host fish in order to metamorphose into their juvenile state. This obligate stage in the unionid freshwater mussel life-cycle was found to cause a stress response in fish infected at high concentrations, where elevated cortisol in stressed fish was found to be coincident with an increase in glochidia metamorphosis to the juvenile stage. Second, both laboratory and field studies were conducted to understand the effects of the Deepwater Horizon oil spill on populations of Gulf killifish (*Fundulus grandis*) in oil-impacted areas in Louisiana, compared to reference sites in Louisiana, Mississippi, and Alabama. We show evidence of exposure to crude oil coincident with contamination from the Deepwater horizon oil spill in field-collected fish collected in 2010 and 2011 from oil-impacted sites, which had divergent gene and protein expression patterns compared to fish from unoiled locations. Further, controlled laboratory exposures of Gulf
killifish embryos to field-collected sediments from oiled locations revealed that fish exposed to heavily oiled sediments as embryos had reduced cardiovascular defects, delayed hatching, reduced overall hatching success, pericardial edema, and were smaller at hatch. Together, these data suggest that fish were exposed to toxins in crude oil for two successive breeding seasons, indicating, that contaminating oil from the Deepwater Horizon oil spill impacts organismal fitness, which may be predictive of long-term effects on Gulf killifish populations, and other biota that inhabit these areas, and also highlights the utility of combining laboratory and field-based techniques to predict the effects of natural and anthropogenic stressors on fish.
Aquatic species are increasingly being subjected to multiple sources of stress exerted by natural and anthropogenic environmental effectors. Naturally occurring fluctuations in water chemistry and availability due to tidal cycles, temperature variation, habitat alteration and destruction, and long term climate change, can cause aquatic species to be subjected to changes in temperature, salinity, acidity, water availability, and other modifications in habitat characteristics that can impact an organism’s ability to survive within its niche [1]. Urban expansion and infrastructure development alters and reduces the natural environment while toxicants from industrial activity accumulate in the environment ranging from an increase of a particular chemical at a point source to complex mixtures of chemicals from multiple sources that pose additional challenges for survival [2]. In order to assess the impacts of these exogenous stressors on aquatic habitats, physiological changes in teleost fish are commonly used as an indication of the effect of stressors on an ecosystem’s biota [3].

Changes in fish physiology due to natural and anthropogenic stressors are determined by measuring fluctuations in behavior [4-6], metabolism [7, 8], gene function [9, 10], endocrine and neural function [1], and reproductive capacity [11, 12]. Variation in such characteristics can suggest sub-lethal alterations in physiological function that might impact overall health and productivity [13-15], and can help predict long term, population-level effects [16, 17]. Such assessments are measured across multiple levels of biological integration from the gene level where changes in expression can be predictive of corresponding protein function [18], through the gross level where these molecular interactions are manifested as anatomical abnormalities.
This combination of multiple measures of physiological variation from the molecular to the tissue level provides a comprehensive mechanism for evaluating the effects of environmental perturbations on fish.

In order to better understand how fish respond to a stressor, controlled laboratory experimentation is often paired with field-based *in situ* evaluations of physiological status. In a laboratory experiment we attempt to control as many environmental variables as possible and examine the response to a single effector. While this technique answers basic biological questions, the practice is counterintuitive when the goal is to understand the environmental condition where numerous variables co-exist. Field-based experiments permit the inclusion of many stressors that are naturally present within the niche of the species being studied, and are likely most indicative of the *in situ* condition. However, a field-based approach brings with it the many sources of environmental variability that enhance variance (i.e. predation, weather patterns, parasites, competition, etc.) [21]. This may alter the outcome of such investigations, such that caution should be exercised when delineating cause-effect relationships observed within the *in situ* condition [21]. It follows that a pragmatic methodology which couples both laboratory and field-based experimentation, is essential in order to accurately predict the effects of natural and anthropogenic stressors on fish health. The following chapters describe a holistic approach that couples laboratory and field-based experimentation to understand the impacts of natural and anthropogenic stressors on fish physiology by assessing molecular, tissue level, and whole-animal measures of response to environmental perturbations.

Chapter two begins with a laboratory-based investigation of a natural stressor that studied the effects of infection by larval unionid mussels on fish physiology. Larval unionid mussels are freshwater species whose larvae, called glochidia, undergo an obligate parasitic stage whereby
they must attach to the gills and fins of a host fish in order to metamorphose into juveniles [22, 23]. Mostly attaching to the gills of fish, glochidia attach and become encysted by epithelial cells of the fish host, where they undergo their transformation to become a free-living juvenile [23]. Since glochidial cysts reduce the effective surface area of the gill and cause a considerable change in tissue morphology in response to infection [24, 25], it was hypothesized that during infection, the fish would undergo a stress response. Since freshwater mussels are the among the most endangered group of animals on the planet, and very little is known about the obligatory relationship between the mussel and the fish host, a series of experiments were designed to increase our understanding of the role of host physiology in unionid mussel survival [26-28].

Using traditional and novel laboratory-based techniques, experiments were designed to determine the effects of laboratory exposure to glochidia on fish hosts. Using controlled infections of host fish, and then subsequently monitoring for changes in their physiological function, it was demonstrated that high levels of glochidial infestation elicit increases in plasma cortisol in fish, indicating that a stress response is occurring in response to infection. Additionally, glochidia attachment to fish hosts were significantly increased when the fish host underwent a stress response; leading to a secondary hypothesis proposing that elements of the host stress response increased the potential for survival during this critical life stage of the unionid mussel’s life cycle. This hypothesis was tested, and it was found that fish with elevated cortisol were better hosts, allowing significantly more glochidia to attach and metamorphosose into free-living juveniles. From these data, it was concluded that fish undergoing stress may serve as better hosts for glochidia as a result of physiological change related to the increase of cortisol. This may hold some adaptive significance with regards to the methods that adult freshwater mussels employ to infect fish, some of which result in high levels of infection, and to
environmental pressures that may alter fish host physiology. Furthermore, this information may become useful in artificial propagation programs that are attempting to increase numbers of endangered mussel species.

The utility of these data will be improved by pairing the results and future experiments with field-based assessments of *in situ* fish physiology and mussel-fish interactions to better predict the environmental condition. Understanding the natural condition of unionid mussels, and the fish that serve them as hosts is difficult using solely laboratory-based experimental regimes. Infection methodology and fish holding conditions were tightly regulated in the experiments described in Chapter two, and were not designed to reflect an environmentally-relevant duplication of the *in situ* condition, but rather to determine a mechanism in fish hosts that act to regulate the metamorphic success of the glochidia. By doing this, it was possible to increase our knowledge of the challenges that these animals are exposed to, and to infer new insights into the relationship between freshwater fish and unionid mussels. Additionally, a single species of fish and mussel was used based on their ubiquitous abundance in freshwater systems and favorable host-parasite relationship; this is a further departure from the natural condition where hundreds of species of both fish and mussel interact in varying degrees of host-parasite specificity [23]. Further understanding of mussel-fish relationships where mussel species may have infrequent interactions with favorable hosts is warranted to determine the nature of such challenges that these species face, and how some species overcome these challenges while others perish. Chapter two outlines background information on unionid mussels and insight into possible future laboratory investigations to understand the mechanisms involved in fish host response to glochidia infection and how this influences the host-parasite relationship in a species-specific and general context. Ultimately this work offers a base of knowledge that could be paired with
field-based experiments that may allow us to better predict the future distribution and abundance of unionid mussel populations.

The remaining chapters describe an investigation that incorporates both field-based and laboratory-based evaluations to determine the effects of an anthropogenic stressor on fish health. The Deepwater Horizon oil platform exploded on April 20, 2010, releasing over 600 million liters of crude oil from the Macondo well, and affected 1040 km of shoreline along the Gulf coast [29, 30]. Remediation efforts were initiated by responsible parties and state and federal authorities, to mitigate the effects of this environmental disaster, and to determine the extent of damage on the ecosystem caused by the presence of crude oil. As oil made landfall, a natural experiment occurred (i.e. experimental conditions were set by factors that were not under our control) where crude oil from the Macondo well became a variable that differentiated affected areas from areas that did not receive landfall of oil. Since crude oil contains chemicals that are toxic to aquatic species, and data from laboratory and field experiments following the Exxon Valdez oil spill revealed population-level effects in fish species, it was hypothesized that similar effects would be seen in areas affected by the Deepwater Horizon oil spill [16, 17, 31-33]. As resources and opportunities to study the effects of the Deepwater Horizon oil spill on fish health became available, an ongoing study began that characterizes the effects using both field-caught fish and laboratory based experimentation.

In Whitehead et al., 2012 (Appendix 1) [34], a preliminary characterization of the potential impact of the Deepwater Horizon oil spill on resident Gulf killifish (Fundulus grandis) populations was described. Whitehead et al., characterized the physiology of adult Gulf killifish collected in situ from six locations in Louisiana, Mississippi, and Alabama in May, June, and August 2010, and examined potential reproductive effects in vitro by exposing Gulf killifish
embryos from a laboratory population to waters collected from each location. These data demonstrated a significant divergence of gene expression in hundreds of genes in liver tissues from fish collected from the heavily-oiled island of Grande Terre, Louisiana compared to those collected from unoiled locations in Mississippi and Alabama. Additionally, cytochrome P4501A (CYP1A) protein, a well described biomarker of crude oil and toxicant exposure (see below) had increased expression in the gills of fish from Grande Terre, Louisiana. Embryonic exposures to field-collected waters showed no visible developmental defects, but demonstrated an increase in CYP1A protein in multiple tissues in embryos exposed to water collected from Grande Terre compared to embryos exposed to water collected from sites in Mississippi and Alabama. These data were paired with analytical chemistry of water, tissue, and sediment samples, as well as satellite-remote sensing data that linked the incidence of physiological abnormalities in Grande Terre, Louisiana fish with the presence of oil from the Deepwater Horizon oil spill; oil that was found predominantly in the sediment at this site based on analytical chemistry [34].

Among the divergently expressed genes Whitehead et al., 2012 [34] found in Grande Terre fish were a number of genes associated with activation of the aryl-hydrocarbon receptor (AhR), including CYP1A. The AhR is in all vertebrates and has endogenous ligands such as indigoids and tryptophan derivatives that have similar structure to xenobiotic ligands, as well as activators such as low density lipoprotein [35-37]. This pathway has been implicated in immune modulation, vascular development, osmoregulation, endocrine signaling, cell cycle control, apoptosis, and metabolism of steroids, fatty acids, vitamins, and prostanoids, in addition to having a major role in xenobiotic and drug metabolism [35-38]. Activation of the AhR results in multiple genes to be activated to ultimately produce protein end products. Of these genes and end products, CYP1A is the most popular indicator of AhR activation because although it is
expressed under normal conditions, expression is far greater when induced by exogenous xenobiotic ligands compared to constitutive expression [39]. It is because of this and a voluminous base of literature solidifying the use of CYP1A as a biomarker of AhR activation and xenobiotic exposure that CYP1A was the primary biomarker of exposure to crude oil used in this preliminary field study.

The results of the Whitehead et al., 2012 [34] field study suggested that fish were exposed to crude oil from the sediment at the Grande Terre, Louisiana field site. Much of the oil from the Deepwater Horizon oil spill made landfall to the west of the Mississippi River, making its way into the Barataria Bay Basin, where many kilometers of shoreline were oiled according to the Shoreline Cleanup and Assessment Technique (SCAT) data compiled by the National Oceanographic and Atmospheric Administration (NOAA). Based on the data provided by NOAA and from our own preliminary field expeditions, an intensive survey of fish populations across the Barataria Basin was initiated in November, 2010 to determine the extent of exposure in fish inhabiting this heavily impacted area. Chapter three includes a focused study on the expression of key biomarkers of exposure to crude oil in Gulf killifish sampled in situ from fourteen locations in Plaquemines Parish, Louisiana within the Barataria Bay Basin. Two AhR-associated biomarkers of exposure to oil, CYP1A and the AhR repressor (AhRR) were utilized as classic indices of exposure [34]. Livers from fish collected at each of the fourteen sites were measured for mRNA transcript abundance of CYP1A and AhRR, and CYP1A protein were measured through an enzyme-linked immunosorbant assay (ELISA) to obtain an overview of exposure to crude oil in the Barataria Bay estuary. These data and a histological survey of CYP1A in the gill, intestine, and head kidney suggest that the most likely route of exposure was
via the gastrointestinal tract from dietary intake of benthic organisms, inadvertent ingestion of sediment, or the drinking of water.

Chapter four revisits Grande Terre over one year after the landfall of oil, and describes extensive laboratory-based studies along with physiological indications of exposure in fish collected in situ to illustrate sustained exposure to crude oil in adult fish and evidence of reduced reproductive capacity. The sampling of Grande Terre in August of 2011 added a fourth time point to the original study, and archived tissues from 2010 field collections and newly-collected tissues were utilized to provide a comprehensive evaluation of genomic and protein-level biomarkers of exposure to oil over time. Furthermore, Gulf killifish embryos were exposed to field-collected sediments from Grande Terre in 2010 and 2011 and from two other locations in Barataria Bay. These early life-stage sediment exposure experiments suggested that there is a reduction in reproductive potential of Gulf killifish living in heavily-oiled sites, and provided substantial evidence of sustained exposure in less-oiled sites. These data show continued exposure of Gulf killifish to toxicants in crude oil for over one year following the initial landfall of oil at Grande Terre island.

Chapter five follows with a short conclusion concerning the importance of combining field-based and laboratory-based studies to determine the effects of both natural and anthropogenic stressors on fish health, and a summary of the findings.
CHAPTER TWO – THE INFLUENCE OF CORTISOL ON THE ATTACHMENT AND METAMORPHOSIS OF *UTTERBACKIA IMBECILLIS* ON BLUEGILL SUNFISH (*LEPOMIS MACROCHIRUS*), [40].

### 2.1 INTRODUCTION

Freshwater unionid mussels are the most endangered animals in North America, with more than 70% of the approximately 300 species in North America threatened or otherwise considered endangered [26-28]. The decline in their numbers in recent years is of significant ecological relevance to the southeastern region of the United States, where biodiversity of freshwater mussels is higher than in any other region of the world [27]. Although the possible causes of this loss in biodiversity are varied [41], the inability of glochidia to undergo metamorphosis on fish hosts likely contributes to this decline.

Freshwater unionid mussels have a unique life cycle, beginning with their development in the gills of parental mussels. When embryonic development is complete, the larvae, which are also called glochidia, are released from the adults and attach primarily to the gills or fins of fish [23]. Upon attachment, epithelial cells of the host migrate to encapsulate the glochidia, forming cyst-like capsules around the larvae [42, 43]. Depending on the mussel species, a glochidium remains within a cyst for days to months [25, 42, 44-46], during which time it may metamorphose into a juvenile. Eventually the juvenile mussel will detach from the host fish to continue its life as a free-living benthic organism. Although the transition of a unionid mussel from the larval to the juvenile stage requires a host, little is known about the physiological mechanisms regulating this host-ectoparasite interaction.

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1 Reprinted with permission from the Marine Biological Laboratory, Woods Hole, MA.
Each species of glochidia has differing levels of host specificity that dictate the fish species it may utilize as a host [23, 43, 47-50]. Some mussel species are generalists capable of metamorphosing on numerous hosts, whereas others are compatible with a much smaller number of host species [23]. Hosts are also capable of acquiring resistance to glochidia following repeated infections [23, 43], which could also alter the compatibility between glochidia and fish species.

Although the underlying basis for host specificity and host resistance to glochidia are largely unknown, local tissue reactions of the host are likely important in mediating these responses [42, 44, 49-51]. The localized tissue response in the fish gill to glochidia infection consists of inflammation, hyperplasia, necrosis, and infiltration of different cell populations to the sites of attachment [25, 44, 49, 50, 52]. Although these effects are seen in all glochidia-infected fish to some degree, their magnitude are greatly exaggerated in resistant or non-host fish [25, 53-55]. These enhanced localized responses may lead to incomplete cyst formation following attachment of glochidia to the fish gill, and subsequently to the premature shedding of these larvae [42, 48, 49, 53]. It is thought that these mechanisms account for the early shedding of glochidia from resistant fish [25, 47]. Understanding the influence of host physiology on these gill level effects may help describe the specific requirements for successful metamorphosis of glochidia on host fish.

The fish gill is the primary organ for maintenance of whole-animal homeostasis in the fish [56, 57]. It is also the primary site for the introduction of potentially pathogenic organisms into the body [58, 59]. Tissue-level responses to glochidia may potentially impair gas exchange, ion homeostasis, and acid-base balance, all of which are physiological functions primarily regulated at the gills [56]. At high levels of glochidia infestation, gill burdens may be severe enough to kill
the fish host [51, 60], jeopardizing the continued development of the glochidia. At low to moderate levels of glochidia infection, it is likely that the host will undergo a stress response, which may affect host immunity, and thus influence glochidia survivorship on the host. It follows that changes in the physiological status of potential fish hosts may ultimately affect the efficacy of glochidia attachment and metamorphosis [28, 61, 62].

The objectives of this study were to investigate the effects of glochidia exposure on various physiological endpoints broadly indicative of host stress, including plasma cortisol, lactate, glucose, sodium, and blood hematocrit. We demonstrated that exposure to high concentrations of glochidia resulted in elevated plasma cortisol in hosts, and that at these high glochidia concentrations, more glochidia attached to hosts and a greater percentage of glochidia metamorphosed into juveniles. We also set out to test the hypothesis that exogenous administration of cortisol to fish could directly affect the ability of glochidia to attach and metamorphose following infection. To our knowledge, this is the first study involving controlled experimental infections measuring the teleost stress response and its effects on glochidia metamorphosis in host fish.

2.2 MATERIALS AND METHODS

2.2.1 Animals
Bluegill sunfish (*Lepomis macrochirus*, Rafinesque, 1819) were obtained from Pond Pro, LLC (Liberty, MS) where fish were reared in small ponds, which had no signs of mussel shells or live animals based on our own inspection after pond draining. All fish were transported to the Department of Biological Sciences Aquatic Facility at Louisiana State University, where the facility was maintained on a 12 hour light cycle. Prior to experimental infections with glochidia, fish were acclimated at 21-23 °C for at least 1 month in 380 liter polyethylene tanks in
artificially-formulated pond water (AFW) [63] under recirculating conditions. Fish were fed 40% protein fish pellets (Cargill Aquaxcel™) at a ration of 3 % body weight per day. For experimental infections, glochidia of the paper pondshell mussel (*Utterbackia imbecillis*, Say, 1829) were utilized. *U. imbecillis* is a host generalist capable of parasitizing a wide array of fish, including the bluegill [22, 42]. This species can be found gravid year round in small ponds in East Baton Rouge Parish, LA. Gravid mussels were collected as needed and transported to Louisiana State University, where they were utilized upon arrival, or held in 19 liter containers for no longer than 3 weeks under static conditions in native pond water at 21-23°C. Mussels were fed a mix of live or freeze-dried algae weekly.

Glochidia were isolated from gravid mussels using a slightly modified protocol from that of Dodd *et al*. 2005. Briefly, the outer demibranchs of each gravid mussel were cut down the midline and flushed into a 1000 mL beaker with a stream of AFW from a plant sprayer. The mixture was briefly stirred until aggregates of glochidia and mucus formed. A pipette was used to transfer these mucus-bound glochidia to a 25 mL beaker. A gentle stream of AFW was focused on the mass to dislodge the glochidia, which were allowed to overflow back into the 1000 mL beaker. Glochidia from each mussel were held in separate 1000 mL beakers and washed twice with AFW. The glochidia from each mussel were assessed for viability by removing a 100 µL aliquot of glochidia suspension from each mussel followed by the addition of KCl crystals. Open glochidia that closed upon addition of KCl were considered viable. The ratio of viable glochidia to total glochidia was used to determine the percentage of viable glochidia for each mussel. Mussels with > 90 % viable glochidia were pooled and utilized for experimental infections.
All animals were housed and handled according to Institutional Animal Care and Use Committee Protocol #07-053. Animal care facilities were managed by the Department of Laboratory Animal Medicine, Louisiana State University, School of Veterinary Medicine and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2.2 Infection procedures
Initial infections were conducted in 19 liter glass aquaria to determine an optimal fish size for subsequent experiments. Sixty fish, ranging in size from 0.26 to 22.92 g, were exposed in small groups ($n \leq 6$) to 4000 glochidia liter$^{-1}$ in AFW for 25 min. Glochidia were kept in suspension by moving water with a 25 mL serological pipette. After infections, fish were killed and gill arches isolated into chilled phosphate-buffered saline (PBS) awaiting analysis of gill infection intensity using a stereomicroscope (Zeiss, SteREO Lumar .V12). Number of glochidia attached to each gill arch was counted to estimate total gill burden.

For all other experiments, an automated infection system was developed to minimize human interaction with the fish. Each system consisted of a 19 liter conical-bottomed tank affixed with a drain to collect glochidia. An air driven plumbing system gently recirculated the glochidia to the top of the tank, then across a baffle to resuspend glochidia evenly throughout the tank. Plumbing was designed to accommodate both recirculating and flow-through capabilities. Fish were allowed to acclimate to the infection systems for 48 hours prior to infection under flow-through conditions. At the start of infections, each system was switched to recirculating conditions and glochidia added as described below. After infection, the chambers were switched to flow-through conditions by flushing with AFW for at least 10 minutes to remove glochidia.
2.2.3 Physiological response to infection

Thirty bluegill (14.06 ± 0.30 g) were randomly divided into 5 groups \((n = 6)\) and acclimated to 5 infection chambers for approximately 48 hours prior to exposure to glochidia at concentrations of 0, 1000, 2000, 4000, and 8000 glochidia liter\(^{-1}\) for 25 minutes. Twenty-four hours after exposure, fish were sampled, and their body mass and fork-length recorded. Blood was collected from the caudal peduncle in ammonia heparinized micro-hematocrit (Ht) capillary tubes (Fisher Scientific, 22-362-566), then centrifuged in a micro-capillary centrifuge (International Equipment Company, Model MB). The Ht of each fish was calculated as the percent of packed red blood cells to total blood volume. Plasma from each fish was collected and flash frozen in liquid nitrogen, and stored at \(-80^\circ\text{C}\) until further processing. Plasma sodium ion concentrations were measured by flame atomic absorption spectroscopy (Varian, AA240FS). Plasma cortisol concentrations were measured by radioimmunoassay (Diagnostic Systems Laboratories, DSL-2100). Plasma glucose was measured using a 96-well glucose assay kit (Cayman Chemicals, 10009582), and plasma lactate was measured with a lactate meter (Nova Biomedical, Lactate Plus).

2.2.4 Influence of infection intensity on metamorphic success and attachment

Twenty-four bluegill (13.33 ± 1.52 g) were divided randomly into 4 groups \((n = 6)\). Each group was infected as described above at concentrations of 1000, 2000, 4000, and 8000 glochidia liter\(^{-1}\). After infections, fish were immediately transferred into a custom-built system designed for monitoring the shedding of glochidia and juveniles from individual fish. Briefly, self-cleaning 2.8 liter tanks (Aquaneering, San Diego, CA) were arranged on a multi-level shelving unit equipped with a recirculating water delivery system capable of distributing water to the individual tanks. Water delivered to each tank was directed across the bottom of the tanks and through an overflow spout, effectively removing >95% of glochidia in a tank within 45 min (data
not shown). Each tank was outfitted with a 100 µm filter to collect debris from the overflow. The contents of each filter were examined daily under a stereomicroscope for the presence of glochidia and juveniles. The number of glochidia attached to the hosts during infection was calculated from the sum of glochidia and juveniles recovered from each fish during the 10 day post-infection period. The total number of mussels recovered from each fish was divided by the mass of each fish to give an estimate of attachment g⁻¹ fish. Metamorphosis success was determined according to the ratio of juveniles to total mussels shed from each fish during the duration of infection. By monitoring the emergence of glochidia and juvenile mussels each day, the time required until the first emergence of juveniles [47] and the total duration during which juveniles were shed from host fish [43] were calculated.

### 2.2.5 Effects of exogenous cortisol on metamorphic success and attachment

Eighteen bluegill sunfish (27.17 ± 0.86 g) were divided into three groups (n = 6). One group of fish was administered a dosage of 0.2 mg g⁻¹ fish of hydrocortisone 21-hemisuccinate (Sigma, H4881), hereafter referred to as cortisol, dissolved in warm coconut oil. Injections were performed intraperitoneally at a volume of 4 µl g⁻¹ fish using a 26 gauge needle. A second group of fish was sham injected with coconut oil at a volume consistent with the cortisol-laden injections. An additional group of non-injected fish was also included, and was otherwise handled identically to the cortisol and sham injected fish. All fish were allowed to recover from injections and handling for 48 hours. All three groups were then infected with 1000 glochidia liter⁻¹ and monitored for 14 days post infection to assess glochidia attachment and metamorphosis success for each fish, as described above.
2.2.6 Statistics
Linear regression was performed using Microsoft Excel. Statistical analysis was performed with XLSTAT Version 2010.3.03. Variables were compared using Kruskal-Wallis, followed by Dunn’s pairwise comparison if they did not meet assumptions of parametric normality. Normally distributed data sets were compared using one-way ANOVA followed by Tukey’s (HSD) pairwise comparison with a 95% confidence interval. All values were presented as means ± SE. Criteria for significant differences were at $p \leq 0.05$ for all comparisons.

2.3 RESULTS
Initial infections demonstrated a relationship between fish mass and gill glochidia burdens, increasing in an exponential fashion (Fig. 2.1A, $R^2 = 0.61$). This relationship was most evident in fish weighing at least 8 g, with glochidia gill burden increasing approximately 4-fold between 8 g to 23 g (Fig. 2.1A and B). Plotting these data on log-log scales linearized this relationship, providing a power function of 2.19 ($R^2 = 0.61$). Based on these data, we chose to conduct subsequent experiments with fish weighing no less than 8 g whenever possible, in order to increase the probability of glochidia accumulation on fish gills during infections.

Fish were infected with glochidia at concentrations ranging from 0 - 8000 glochidia liter-1 for 25 min using automated infection chambers, then assessed for various hematological endpoints at 24 hours post infection. There was a significant increase in plasma cortisol in fish exposed to 2000, 4000, and 8000 glochidia liter-1 compared to fish infected with 0 or 1000 glochidia liter-1 ($P < 0.03$) (Fig. 2.2, Table 2.1). In comparison, there were no significant differences between treatments in plasma glucose, lactate, and sodium (Table 2.1). There was no clear pattern of effect in blood Ht with regards to glochidia infection concentration. Ht was significantly elevated in the 0, 2000, and 8000, glochidia liter-1 exposed fish compared to the 1000 and 4000 glochidia liter-1 infected fish (Table 2.1).
Figure 2.1 A–B. Relationship between fish mass and infection in the gill for fish infected with 4000 glochidia liter\(^{-1}\). Sixty fish were exposed in small groups to 4000 glochidia liter\(^{-1}\) for 25 minutes. The number of glochidia attached to the fish gills was enumerated using stereomicroscopy. (A) Non-linear regression of the number of attached glochidia on the gills of each fish versus fish mass (g). (B) Same data as A plotted on log-log scales. Circles represent the data for fish used in establishing the line of best-fit through the data. Triangles represent the data for fish that were excluded in establishing the line of best-fit (see text for more details). \(R^2\) represents the correlation coefficient for the line of best-fit calculated using non-linear regression.

Table 2.1. Effects of glochidia infection on hematological variables and shedding dynamics.

<table>
<thead>
<tr>
<th>Infection (glochidia (1^+))</th>
<th>Hematocrit</th>
<th>Na (mmol (1^+))</th>
<th>Glucose (mg/dl)</th>
<th>Lactate (mol (1^+))</th>
<th>Cortisol (ng/ml)</th>
<th>Time until juvenile shedding (days)</th>
<th>Duration of juvenile shedding (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 gl/l</td>
<td>0.260 ± 0.013</td>
<td>151.01 ± 5.58</td>
<td>53.67 ± 5.27</td>
<td>1.38 ± 0.17</td>
<td>24.5 ± 3.85</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>1000 gl/l</td>
<td>0.250 ± 0.007</td>
<td>150.20 ± 4.17</td>
<td>54.41 ± 7.32</td>
<td>1.24 ± 0.27</td>
<td>31.7 ± 12.13</td>
<td>6.00 ± 0.00</td>
<td>2.20 ± 0.20</td>
</tr>
<tr>
<td>2000 gl/l</td>
<td>0.267 ± 0.015</td>
<td>142.85 ± 4.33</td>
<td>52.40 ± 6.17</td>
<td>1.52 ± 0.23</td>
<td>66.7 ± 20.85*</td>
<td>5.83 ± 0.17</td>
<td>3.17 ± 0.17</td>
</tr>
<tr>
<td>4000 gl/l</td>
<td>0.258 ± 0.007</td>
<td>148.28 ± 6.21</td>
<td>46.21 ± 4.31</td>
<td>1.28 ± 0.35</td>
<td>93.2 ± 20.79*</td>
<td>5.33 ± 0.21*</td>
<td>3.67 ± 0.21*</td>
</tr>
<tr>
<td>8000 gl/l</td>
<td>0.286 ± 0.011*</td>
<td>141.04 ± 4.27</td>
<td>44.46 ± 3.09</td>
<td>1.13 ± 0.14</td>
<td>84.4 ± 9.18*</td>
<td>5.33 ± 0.21*</td>
<td>3.67 ± 0.21*</td>
</tr>
</tbody>
</table>
Figure 2.2. Plasma cortisol concentrations of host fish sampled 24 hours after infection with glochidia ranging in concentrations from 0 - 8000 glochidia liter$^{-1}$ for 25 minutes. Values represent the mean plasma cortisol ± SE ($n = 6$). Bars with asterisks are statistically different from the 0 and 1000 liter$^{-1}$ infection concentration ($P < 0.03$).

A separate group of fish was infected with glochidia at concentrations ranging from 1000 to 8000 glochidia liter$^{-1}$ in order to assess the pattern of shedding of immature glochidia and mature juveniles from hosts over time (Fig. 2.3). These data were used to calculate the mean time required for the first appearance of juvenile mussels from hosts (Table 2.1). Juvenile mussels began to emerge from host fish approximately 0.6 days sooner when fish were infected with 4000 and 8000 glochidia liter$^{-1}$ compared to 1000 glochidia liter$^{-1}$ ($P = .027$) (Table 2.1, Fig. 2.3). Once the first juvenile mussels began to emerge, the average duration of time during which juveniles were shed from hosts was significantly longer by approximately 1.5 days for fish infected at 4000 and 8000 glochidia liter$^{-1}$ compared to fish infected at 1000 glochidia liter$^{-1}$ ($P < 0.002$) (Table 2.1, Fig. 2.3).
Figure 2.3. Number of glochidia and juvenile mussels recovered from host fish after exposure to A) 1000, B) 2000, C) 4000, and D) 8000 glochidia liter$^{-1}$ for 25 minutes. Bars represent the mean number of glochidia or juveniles recovered each day ± SE ($n = 6$ per treatment) post infection for 10 days.
These data (Fig. 2.3) were used to calculate the number of attached glochidia per fish as attachment g⁻¹ fish (Fig. 2.4A) and metamorphosis success (Fig. 2.4B). Mean number of attached glochidia g⁻¹ fish mass was significantly elevated in hosts infected with 2000, 4000 and 8000 glochidia liter⁻¹ compared to those infected at 1000 glochidia liter⁻¹ ($P < .001$) (Fig. 2.4A). Mean metamorphosis success was significantly elevated from 48.8 ± 12 % in fish infected at 1000 glochidia liter⁻¹ to an average of 79.9 ± 1 % in infection concentrations at infection intensities at or above 2000 glochidia liter⁻¹ ($P < 0.01$) (Fig. 2.4B).

Experiments were conducted to assess whether cortisol supplementation of hosts prior to infection could influence the attachment and the subsequent metamorphosis of glochidia following a standard 1000 glochidia liter⁻¹ infection, a concentration that was found previously not to increase endogenous plasma cortisol (Fig. 2.2). The first appearance of juveniles was observed on day 6.0 ± < 0.01 for all sham-injected and cortisol-injected fish, and the first appearance of juveniles occurred in the control group on day 7.0 ± < 0.01 post infection (Fig. 2.5). The average duration of juvenile shedding for the cortisol injected fish lasted 5.5 ± 0.43 days (Fig. 2.5). This value was significantly different than the duration of 3.0 ± 0.41 days calculated from the control fish ($P = 0.003$). There was no statistical difference between the cortisol injected and sham-injected fish ($P = 0.16$). The mean number of attached glochidia g⁻¹ fish was 49.9 % higher in cortisol injected fish compared to control fish ($P = 0.01$) (Fig. 2.6A). The number of attached glochidia was 34.9 % higher in cortisol injected fish compared to sham injected fish ($P = 0.12$). Additionally, mean metamorphosis success was significantly elevated to 90 ± 1.12 % in fish that were administered cortisol injections compared to both control and sham injected fish, which had metamorphoses values of 62 ± 6.18 % and 60 ± 6.00 %, respectively ($P < 0.005$) (Fig. 2.6B).
Figure 2.4. Glochidia attachment, metamorphosis success, and the percentage of glochidia shed during the first 48 hours post infection for fish exposed to 1000, 2000, 4000, and 8000 glochidia liter⁻¹ for 25 minutes. All values are expressed as means ± SE (n=6 per treatment). Bars with asterisks are statistically different from the 1000 glochidia liter⁻¹ infection concentration. (A) Values represent the number of attached glochidia g⁻¹ fish ($P < 0.001$). (B) Values represent the metamorphosis success expressed as a percentage of the total number of attached glochidia ($P < 0.01$). (C) Values represent the average percentage of glochidia shed in the first 48 hours as a percentage of the total number of attached glochidia collected ($P < 0.001$).
Figure 2.5. Number of glochidia and juvenile mussels recovered from host fish after exposure to 1000 glochidia liter⁻¹ for 25 minutes for A) fish serving as non-injection controls, B) fish given an i.p. injection of coconut oil (sham injected) 48 hours prior to infection, and C) fish given an i.p. injection of cortisol dissolved in coconut oil 48 hours prior to infection (see text for more details). Bars represent the mean number of glochidia or juveniles recovered each day ± SE (n = 6 per treatment) post infection for 14 days.
Figure 2.6. Glochidia attachment, metamorphosis success, and the percentage of immature glochidia shed from non-injection control fish, or from fish either injected with coconut oil (sham injected), or injected with cortisol dissolved in coconut oil, 48 hours prior to exposure to 1000 glochidia liter$^{-1}$ for 25 minutes. All values are expressed as means ± SE (n=6 per treatment). Bars with asterisks represent statistical difference between the cortisol injected treatment group compared to the control (non-injected) and/or sham (coconut oil injected) treatment groups determined using Kruskall-Wallis, followed by Dunn’s pairwise comparison (panel A & B) or Tukey’s multiple range test (panel C). (A) Values represent the number of attached glochidia g$^{-1}$ fish ($P = 0.01$) for cortisol injected fish compared to control fish. (B) Values represent the metamorphosis success expressed as a percentage of the total number of attached glochidia ($P \leq 0.004$) for cortisol injected fish compared to control and sham injected fish. (C) Values represent the mean percentage of glochidia shed in the first 48 hours as a percentage of the total number of attached glochidia ($P \leq 0.02$) for cortisol injected fish compared to control and sham injected fish.
During the first 48 hours post infection, shedding of glochidia was at its peak for all treatment groups. In order to identify differences in the shedding during this time, the number of mussels shed during the first 48 hours was examined. The percentage of the total that were shed as
glochidia in the first 48 hours post infection was approximately 47 ± 9 % for fish infected with 1000 glochidia liter⁻¹, compared to approximately 13% to 18% for fish infected between 2000 to 8000 glochidia liter⁻¹ (P = 0.001) (Fig. 2.4C). The percentage of the total number of attached mussels shedding as immature glochidia in the first 48 hours post infection was approximately 7 ± 1 % for fish injected with cortisol compared to approximately 30 % for control and sham injected fish (P < 0.03) (Fig. 2.6C).

2.4 DISCUSSION

All species of North American unionid mussels must undergo an obligatory parasitism of specific hosts in order to metamorphose from glochidia into free-living juveniles [64]. After attachment to fish, epithelia cells and connective tissue migrate to encapsulate a glochidium, forming a cyst that protects the larval mussel from the aquatic environment. Cyst formation, which is likely an intrinsic reaction of the gill epithelium to invasion by a foreign object, is remarkably fast in fish [65, 66]. In most cases, glochidia utilize the gill epithelium of fish as their primary site of infection, although for some species, other external structures such as fins, may also serve as important substrates for attachment [23]. Since the gills receive the entire cardiac output of the fish and play such a pivotal role in maintaining overall health, any gill injury or pathogen that enters through the gill can have far-reaching effects on the physiology of the animal.

In the present study, we evaluated the effects of glochidia exposure at 24 hours post infection on various physiological endpoints in bluegill. We found that glochidia had few effects on the physiology of bluegill following acute exposure to concentrations of up to 8000 glochidia liter⁻¹. However, plasma cortisol was found to be a sensitive indicator of effect in fish hosts, becoming significantly elevated at infection concentrations at or above 2000 glochidia liter⁻¹. Cortisol, the
primary corticosteroid in fish [67], can be induced within minutes by a variety of physiological and environmental stressors [1]. Unlike other stress-induced factors such as catecholamine release, which are only transiently affected, cortisol stimulation can persist over time, making it a suitable marker of stress in fish [1, 68]. Although cortisol is capable of affecting carbohydrate metabolism [1, 7, 69], we found no effect of glochidia exposure on either plasma glucose or plasma lactate. However, it remains unclear whether cortisol levels were simply insufficient to stimulate these secondary responses in hosts, or whether these factors recovered over the 24-hour post infection period [70]. Following infection, glochidia typically take hours to become completely encapsulated. Despite this, damage ensuing from glochidia attachment would be expected to reduce plasma Na\(^+\) in hosts due to ion loss to the dilute fresh water. In contrast, we found no effect of glochidia infection on this critical plasma osmolyte, suggesting either that the level of gill damage was relatively minor or that any ionoregulatory impairment during cyst formation was quickly remedied. Certainly, epithelial remodeling in fish is fast compared to that observed in most other animals, presumably due to the inherent risks of failure to maintain ion balance in an aquatic environment [65, 66].

Two of the more notable results of this study were that hosts with elevated plasma cortisol also had a greater number of glochidia attach gram\(^{-1}\) fish and that a greater proportion of these attached glochidia successfully underwent metamorphosis. The timing of glochidia and juvenile shedding indicated that this effect on metamorphosis was due to events taking place in the first 2 days after infection (see below). This was followed by an interim period during which few larvae or juveniles excysted from their hosts. The bulk of the juveniles emerged during a final pulse lasting 2-3 days. In addition, there was also a slight treatment effect on the time required to metamorphose (Table 2.1). Fish infected with 1000 glochidia liter\(^{-1}\) lost 47 % of all attached
mussels within 2 days after infection. Worth reiterating is that fish infected at a concentration of 1000 glochidia liter\(^{-1}\) did not experience an increase in plasma cortisol relative to non-exposure controls (Fig. 2.2). This is in stark contrast to the 14-18 % of attached mussels that were lost from the hosts within 48 hours after infection with larval exposures at or greater than 2000 glochidia liter\(^{-1}\) (Fig. 2.4c). In other words, cortisol appeared to enhance glochidia survivorship past two days post infection. Furthermore, for glochidia passing through this critical stage, the majority went on to successfully metamorphose into juveniles. In fact, there was little change in metamorphosis success rates between treatment groups (89 – 96 % for all treatment groups) when the number of mussels shed in the first 48 hours were removed from the total number of mussels during calculations of metamorphosis success. These data suggest that cortisol-related mechanisms active during the first 48 hours are largely responsible for the successful metamorphosis of glochidia to the juvenile stage. It also suggests that high infection intensity can induce an increase in plasma cortisol that can act to stimulate glochidia metamorphosis. The results of this study are in accordance with others that have demonstrated that a large proportion of attached glochidia fail to metamorphose into juveniles, tending to slough prematurely from fish shortly following infection [25, 47]. It appears that these early shedding events are largely responsible for the inability of glochidia to persist on non-host fish species or on fish which have developed resistance to glochidia after repeated infections [25, 47].

In order to investigate more closely this potential link between plasma cortisol and glochidia attachment and/or metamorphosis, glochidia attachment was monitored after alteration of host physiology. After administering a physiologically-relevant dose of cortisol, fish were exposed to glochidia at a concentration that we had previously found not to elicit a stress response (i.e. 1000 glochidia liter\(^{-1}\)). Using this methodology, we were able to verify that hosts with elevated
cortisol had significantly more glochidia attached gram$^{-1}$ fish, and that these attached glochidia had a greater probability of successfully undergoing metamorphosis (Fig. 2.6A, B). It is not surprising that an increase in cortisol could be associated with an increase in attachment of glochidia. Cortisol is known to increase the susceptibility of fish to bacterial and fungal infections and makes hosts less resistant to ectoparasitic infection [71-73]. It has also been reported to induce the metamorphosis of glochidia on non-host fish [74]. It follows that stimulatory effects of cortisol could be operating through similar mechanisms on host fish.

In general, cortisol administration reduces hyperplasia and inflammation, which are both pathologies associated with activation of the innate immune system. Stimulation of host immunity stemming from a change in leukocyte distribution near cysts has been proposed as a mechanism for rejection of attached glochidia [25]. Eosinophilic granular cells in and around the glochidia cyst are reported as being fewer in number in the gills of more suitable host fish [25]. The presence of this cell type around areas of infection is pervasive in vertebrates, and has been described numerous times in fish infected with ectoparasites. Additionally, inflammatory responses are generally less pronounced in fish that are suitable hosts. This parallels the presence of an inflammatory response in coho salmon (Onchorhynchus kisutch) resistant to the ectoparasite Lepeopthereirus salmonis, or the lack of this response in Atlantic salmon (Salmo salar), which are susceptible to these parasites [75]. However, the immunosuppressant properties of cortisol are only a part of a wide range of actions elicited by this hormone. In addition to a decrease in immunity, the acute effects of this release of circulating hormones are manifold and include an increase in metabolism, which can lead to increased ventilation at the gill [1]. This increase in ventilation could lead to increased accumulation of glochidia in the gill by bringing higher numbers of the larvae into direct contact with the respiratory surface. In fact,
glochidia attachment has been classically used as a method of assessing ventilation rates in fish [24]. It appears that understanding the relationship between fish mass, gill surface area, and gill ventilation is integral to understanding the potential of a gill to act as a suitable surface for glochidia attachment. As we have found when performing initial infections to determine the optimal fish size for these experiments, attachment of *U. imbecillis* to the gills of bluegill sunfish positively correlates with fish mass. However, at this time, we have insufficient data available to describe this relationship in detail.

The physiological status of the host, including variations in hormone levels (i.e., plasma cortisol) [76], acid-base status [77-80] and ion balance may influence the capacity of the fish gill to undergo epithelial remodeling during cyst formation. These factors may influence the fish gill directly, or may elicit classical stress responses, which may indirectly influence fish gill remodeling. Furthermore, handling and confinement associated with initial infections were suspected of inducing considerable stress on experimental animals [1, 7, 71, 81]. Efforts to reduce human interaction were attempted in all instances, where possible. The steps leading up to infection were implemented with minimal direct contact to the fish. However, transfer from the infection chambers to the individual tanks for monitoring metamorphosis was favored over monitoring the fish as a group, but nonetheless, produced only a negligible increase in plasma cortisol.

To date, we have found that most of the research on early life stages of unionid mussels has focused on understanding the ecology of these animals [28]. Due to the threatened status of unionid mussels, conservation strategies for imperiled species have included the establishment of artificial propagation efforts. Identifying natural fish hosts that can be used in artificial propagation programs is important to these activities, and is critical to the management and
protection of unionid mussels. Carefully monitoring metamorphosis on individual fish can characterize the degree of compatibility between fish hosts and mussels species. Using modifications of standard techniques for laboratory infection and subsequent monitoring of metamorphosis of glochidia on individual fish [42, 43], we have described a possible role of fish stress on the efficacy of glochidia attachment and metamorphosis.

It is currently unknown what consequences that a reduction in fish stress during infection will have on the acquisition of immunity from multiple exposures to glochidia, although it is likely that low level infections will act to prime the immunological memory, and that both administered and stress related endogenous cortisol could act to lessen this adaptive response, resulting in an increase in successful metamorphosis. Additionally, the stress associated with high levels of infection could also influence the degree of resistance that accumulates after a fish is infected multiple times. It is likely that stress free infection systems could be applied to multiple infection studies to reduce handling and crowding stress associated with traditional laboratory infection methodology. Obtaining a better understanding of the physiological effects of glochidia attachment and how host physiology influences glochidia attachment and metamorphosis are likely to provide useful information for conservation efforts.
CHAPTER THREE – INDICATIONS OF CRUDE OIL EXPOSURE IN GULF KILLIFISH (FUNDULUS GRANDIS) COLLECTED IN SOUTHERN LOUISIANA MARSH FOLLOWING THE DEEPWATER HORIZON OIL SPILL.

3.1 INTRODUCTION

The Deepwater Horizon oil spill (DWOS) occurred on April 20, 2010 resulting in the release of over 600 million liters of light crude oil into the Gulf of Mexico, approximately 80 kilometers from the Louisiana coast [82]. Shoreline Cleanup and Assessment Technique (SCAT) operations conducted by the National Oceanographic and Atmospheric Administration (NOAA) documented that over 1,040 km of Gulf of Mexico shoreline was oiled, with approximately 690 km of this occurring in southern Louisiana [30]. One of the most affected areas in Louisiana was within the Barataria Basin, which covers approximately 5720 km$^2$ of estuarine marsh and open water [83]. The oil released as a result of the DWOS was a light crude with a composition containing over 50% low molecular weight hydrocarbons that enable more rapid weathering from evaporation, biodegradation, and dissolution compared to heavier crudes [84]. Due to its chemical composition, the addition of dispersants, high temperatures of Gulf of Mexico surface waters, high numbers of oil-degrading bacterial species, the depth of the well, and the distance between the well and the shoreline, significant weathering of the oil occurred prior to landfall, reducing the concentration of low molecular weight polycyclic aromatic hydrocarbons (PAHs) that are toxic to fish and other organisms [84]. However, even at low concentrations, PAHs in crude oil such as Benzo[a]pyrene (BaP), naphthalene, chrysene, fluorathene, and others identified by the World Health Organization as priority pollutants can exert their toxicities on organisms living in areas where crude oil is present [85].
Due to the high density of oiled locations in this area, and the potential for long-term impacts of crude oil, several studies have begun to characterize the effects of DWOS oil on the biota inhabiting the Barataria Basin [34, 83, 86, 87]. PAHs exert their toxicity through the aryl-hydrocarbon receptor (AhR), a ligand-activated transcription factor found ubiquitously in a diverse number of organisms [35, 85]. PAHs bind to the AhR in the cytosol, which ultimately results in the production of xenobiotic metabolizing enzymes and transporters, including phase I, II, and III components of metabolism, and elimination of xenobiotics [36]. Thus, exposure to AhR-active PAHs present in crude oil is often assessed by measuring AhR pathway-related molecules [2, 88-90]. Among these indicators of exposure to PAHs and subsequent activation of the AhR pathway, cytochrome P4501A (CYP1A) enzyme is the most common indicator of exposure because of its sensitivity [39, 88, 90-92].

The liver is the primary site of xenobiotic metabolism and hepatic CYP1A has been historically the most commonly-used endpoint in measuring exposure to PAHs in both laboratory and field studies [92]. CYP1A expression in other tissues can also be utilized to determine exposure to PAHs, and can aid in determining the route of exposure in fish [93]. Direct exposure to PAHs dissolved in the water column can occur through the gills, which are in direct contact with the water, or through the intestine as seawater fish drink to regulate osmotic balance or during ingestion of PAH-laden food [76]. As such, the gill and intestine both represent major routes of PAH exposure, but also perform first pass metabolism of PAHs prior to systemic transport [94, 95].

In the study described herein, 14 sites were chosen within the Barataria Basin based on publically-available SCAT data, ranging from field sites receiving little to no oiling, to those heavily oiled in the aftermath of the Deepwater Horizon oil spill. Gulf killifish were collected
from these field sites to assess the extent of oil exposure in a local species *in situ*. As an indicator of oil exposure, the cellular location and relative abundance of CYP1A protein expression was monitored in the gill, head kidney, and intestine of fish. Targeted gene expression and ELISAs were also performed with liver tissues for the same purpose. This study will draw relationships between the extent of biological effects and relative oiling based on SCAT data. These conclusions will help infer possible routes of crude oil exposure in field-collected Gulf killifish in southern Louisiana marsh, and the extent to which biological measures of exposure are predicted by visual assessment of oil exposure based on SCAT monitoring activities.

3.2 Methods

3.2.1 Collection of adult fish

Gulf killifish were collected from 14 locations, chosen based on SCAT data that assessed the degree of oiling, in order to monitor remediation efforts. Detailed SCAT assessment of this region designated shoreline oiling into categories based on visual observations of SCAT team members. Reference and oiled sites were chosen based on available SCAT oiling survey data at the time of planning for sampling trips (Table 3.1) (www.gomex.ema.noaa.gov). At the time of planning, available data indicated that reference sites chosen for sampling were unoiled. As more data became publically available, it was found that some sites chosen as reference sites had SCAT values that indicated the presence of visible oiling near the site (i.e. R1-R3 and R7). These sites remained designated as reference sites for this report.

Fish were collected using wire minnow traps deployed along the shoreline approximately every 5 meters, all within 3 meters of the shoreline. Twenty-five fish were collected and removed from the traps at each site, and sampled *in situ* following measurement of length and
mass (data not shown). Livers from eight of the fish per site were dissected and placed in RNAlater (Ambion) and kept on ice for transport, then stored at -20 °C for mRNA analysis, and livers from 12 more fish per site were flash frozen in liquid nitrogen and stored at -80 °C until use for quantification of CYP1A protein, as described below. The remaining five fish per site were fixed whole in Z-fix zinc-buffered formalin (Ameresco), after opening the peritoneal cavity to allow penetration of fixative for histological processing of tissues, as described below.

Table 3.1. Sample locations, date of sampling, and maximum oiling observations.

<table>
<thead>
<tr>
<th>Site I.D.</th>
<th>Location Name</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Sampling Date</th>
<th>Average SCAT Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>Eastern Barataria Bay</td>
<td>29.424640°</td>
<td>-89.825160°</td>
<td>11/8/2010</td>
<td>Very light</td>
</tr>
<tr>
<td>R4</td>
<td>Northwest Bay Sansbois</td>
<td>29.474961°</td>
<td>-89.779056°</td>
<td>12/30/2010</td>
<td>No oil observed</td>
</tr>
<tr>
<td>R5</td>
<td>Southwest Wilkinson Bay</td>
<td>29.463360°</td>
<td>-89.932190°</td>
<td>11/8/2010</td>
<td>No oil observed</td>
</tr>
<tr>
<td>R6</td>
<td>West Wilkinson Bay</td>
<td>29.475360°</td>
<td>-89.933780°</td>
<td>11/8/2010</td>
<td>No oil observed</td>
</tr>
<tr>
<td>R7</td>
<td>Southeast Mud Lake</td>
<td>29.460794°</td>
<td>-89.981086°</td>
<td>12/30/2010</td>
<td>Moderate</td>
</tr>
<tr>
<td>O1</td>
<td>South Wilkinson Bay</td>
<td>29.458204°</td>
<td>-89.909429°</td>
<td>11/11/2010</td>
<td>Heavy</td>
</tr>
<tr>
<td>O2</td>
<td>Southeast Bay Jimmy</td>
<td>29.445140°</td>
<td>-89.880103°</td>
<td>10/30/2010</td>
<td>Light</td>
</tr>
<tr>
<td>O3</td>
<td>Northwest Bay Batiste</td>
<td>29.479324°</td>
<td>-89.863246°</td>
<td>10/30/2010</td>
<td>Light</td>
</tr>
<tr>
<td>O4</td>
<td>Bayou Dulac</td>
<td>29.455440°</td>
<td>-89.805176°</td>
<td>10/30/2010</td>
<td>Heavy</td>
</tr>
<tr>
<td>O5</td>
<td>South Wilkinson Bay</td>
<td>29.464950°</td>
<td>-89.911604°</td>
<td>11/10/2010</td>
<td>Heavy</td>
</tr>
<tr>
<td>O6</td>
<td>North Barataria Bay</td>
<td>29.436544°</td>
<td>-89.884232°</td>
<td>11/10/2010</td>
<td>Moderate</td>
</tr>
<tr>
<td>O7</td>
<td>Saint Mary’s Point</td>
<td>29.447160°</td>
<td>-89.937930°</td>
<td>11/11/2010</td>
<td>Heavy</td>
</tr>
</tbody>
</table>

*Average SCAT oiling observations within 100 meters of sites reported from September 17, 2010. ([http://gomex.ermo.noaa.gov](http://gomex.ermo.noaa.gov))

3.2.2 mRNA

Two mRNA transcripts were measured in liver homogenates from fish collected from each location (N = 6-8). CYP1A mRNA was measured as an indicator of AhR induction and aryl-hydrocarbon receptor repressor (AhRR) mRNA measured as an indicator of regulation of AhR signaling. Total RNA was isolated using the TRIzol method according to the directions provided by the manufacturer (TRIzol, Invitrogen), and quality was verified using microcapillary gel
electrophoresis (Experion, Bio-rad Laboratories). cDNA synthesis was completed using the SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) to generate cDNA for each sample. Specific gene expression levels were analyzed by quantitative real-time PCR with a Biorad iC5 detection system using selected primers sets (Table 3.2) and RT² SYBR green/fluorescein master mix. The quantity of these mRNAs was expressed as fold-changes in gene expression compared to 18S expression. For data analysis, gene expression fold change was measured using the delta delta C\(_t\) method (i.e. the Pfaffl method) [96].

**Table 3.2.** Primer sequences and RT-PCR operation data.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession #</th>
<th>Primer Sequence (5’ → 3’)</th>
<th>Tm °C</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhRR</td>
<td>AF443441.1</td>
<td>F: TTG TCT CGA AGC TGT ATG GCT CGT R: ATC TTA ATG GGC GGC ATT TCA GGC</td>
<td>57</td>
<td>124</td>
</tr>
<tr>
<td>CYP1A</td>
<td>AF 026800.1</td>
<td>F: AAG CAA GAG GGA GAG AAG GTC CTT R: TGT GCT TCA TCG TGA GGC CAT ACT</td>
<td>57</td>
<td>150</td>
</tr>
<tr>
<td>18S</td>
<td>M91180.1</td>
<td>F: TTC GTA TTG TGC CGC TAG AGG TGA R: TTC GAA CCT CCG ACT TTC GCT CTT</td>
<td>57</td>
<td>125</td>
</tr>
</tbody>
</table>

### 3.2.3 ELISA

CYP1A protein content in the livers was quantified using enzyme-linked immunosorbant assays (ELISA) using monoclonal antibody (mAb) C10-7 using methods modified from Rice and Rozall, 1998 [97]. Twelve livers from each sampling site were thawed on ice, weighed, and individually-homogenized in ice-cold homogenization buffer (0.25 M sucrose, 0.05 M Tris-base, 1mM EDTA, 1 mM DTT, 0.1 mM PMSF). Homogenates were spun at 10,000 g at 4°C for 20 minutes. The upper layer of fat from each tube was aspirated, and supernatants were transferred to new tubes and spun at 100,000 g for 70 minutes. The resulting supernatants from the second spin (cytosolic fraction) were removed and stored for future use, and the microsomal pellets were resuspended in a 5% glycerol resuspension buffer (0.25 M sucrose, 0.01 M HEPES, 0.1 mM EDTA, 0.1 mM DTT, 0.2 mM PMSF), aliquoted, and stored at -80°C until use. Ten additional
fish were collected from an unoiled location at the Louisiana Universities Marine Consortium (LUMCON) facility in Cocodrie, LA, as above, and were subsequently injected with benzo(a)pyrene (BaP), a potent inducer of the AhR pathway, for use as positive controls. Positive control fish were acclimated for at least one month, then given intraperitoneal injections of BaP (50 mg/kg), and sacrificed at 48 hours after injection. Livers were collected and microsomal fractions were prepared (as above). Liver microsomal fractions from positive control fish were homogenized to serve as both a positive control and to control for plate-to-plate variation. Microsomal protein content was determined using the bicinchoninic acid protein assay (Thermo Scientific, Pierce). Microsome samples from each liver were diluted with phosphate buffered saline (PBS) to a final protein concentration of 50 µg, and dispensed in triplicate to poly-l-lysine coated 96-well plates. Plates were incubated overnight at 4°C, then washed three times in PBS with 0.05% Tween-20, then blocked with PBS with 3% fetal bovine serum to prevent non-specific binding. Plates were washed again prior to incubating with monoclonal antibody C10-7 for one hour at room temperature. After incubation, plates were washed and incubated for 60 minutes with goat-anti mouse immunoglobulin (IgG) linked to alkaline phosphatase (Sigma-Aldrich). Plates were then washed and visualized at 405 nm after addition of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) (Sigma-Aldrich) as a substrate for the alkaline phosphatase reaction. Plates were measured every ten minutes for one hour then every thirty minutes for the next hour. Slopes of the reaction rates for each sample were calculated during the linear binding phase. Each plate had microsomes from BaP-induced positive control fish dispensed in quadruplicate to account for plate-to-plate variation in optical density, and to serve as a positive control for comparison. Tissue protein content was expressed relative to the positive control values which were normalized to a value of one.
3.2.4 IHC of gill, head kidney, and intestine

Whole fish were dissected to remove the gills, head kidneys, and intestines preserved in Z-fix. Tissues were dehydrated in standard fashion, through a graded series of ethanol before clearing in Histochoice Clearing Agent (Ameresco), prior to embedding in Paraplast X-tra (Fisher Scientific) embedding media. Embedded tissues were cut to 4-5 µm sections on a Microm HM 330 microtome (Heidelburg), and adhered to poly-l-lysine coated glass microscope slides and stored at room temperature until staining. Slides were then rehydrated and processed according to Whitehead et al., 2012 [34] for immunohistochemical localization of CYP1A using mAb C10-7. Briefly, the Vectastain ABC immunoperoxidase system combined with NovaRed (Vector Laboratories) was used to probe mAb C10-7 to indicate CYP1A protein as a deep red color, prior to counterstaining nuclei in blue with Hematoxylin QS (Vector Laboratories). All sections were processed identically, including incubation times with antibodies and hematoxylin staining.

Antibody-probed slides were subjectively scored based on the presence, absence, and intensity of immunoreactivity for CYP1A protein (red color) in cells that characteristically express CYP1A upon exposure to AhR-activating ligands, in the gill, head kidney, and intestine [93]. Multiple images of each tissue were taken at random and were all viewed to create a combined subjective score for each tissue. Gills were assessed for the presence or absence of CYP1A staining to a) pillar cell and b) other structures, including epithelial cells, mucus cells, and vascular endothelial cells, head kidneys were monitored for the absence or presence of CYP1A staining to a) tubules, and b) vascular endothelia, and intestine were scored similarly for CYP1A staining to a) mucosal layer cells, and b) sub-mucosal cells. For each of these six structures, CYP1A staining was given a value ranging from 0-3, based on negative (0), mild (1), moderate
(2), and strong (3) CYP1A staining intensity (Table 3.3) [93]. Category scores were summed for each tissue for a combined total score of six.

**Table 3.3.** Description of criteria used for determining degree of immunoreactivity for CYP1A protein in gill, head kidney, and intestine, based on categories of major sites of CYP1A protein expression and relative staining.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Category Number</th>
<th>Description</th>
<th>Criteria</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gill</td>
<td>1</td>
<td>Gill Pillar Cells</td>
<td>No staining observed</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Some pillar cells stained</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Most pillar cells stained with some saturated</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pillar cells saturated with stain</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Other Gill cells (epithelial cells, mucus cells, vascular endothelial cells)</td>
<td>No staining observed</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Light pink</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Observation of moderate staining of some non-pillar cells</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pervasive staining of epithelial cells, mucus cells</td>
<td>3</td>
</tr>
<tr>
<td>Head Kidney</td>
<td>1</td>
<td>Tubules</td>
<td>No staining - light pink</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Light pink - red</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Red with some tubules saturated dark red</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Majority of tubules saturated red</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Vascular Endothelial Cells</td>
<td>No staining</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Light pink</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Red</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Majority of VECs saturated dark red</td>
<td>3</td>
</tr>
<tr>
<td>Intestine</td>
<td>1</td>
<td>Mucosal Layer</td>
<td>No staining - light pink</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Some staining of mucosal layer</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Consistent, light staining in mucosal layer</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Majority of mucosal layer saturated red</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Vascular Endothelial Cells</td>
<td>No vascular endothelial cells stained</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slight staining of some vascular endothelial cells</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Moderate staining of vascular endothelial cells</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Heavy staining in vascular endothelial cells</td>
<td>3</td>
</tr>
</tbody>
</table>
3.2.5 SCAT value comparisons

For comparison between visible oiling and values produced from subjective scoring of IHC, numerical values were assigned to SCAT visible oiling color designations for heavy oiling (red), moderate oiling (orange), light oiling (yellow), very light oiling (green), tar balls (grey), and no visible oiling (blue). Numbers (6-1 respectively) were assigned to the colors as defined in Table 3.4. Values representative of the visible oiling SCAT color designations from within 100 meters of each sampling location were averaged based on the presumed limited home range of *F. grandis*, based on experimental data of its sister species, *Fundulus heteroclitus* (Table 3.4) [98]. For CYP1A mRNA and protein data, and for AhRR mRNA data, fish from oiled sites had significantly higher values compared to those collected from reference sites (see below), so average SCAT values were scaled to the average score for oiled fish in each category to fit the y-axis in each graph, and divided into six equal integers to represent the six categories of oiling.

3.2.6 Imaging

Slides were observed and imaged on a Nikon Eclipse 80i microscope using Nikon DSFi1 camera and NIS-Elements BR 3.10 software. Images were balanced globally in Photoshop CS3® (Adobe) using the curves tool to white balance or the levels function. No additional adjustments were made locally to any images or portions thereof.

3.2.7 Statistics

Means from mRNA levels and relative CYP1A protein expression were evaluated with XLSTAT ver. 2012.6.08 and were compared using the Kruskal-Wallis test, followed by Dunn’s pairwise comparison using a 95% confidence level. Linear regression was used to determine correlations between SCAT data and means from mRNA, relative CYP1A protein, and tissue scores for IHC at a 95% confidence level.
Table 3.4. Relative oiling at each field site based on the average SCAT classification within 100 meters from the site of fish collections. Each classification is indicated with + symbol.

<table>
<thead>
<tr>
<th>Site ID</th>
<th>SCAT average</th>
<th>SCAT average designation</th>
</tr>
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<tbody>
<tr>
<td>R1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O3</td>
<td>+</td>
<td>+</td>
</tr>
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<td>O4</td>
<td>+</td>
<td>+</td>
</tr>
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<td>O5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O7</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

### 3.3 RESULTS AND DISCUSSION

#### 3.3.1 CYP1A protein levels and mRNA levels of CYP1A, AhRR, and COX-2

SCAT data is potentially useful in formulating field sampling plans since visible oiling observations are clear indicators that toxicants such as PAHs may be present. Average AhR-mediated CYP1A protein and CYP1A and AhRR mRNA transcript abundance was compared to relative SCAT visible oiling data collected within 100 meters of the center of each site (Figure 40).
3.1 A-C). Average liver CYP1A protein from heavily oiled sites (O1-O7) had significantly elevated liver CYP1A protein and mRNA levels compared to the average CYP1A protein found in fish collected from reference locations (R1-R7) (P ≤ 0.007) (Figure 3.1 B and D). This was not surprising since field observations at the time of sampling were mostly consistent with SCAT data that indicated heavy oiling at all sites designated herein as heavily oiled sites, and although somewhat variable, little to no oiling was reported at the reference sites. Average AhRR mRNA was also significantly increased in fish from oiled locations (P ≤ 0.05) (Figure 3.1 E-F). It should be noted that variation in the expression of AhR-mediated CYP1A protein and mRNA and AhRR mRNA in these field-caught fish was found, and is suggestive of varying distribution and abundance of unseen oil beneath the surface that may be present at each location. Further analysis of this data is warranted to describe this relationship in detail. Overall, the correlation between SCAT visible oiling data and the averages of the above data from oiled versus reference sites suggests that there is utility in using SCAT data as a predictor of potential exposure to toxicants in crude oil.

3.3.2 IHC localization of CYP1A in gill, head kidney, and intestine.

Average subjective scoring of gill tissues for all fish gills collected from oiled sites were higher than the average gill score from reference site fish (P=0.016), although no clear relationship was evident between immunolocalization of CYP1A in the gills and SCAT data for these sampling locations (R^2=0.050, P=0.441) (Figures 3.2-3.3). Increase in CYP1A protein was seen mostly in the pillar cells of gills from sites where SCAT data reported visible oiling. Increase in pillar cell CYP1A protein is an indication of AhR-mediated activity and suggestive of an increase in exposure at these sites [93, 95]. Few fish showed signs of CYP1A in the epithelial cells of the gills, or elsewhere along the gill filament. Gill tissues from O7 (St. Mary’s Point)
had the highest scores, which likely indicate water-borne exposure to PAHs at this heavily-oiled site [95, 99]. No other site had notable CYP1A protein expression in the gills other than at site R2, a lightly oiled site near Bayou Dulac, which serves as a main thorough-fare for boat traffic coming from Port Sulphur into Bay Batiste and Barataria Bay.

Figure 3.1 A-F. AhR-mediated protein and mRNA and SCAT visible oiling data. (A-C) Relative concentration of CYP1A protein, CYP1A mRNA, and AhRR mRNA (black bars) (N=7-8), respectively, at 14 locations in the Barataria Basin, and the relative visible oiling based on SCAT data available for each location (striped bars). (C-D) Average CYP1A protein, CYP1A mRNA, and AhRR mRNA values for fish exposed in situ to varying degrees of oiling in the field (N=50-52). P<0.05. Error bars indicate standard error of the mean. SCAT visible oiling values were averaged when multiple observations existed within 100 meters of a sampling site, and scaled to the average of all oiled site value for A-C to fit to y-axis. See text for further details.

Head kidney tissues from designated oiled sites had a marked increase in CYP1A protein in the tubular epithelium and an increase in the staining of the vascular endothelial cells (Figure

42
Average scores for oiled sites were elevated compared to reference sites ($P=0.021$) indicating that these fish were likely exposed to systemic AhR-active PAHs that escaped the first pass metabolism of the gills and intestine. Some reference site fish showed mild to moderate staining of CYP1A, albeit to a much lesser extent than the fish from oiled sites (Figure 3.3). Basal levels of CYP1A protein in the tubules of the head kidney were common in fish from most locations, although this was highly variable between fish (data not shown). CYP1A protein in the vascular endothelial cells is a hallmark of exposure to PAHs and AhR-inducing ligands, and upregulation of CYP1a expression is ubiquitous in this tissue, making it an important indication of exposure [93]. The presence of CYP1A-positive cells in the head kidney showed slightly more correlation to the SCAT survey data than the gill tissues ($R^2=0.093$, $P=0.290$), and further data analysis will be required to define this relationship.

Intestinal tissue was the most sensitive indicator of exposure to PAHs. CYP1A protein levels in the mucosal cells of the intestine correlated to the degree of oiling determined by SCAT surveys during the time of sampling of these fish ($R^2=0.482$, $P=0.006$), and average scores for oiled sites were significantly elevated compared to intestines from reference sites ($P<0.001$) (Figure 3.4 and 3.2C). Intestinal tissue sampled from oiled locations had far more pervasive and darker staining in the mucosal layer compared to fish sampled from reference sites (Figure 3.2C). From this information and the relatively low levels of CYP1A protein in the gills compared to the intestine and head kidney, there is evidence that the uptake of PAHs is occurring from contaminated sediment and likely from benthic food sources. First pass metabolism of xenobiotics can occur as they cross the intestinal epithelium reducing the systemic load of PAHs that the liver and head kidneys receive [88, 94]. The reduction in circulating PAHs would indeed affect the detection of AhR-related biomarkers that would be detectable in the liver, head kidney,
and gill. This could also account for the increased variability of liver mRNA levels of CYP1A and AhRR that was observed within and between sites depending on the route of exposure and bioavailability of the toxicants. Anecdotal evidence was observed of increased CYP1A protein in the anterior regions of the intestine where food is absorbed compared to the posterior portions where water absorption in marine and brackish killifish is observed. These data together support the original hypothesis that fish at oiled sites were exposed to AhR-inducing ligands through the intestine at the time of sampling when these sites retained heavy oiling in the marsh grass and in sediments.

Figure 3.2 A-F. CYP1A immunolocalization staining intensity scores for gill, head kidney, and intestinal tissues and SCAT visible oiling data. (A-C) Average subjective staining intensity scores for gill, head kidney, and intestine, respectively, at 14 locations in the Barataria Basin (N=4-5), and the relative amount of visible oiling recorded near each location during SCAT surveys. (D-F) Average scores of all reference versus all oiled site fish in gill, intestine, and head kidney tissues (N=3-5). P<0.05. Error bars indicate standard error. SCAT scores for each graph (A-C) were scaled to the average score for oiled fish in each tissue, in order to match the y-axis.
Figure 3.3 A-C. Distribution of CYP1A protein (red staining) in gills, head kidney, and intestine from adult killifish collected from seven reference and seven oiled sites across Barataria Bay, Louisiana. (A) Increase in CYP1A in the gill lamellae (chevrons) and in the epithelia of the interlamellar regions of the gill filament (star) as well as an increase in hyperplasia in the gill lamellae and in the interlamellar regions of the gill filaments was found in fish from oiled sites (O1-O7) compared to fish from unoiled or lightly oiled sites (R1-R7). (B) Increased staining in epithelial cells of the kidney tubules (arrow heads) and an increase in CYP1A-positive vascular endothelial cells (arrows) were found in fish from oiled sites compared to unoiled or lightly oiled sites. (D) Intestinal tissues show an increase in CYP1A in the mucosa and in CYP1A-positive vascular endothelial cells (arrows) in the lamina propria and submucosa. All tissues sectioned at 4 µm and imaged with a 20X objective. Arrows = vascular endothelial cells, chevrons = gill filaments, asterisks = bucopharyngeal cavity, arrow heads = kidney tubules. Scale bar = 50 µm. All slides were counterstained with hematoxylin (blue).
Figure 3.3 A-C.

Analytical chemistry results published in Whitehead et al., 2012 [34] that indicate that the crude oil is highly elevated in the sediments compared to the water, as of August 2010. Although SCAT visible oiling observations are semi-quantitative and lack analytical chemistry, correlations between visible oiling and biological effects exist. Specifically, SCAT data more closely correlated with the degree of staining seen in the intestinal tissue compared to the gills or head kidney, and further investigation of the effects of weathered oil in Barataria Bay sediments on the health of aquatic species is merited to determine the route of exposure, and to determine the extent of damage and rate of recovery in this region. Additional utilization of physiological
indices of health such as immunological modulation, physical performance, and reproductive capacity to predict the effects of crude oil on resident species is warranted. The associations between these indices of health and the presence and abundance of crude oil provide benchmark data for comparison for remediation efforts, and for future responses to anthropogenic alteration of habitat.

Figure 3.4. Correlation between CYP1A immunolocalization staining intensity scores for intestinal tissues at 14 locations in the Barataria Basin (N=4-5), and the relative amount of visible oiling recorded near each location during SCAT surveys (SCAT Score). SCAT visible oiling values were averaged when multiple observations existed within 100 meters of a sampling site, and scaled to the average of all oiled site value for A-C to fit to y-axis (See text for further details). R² represents the correlation coefficient for the line of best-fit calculated using non-linear regression.
CHAPTER FOUR - MULTI-TISSUE MOLECULAR, GENOMIC, AND DEVELOPMENTAL EFFECTS OF THE DEEPWATER HORIZON OIL SPILL ON RESIDENT GULF KILLIFISH (*FUNDULUS GRANDIS*).

4.1 INTRODUCTION

As a result of the explosion of the Deepwater Horizon oil platform, as much as 700 million liters of crude oil were released from the Macondo well [29]. Despite cleanup efforts, this oil was widely distributed along shorelines of Louisiana, and to a lesser extent, Mississippi, Alabama, and Florida. During the initial response and cleanup effort, visible oil was reported on the water surface, along beaches, and in marshes as early as May 2010, which coincided with the spawning season for many marine and estuarine fish species. By August 2010, much of the surface oil slick had dissipated or was removed, and visible oil on the beaches and marsh became far less noticeable. However to this date, a considerable amount of weathered oil likely remains deposited in the sediment, serving as a reservoir for persistent toxic exposure to resident species [17, 34, 88]. Since crude oil contains chemicals that are toxic to fish such as polycyclic aromatic hydrocarbons (PAHs), it is probable that residual oil from the Deepwater Horizon oil spill (DWOS) will affect populations of fish living and breeding in contaminated locations.

Fish deaths from toxic acute exposure to PAHs certainly occur, and are often used as a measure of the effects of oil on fish populations [16]. However, sublethal effects of PAH exposure such as impairment of corticosteroid secretion, immune dysfunction, gill damage, impaired growth and reproduction, and reduced cardio-respiratory capacity are capable of reducing an animal’s ability to effectively respond to environmental stressors [17, 33, 89, 100-106]. Furthermore, well-characterized and widespread developmental abnormalities in larval fish such as craniofacial defects, edema, reduced size, and cardiovascular defects result in a
decrease in fitness that can persist into adulthood, affecting the productivity and survivorship of a population [16, 17, 32, 107, 108]. Multiple studies following the Exxon Valdez oil spill (EVOS) linking crude oil to biological effects in fish were reported, providing benchmarks for the evaluation of remediation efforts and recovery status of affected areas [16]. Examination of adult fish for biological effects and molecular markers of exposure to oil, along with developmental endpoints in embryos and larval fish both in oiled field sites and in laboratory-based exposures, were predictive of population-level impacts in fish populations [16, 17]. Few reports have emerged to date using a resident species either in field or laboratory-based bioassays of exposure and effect in areas known to be affected by the DWOS.

This study follows the first report to describe the biological effects in populations of resident killifish exposed \textit{in situ} to crude oil from the DWOS [34]. Here we present evidence of exposure to xenobiotics in multiple tissues of adult fish; effects that persist for over one year following the landfall of DWOS oil. We also show developmental defects in embryos exposed to sediments collected from oil-impacted sites in coastal Louisiana in 2010 and 2011. Collectively, the long-term exposure of these fish to persistent PAHs bound in the sediment and the reduction in fitness of developing embryos due to early-life exposure to these sediments could be predictive of persistent population impacts as seen after the EVOS [17].

4.2 MATERIALS AND METHODS

4.2.1 Sampling of adult fish.
Adult Gulf killifish, an abundant, non-migratory baitfish, were collected using wire minnow traps during four trips between May 2010 and August 2011, a timeframe that would have coincided with the peak spawning period of this species in the field (Table 4.1) [34]. The first three trips in 2010, as described in Whitehead \textit{et al.}, 2012 [34], consisted of five sites identified
as unoiled reference sites and a sixth site (Grand Terre Island, LA) directly impacted by contaminating oil. Here we utilize remaining tissues from that study collected at two of the five reference sites, Bay St. Louis, MS (BSL) and Bayou La Batre, AL (BLB), and the oiled location, Grande Terre Island, LA (GT) on trip one (T1) prior to oiling, on trip two (T2) at the peak of oiling, and on trip three (T3) after peak oiling. Tissues from a fourth trip (T4) were collected from GT in August 2011, one year after T3.

**Table 4.1.** Field sites, locations, and sampling dates of sampling trips for collection of adult fish (modified from Whitehead *et al.*, 2012 [34] – See Appendix 1) to include additional 2011 sampling trip.

<table>
<thead>
<tr>
<th>Site name</th>
<th>Location (latitude, longitude)</th>
<th>Sampling date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grand Terre Island, LA (GT)</td>
<td>29.272902°, -89.944850°</td>
<td>Trip 1: 5/9/2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trip 2: 6/28/2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trip 3: 8/30/2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trip 4: 8/28/2011</td>
</tr>
<tr>
<td>Bay St. Louis, MS (BSL)</td>
<td>30.256338°, -89.415544°</td>
<td>5/1/2010</td>
</tr>
<tr>
<td>Bayou La Batre, AL (BLB)</td>
<td>30.378452°, -88.246505°</td>
<td>5/1/2010</td>
</tr>
<tr>
<td>Belle Fontaine Point, MS (BFP)*</td>
<td>30.349731°, -88.748094°</td>
<td>5/2/2010</td>
</tr>
<tr>
<td>Mobile Bay, AL (MB)*</td>
<td>30.678825°, -87.994461°</td>
<td>5/5/2010</td>
</tr>
<tr>
<td>Fort Morgan, AL (FMA)*</td>
<td>30.233681°, -87.961344°</td>
<td>5/5/2010</td>
</tr>
</tbody>
</table>

* Not included in present study

**4.2.2 Transcriptomics.**

Gill tissues were excised immediately from five field-captured male fish of the same approximate length (8mm), preserved in RNA-later (Ambion, Inc.), and stored at -20°C until nucleic acid extraction. Microarray data collection was as reported in Whitehead *et al.*, 2012 [34]. Briefly, total RNA was extracted using Trizol reagent (Invitrogen; Life Technologies Corp.), antisense RNA (aRNA) was prepared (Ambion Amino Allyl Messageamp II aRNA amplification kit), then aRNA coupled with Alexa Fluor dyes (Alexa Fluor 555 and 647; Molecular Probes) and hybridized to custom oligonucleotide microarray slides (Agilent eArray design ID 027999). The microarray was designed from *F. heteroclitus* expressed sequences and includes probes for 6800 target sequences. This same microarray platform was previously used
in studies of PCB exposures in *F. heteroclitus* [109], in a field study of the liver response to the DWOS in *F. grandis* [34], and a laboratory study of gill and liver responses to weathered Louisiana crude oil in *F. grandis* (A.W., unpublished data).

Raw data were sequentially normalized by lowess, mixed model analysis, and quantile methods in JMP Genomics (SAS, Inc.), then log$_2$ transformed. Statistical analysis was performed using mixed models in JMP Genomics, where main effects were specified as "time" (including our 4 sampling time-points) and "site" (including our 3 field sites), including an interaction term. Five biological replicates were included within each treatment. A transcriptional response that was different between sites throughout the oiling event was identified by statistically significant (p<0.01) time-by-site interaction. Gene ontology enrichment analysis was performed using David Bioinformatics Resources [110], and gene interaction network analysis was performed using Ingenuity Pathway Analysis software (Ingenuity Systems, Inc.).

### 4.2.3 Immunohistochemistry.

All tissues were processed as described in Whitehead *et al.*, 2012 [34] for immunohistochemical visualization of protein abundance and distribution of cytochrome P4501A (CYP1A), a marker of exposure to AhR-active PAHs, using monoclonal antibody (mAB) C10-7 [89, 91, 93]. Tissues adhered to poly-l-lysine coated slides were probed with mAb C10-7 using Vectastain ABC immunoperoxidase system (Vector Laboratories). CYP1A was visualized with NovaRed (Vector Laboratories), and counter-stained with Hematoxylin QS (Vector Laboratories).
4.2.4 Sediment collection and embryo exposures.

Sediment was collected from GT on June 16, 2010, coincident with peak oiling, and approximately one year later on August 28, 2011, when much of the visible oil had dissipated (Table 4.2). A second location was sampled on August 16, 2011 on a small island in South Wilkinson Bay, LA (WB) where visible oiling was reported from July 2010 to December 2012 by the National Oceanographic and Atmospheric Administration (NOAA) during the Shoreline Cleanup and Assessment Technique (SCAT) surveys (www.gomex.erma.noaa.gov). A third location was chosen in the north of Bay Sansbois (NBS) on August 3, 2011 where no oil was reported by SCAT surveys and this sample served as our non-oiled control sediment (see below).

Table 4.2. Field sites, locations and sampling dates of sampling trips for collection of sediments for exposures of Gulf killifish embryos to field-collected sediments.

<table>
<thead>
<tr>
<th>Site name</th>
<th>Location (latitude, longitude)</th>
<th>Sampling date</th>
</tr>
</thead>
<tbody>
<tr>
<td>South Wilkinson Bay (WB)</td>
<td>29.458204°, -89.909429°</td>
<td>not sampled, 8/16/2011</td>
</tr>
<tr>
<td>Bay Sansbois (NBS)</td>
<td>29.475767°, -89.779367°</td>
<td>not sampled, 8/3/2011</td>
</tr>
</tbody>
</table>

Sediments were collected using a stainless steel sediment sampler (WILDCO®). Approximately 10cm sediment cores were removed along the shoreline every 0.5m, and combined and mixed in an aluminum container, then aliquoted into 950ml amber glass bottles with polytetrafluoroethylene (PTFE) lined lids. Samples were stored on ice for transport to Louisiana State University. Sediments collected in 2010 were stored at -20°C until collection of 2011 sediments, when they were stored with the later at 4°C until use.
4.2.5 Sediment analytical chemistry.
Analytical chemistry of sediments used in embryo exposures was conducted as reported in Whitehead et al., 2012 [34]. Briefly, sediments were solvent-extracted, and extracts analyzed by gas chromatography interfaced with a mass spectrometer. Spectral data were analyzed by Chemstation Software (Agilent Technologies, Inc.).

4.2.6 Embryo collection.
Male and female Gulf killifish were collected from Cocodrie, Louisiana (29.254175°, -90.663559°) and held in a 1500l recirculating tank containing 10ppt artificially-formulated seawater (AFS) (Instant Ocean). Embryos were collected on Spawntex spawning mats (Aquatic Ecosystems), which were placed in the tanks at sundown, and removed one hour after sunrise. Eggs were removed and held in a 950 ml Pyrex® dish until use.

4.2.7 Sediment exposures.
Laboratory mesocosms were prepared by first saturating sediments with 10ppt AFS prior to adding 150 ± 5ml of this mixture to graduated 950 ml Pyrex® dishes. This mixture was then overlaid with 75ml AFS. Suspended sediment was allowed to settle for one hour prior to insertion of sampling baskets containing embryos (see below).

Sampling baskets were constructed using virgin polytetrafluoroethylene (PTFE) pipe stock (4B Plastics, Baton Rouge, LA) and mesh with 250µm openings (Macmaster-Carr). Two interlocking rings were machined (4B Plastics, Baton Rouge, LA) and fitted together to clamp PTFE mesh tightly across the rings to create a filter basket that rested on the sediment for the duration of the exposure, and elevated the embryos approximately 5 mm above the sediment-water interface.

At the start of experiments, 20-54 embryos were randomly distributed to each PTFE basket, which was positioned on top of sediment within a Pyrex® dish containing sediment. Four
Mesocosms were created for GT sediments from 2010 ($N=146$), five from GT sediments from 2011 ($N=132$), three from WB sediments collected in 2011 ($N=60$), and five from NBS sediments collected in 2011 ($N=132$). Mesocosms were placed on an orbital shaker at 29RPM to simulate tidal and wind movement of water, without causing an observable increase in turbidity. Animals were kept at room temperature (20-22°C) on a natural light cycle. Hatching of Gulf killifish embryos typically occurs between days 10-14 post fertilization at this temperature, so embryos were observed daily for mortality and hatching for 21 days prior to termination of an experiment. Every other day, 25ml of water was removed from the mesocosms and replaced with fresh 10ppt AFS. On day eight, the filter baskets were briefly removed from the mesocosms and placed under a stereomicroscope, where the heartbeats of eight embryos per treatment were measured. Each day, newly-hatched larvae were preserved in either Z-Fix buffered zinc formalin fixative (Ameresco) for histological processing as above, or in RNAlater (Ambion, Inc.) for future genomics work. Additional exposures using NBS 2011, WB 2011, and GT 2010 sediments were conducted under identical conditions to measure larval length at hatch. Our supply of GT sediment from 2011 was depleted at the time of these exposures, thus GT 2011 sediment was not included in this test. Larval length at hatch was measured using Zeiss AxioVision 4.8 software on a Zeiss Lumar® stereomicroscope. Embryonic heartbeat mortality, hatching success, and larval length were evaluated using XLSTAT ver. 2012.6.08, which were compared using the Kruskal-Wallis test, followed by Dunn’s pairwise comparison. Cumulative daily hatching success was analyzed using generalized linear mixed models (GLMM) with the GLIMMIX procedure (SAS, Inc.) to compare daily hatching between treatment and day.
4.2.8 Imaging.
Microscope slides were observed and imaged on a Nikon Eclipse 80i compound microscope using a Nikon DS-Fi1 camera and NIS-Elements BR 3.10 software. Images were balanced globally in Photoshop CS3 (Adobe) for levels using the curves tool for white balance or the levels function.

4.3 RESULTS AND DISCUSSION

4.3.1 Genome expression analysis.
In gill, 374 genes were divergently expressed between field sites throughout the time-course of the oiling event (significant time-by-site interaction; p<0.01). Of these, the vast majority (94%) were different in their expression between the oil-exposed site (GT) relative to the two reference sites (BSL and BLB). That is, the response at the oiled GT site was the outlier for 94% of the genes that showed site-dependent expression; whereas, for only 6% of these genes, the outlier was one of the reference sites. The departure in expression at GT from other field sites occurred primarily at the second sampling time-point (Figure 4.1A and 4.1C). This indicates that divergence in genome expression is tightly associated with the timing and location of major oiling events in the field. This is consistent with the divergence in genome expression in the liver, which was also tightly coupled with the timing and location of oil contamination (Figure 4.1 and Whitehead et al., 2012 [34]).

Gene ontology categories that were significantly enriched within the set of oiling-associated genes in gill include "response to wounding", "inflammatory response", "acute phase response", and "cytochrome P450". PCBs are mechanistically-related to the toxic components of oil (PAHs), insofar as toxicity is largely mediated through the aryl hydrocarbon receptor (AhR) signaling pathway [111]. Genes that are PCB dose-responsive in killifish [109] are significantly enriched within this set of oil-associated genes in the gill (p<0.01, Fisher's exact test). Up-
regulation of a canonical set of genes is diagnostic of activation of the AhR pathway. Gene
targets of the activated AhR pathway are among the genes that have oil-associated expression,
including CYP1A1, CYP1B1, GCHFR, CYB5, and NUPR1. Among the top five biological
functions implicated by network and pathway analyses for gill and gill-only genes (Figure 4.1D,
pink + green genes) were “dermatological diseases”, “immunological disease”, and “cancer”.
Acute phase response signaling was also implicated (p<0.0001) among the top canonical
pathways for gill tissue-specific genes (Figure 4.1D, pink genes). Inflammatory signaling is
becoming increasingly recognized as an important mechanism mediating the toxic effects of
AhR agonists [39]. The molecular mechanism of AhR ligand-activated inflammation is cell-type
specific [39], where the inflammatory response is facilitated by cytokines (e.g., TNF) and
chemokines, both of which are implicated in the gill-specific gene cluster (Fig. 4.1D). Such AhR
activation can mediate immune modulation [35], which may increase health risks for animals
encountering persistent pathogen challenge in the wild. These patterns of expression provide
clear evidence that killifish were exposed to the toxic components of oil, and that gill is a
sensitive target of such exposure.

Patterns of liver gene expression (from Whitehead et al., 2012 [34]) were compared with
patterns in gill to uncover expression responses that were unique between tissues, and common
between tissues. More genes were diagnostic of the oiling event in the liver than in the gill (434
versus 248 respectively (Figure 4.1A and 4.1B)). However, the degree of up- or down-regulation
of genes in response to the oiling event was more dramatic in gill than in liver tissue (Figure
4.1C). Most of the genes that showed statistically significant divergence in expression only in
the liver showed the same trend in gill (Figure 4.1A, bottom panel), though the reciprocal pattern
(correlation between liver and gill patterns for genes with significant response only in the gill)
was not apparent (Figure 4.1A, top panel). This more dramatic transcriptional response in the
gill may be reflective of this organ's direct contact with the contaminated external environment.
In contrast, the liver is not in direct contact with the environment, and its attenuated response
relative to the gill may be reflective of mechanisms of chemical uptake at epithelia and the
complex internal dynamics of metabolism.

**Figure 4.1 A-D.** Patterns of expression for the genes that showed site-dependent expression
throughout the oiling event (genes with significant time-by-site interaction). (A) Clusters of
genes with gill-specific (top cluster), liver-specific (bottom cluster), and common (middle
cluster) expression response between tissues, where columns are mean expression for a
treatment, rows are genes, and color of cells indicate fold up-regulation (yellow) or down-
regulation (blue) relative to pre-oil controls per site. Columns are organized by consecutive
time-points within sites, where sites are Grand Terre (GT), Bay St. Louis (BSL), and Bayou La
Batre (BLB). Three consecutive time-points are pre-oil, peak oil, and post-oil (left to right) in
2010. Gills were sampled at a fourth time-point at the GT site one year later (August
2011). (B) Venn diagram indicating number of oil-associated genes expressed per tissue. (C)
Plots of principal component 1 (PC1) from principal components analysis of the trajectory of
transcriptome change through time and between sites for liver (open symbols) and gill (closed
symbols). Red, green, and blue represent the trajectories of time-course response (base through
head of arrows represent first through last sampling times) at sites GT, BSL, and BLB,
respectively. Top, middle, and bottom panels represent genes that were gill-specific, common to
both tissues, and liver-specific, respectively, mirroring the clusters represented in the
heatmap. In brackets is the proportion of variation accounted for by PC1. (D) Interaction
networks for genes that were gill-specific (pink), liver-specific (blue), or common between
tissues (green), in their transcriptional response. Bold symbols represent genes included in the
analysis, whereas other genes form connections with one degree of separation. The common
(green) set of genes are separated into three clusters, where the middle cluster is equally
connected to the gill and liver networks, but the left and right clusters primarily associate with
the liver or gill clusters, respectively.
Figure 4.1 A-D.
4.3.2 Immunohistochemistry of adult fish sampled *in situ*.

Immunolocalization of CYP1A in gill, liver, intestine, and head kidneys from fish collected *in situ* from three locations (GT, BSL, BLB) during peak oiling, one month after peak oiling, and one year after peak oiling (Table 4.1) reveals a pattern of CYP1A protein expression indicative of exposure to crude oil at GT. GT fish collected after the landfall of oil (T2-T4) had elevated CYP1A protein within the interlamellar region of the filament and in the pillar cells of the lamellae compared to fish gills collected prior to oiling (T1), or fish collected from the reference sites (Figure 2A and Whitehead et al., 2012 [34]). Increased hyperplasia along the filamental and lamellar epithelia of the gill was also present in GT fish post-oil compared to fish from GT pre-oil and reference sites. Liver tissues from GT fish collected during T3 and T4 also had elevated CYP1A protein, in contrast to fish collected during T2, when CYP1A was less abundant (Figure 4.2B) compared to fish collected from reference sites. Distribution and abundance of CYP1A protein in the head kidney (Figure 4.2C) and intestine (Figure 4.2D) also suggest exposure to PAHs in crude oil in GT fish collected after the arrival of oil (T2-T4), when compared to fish from reference sites. CYP1A expression, as found pervasively in the vascular endothelial cells of intestine and head kidney of GT fish, is a hallmark of AhR pathway activation [88, 93, 99]. Head kidney tissues also had increased CYP1A protein localized in the tubular epithelial cells, while intestinal epithelial cells were heavily stained in all GT fish collected after the arrival of oil, in contrast to the near absence of staining in head kidney and intestine from fish collected from BSL and BLB. CYP1A expression was detected in some reference fish tissues, consistent with the endogenous CYP1A protein expression in most tissues associated with diverse biological functions [93, 112]. Although CYP1A can be induced by multiple anthropogenic and natural environmental stressors and endogenous AhR ligands,
CYP1A expression is far greater when induced by exogenous xenobiotic ligands compared to constitutive expression, and the relative increase in CYP1A protein distributed throughout multiple tissues in GT fish is consistent with exposure to PAHs coincident with the arrival of contaminating oil (Figure 4.2) [39].

The persistence of increased CYP1A protein expression in GT fish more than one year after initial oiling is not surprising since crude oil can remain bound in sediments, facilitating the slow and long-term release of PAHs from sediments and from benthic food sources [16, 88]. By examining sensitive biological responses in resident species with high home-range fidelity (such as the Gulf killifish), it is possible to determine the distribution and persistence of exposures to the toxic components of oil across space and time although future studies should take into account sex, breeding potential, and other variables known to influence teleost response to crude oil exposure [89, 113].

Typically, responses of liver are used for estimating exposure to PAHs due to its capacity for xenobiotic transformation of blood-borne toxicants, although the gill, intestine, and head kidney are also sensitive indicators of exposure to AhR-inducing chemicals, and have become increasingly popular for their utility in determining exposure to PAHs [93, 112, 114, 115]. However, assessing multiple tissues for CYP1A distribution can provide insights into the route of exposure based on the differential expression between organs and their functional roles. The gill and intestine are transport epithelia capable of metabolizing AhR-inducing xenobiotics, including PAHs [99, 116, 117]. Elevated CYP1A in the gill can indicate water-borne exposure to PAHs, whereas CYP1A increase in the intestine can indicate dietary uptake and metabolism of PAHs as they cross the intestinal epithelium [88, 99, 115]. Additionally, fish in hyperosmotic environments drink to absorb water across the posterior intestine leading to the accumulation of
PAHs via the gastrointestinal tract [88]. CYP1A expression in the head kidney is indicative of exposure of a central part of the teleost immune system to immunotoxic PAHs and also suggests systemic circulation of PAHs [118, 119]. The up-regulation and distribution of CYP1A protein throughout the gill, intestine, head kidney, and liver seen in GT fish, in contrast to that seen in fish collected from reference sites, is consistent with exposure to PAHs in contaminating oil, and with persistence of PAHs in sediments enabling long-term exposure to resident biota [16, 88].

4.3.3 Developmental impacts of field-collected sediments.

Total PAH (tPAH) and alkane content in GT sediments from 2010 and 2011, and WB sediments from 2011 were elevated compared to NBS sediments (Table A2.1-A2.2 and Figure A2.1-A2.3). SCAT data confirms visible oil at GT and WB throughout December 2012, while no oil was reported at NBS. Based on analytical chemistry data, GT sediments collected during 2010 had almost a 10-fold higher tPAH concentration compared to sediments collected at that site a year later. WB sediments collected in 2011 contained approximately 21% less tPAHs than do GT 2011 sediments collected at the same time, whereas NBS had negligible tPAH content that was less than 1% of the tPAHs found in WB sediments.
Figure 4.2 A-D. Distribution of CYP1A protein (burgundy staining) in gills, liver, head kidneys, and intestines from adult killifish collected from Bay St. Louis (BSL), Bayou La Batre (BLB), and Grande Terre (GT) at four time points (T1-T4) beginning prior to landfall of oil in May 2010 (T1), at the peak of oiling in June 2010 (T2), after peak oiling in August 2010 (T3), and one year after landfall of oil in August 2011 at GT only (T4). (A) Increase in CYP1A in the gill lamellae (chevrons) and in the epithelia of the interlamellar regions of the gill filament (star) as well as an increase in hyperplasia in the gill lamellae and in the interlamellar regions of the gill filaments. (B) Livers from GT showed the highest expression of CYP1A at T3 and T4 time points, and an increase in CYP1A was observed in BSL fish at T3. (C) Increased staining of epithelial cells of the kidney tubules (arrow heads) and an increase in CYP1A-positive vascular endothelial cells were found in GT fish. (D) GT intestinal tissues show an increase in CYP1A in the epithelial cells and CYP1A-positive vascular endothelial cells (arrow heads) in the lamina propria and submucosa. All tissues sectioned at 4 μm thickness and imaged with a 20X objective. Arrows = vascular endothelial cells, chevrons = gill filaments, asterisks = buccopharyngeal cavity, arrow heads = kidney tubules. Scale bar = 50μm. All slides were counterstained with hematoxylin (blue). Gill tissues from T1, T2, and T3 are the same tissues used in Whitehead et al., 2012 [34].
Throughout the 21-day exposure of embryos to these sediments, many of the GT sediment-exposed embryos failed to hatch (Figure 4.3 A-B). Beginning at day 13, embryos exposed to GT 2010 and GT 2011 sediments had significantly less hatching events compared to embryos
exposed to WB or NBS sediments (P<0.05). Accordingly, percent hatch within 21 days post-fertilization for embryos exposed to GT sediments collected in 2010 and 2011 was significantly reduced compared to those exposed to sediments collected in 2011 from NBS or WB (P<0.05). In comparison, there was no significant change in embryonic mortality between treatments. Those embryos that did hatch in the GT sediments were significantly smaller, had pronounced bradycardia during embryonic development (P<0.05) (Figure 4.4 A-B), and had poor vigor at hatch. Yolk-sac and pericardial edema were observed in fish exposed to GT sediments, but not observed in WB or NBS larvae (data not shown). These data are consistent with the characteristic developmental impairments associated with early life-stage exposures to crude oil and PAHs, and are correlated with an increase in PAHs (and alkanes) in the sediments, coincident with oiling attributed to the DWOS (Table A2.1-A2.2 and Figure A2.1-A2.3) [16, 20, 34, 120]. Sources of variation between sediments may influence the developmental trajectory of organisms that are bred in these locations. However, the effects seen in these developing fish indicate that resident developing Gulf killifish embryos were exposed to crude oil from these sediments for at least two breeding seasons, and that this exposure may affect future population demographics at locations where crude oil is present.
Figure 4.3 A-B. Hatching success and mortality of embryos exposed to sediments collected from an unoiled reference location in North Bay Sansbois in 2011 (NBS 2011), from a mildly oiled location in Wilkinson Bay in 2011 (WB 2011), and a heavily oiled location at Grande Terre Island in 2010 and 2011 (GT 2010 and 2011). Embryos were fertilized and observed daily for 21 days for mortality and hatching. (A) Percentage of hatched and unhatched larvae and percentage of mortalities. Unhatched embryos are defined as those that did not hatch by day 21 post fertilization. Mortalities are animals that died within the 21 day period. Asterisks indicate significant difference compared to the NBS reference sediment exposure (P≤0.001). (B) After day 13, embryos exposed to GT 2010 and GT 2011 sediments hatched significantly less compared to embryos exposed to WB or NBS sediments. Error bars indicate standard error and are one sided to prevent overlapping and crowding of symbols. Asterisks indicate a significant difference compared to the NBS reference sediment exposure (P≤0.05).

4.3.4 Immunohistochemistry of sediment-exposed larvae.
Larvae collected at <24 hours post hatch had elevated CYP1A protein when exposed to oiled sediments (WB 2011, GT 2010, and GT 2011) compared to larvae exposed to unoiled (NBS) sediment (Figure 4.5 A-B and Figure A2.4). Elevated CYP1A protein in oiled sediment-exposed larvae was found in vascular endothelial cells throughout the body, and in the gill, buccopharyngeal epithelium, liver, head kidneys, and heart (Figure 4.5B). NBS sediment-exposed fish showed slightly elevated CYP1A in kidney tubules, heart, and liver tissue, although elevated expression of CYP1A was not observed in other tissues. Larvae exposed to GT
sediments that had substantially more CYP1A protein than the other sediment-exposed fish, including stronger staining of the external epithelial surfaces. Staining in the external epithelia was present in WB larvae, albeit to a much lesser extent than that of GT sediment-exposed larvae. This is not surprising, since PAH and alkane concentrations were elevated in WB sediments at levels just below that of GT 2011 sediments (Table A2.1-A2.2 and Figure A2.1-A2.3). Despite the increased CYP1A expression in WB sediment-exposed larvae, no bradycardia or developmental effects were noted in these animals (Figure 4.4 A-B). It is likely that the dosage of toxicants needed to elicit observable phenotypic effects is higher than that found in WB sediments, but lower than that found in GT sediments collected at the same time in 2011, or that other physical or chemical characteristics of these sediments contribute to the effects seen in these animals.

**Figure 4.4 A-B.** Early life-stage phenotypic effects of embryos exposed to sediments collected from an unoiled reference location in North Bay Sansbois in 2011 (NBS 2011), from a mildly oiled location in Wilkinson Bay in 2011 (WB 2011), and a heavily oiled location at Grande Terre Island in 2010 and 2011 (GT 2011 and 2010). (A) Larval length at ≤24 hours post hatch was significantly lower for GT 2010 sediment exposed fish. GT 2011 exposed fish were unavailable for length measurements. (B) Heart rate of embryos at 8 DPF. Error bars indicate standard error. Asterisks in both graphs indicate significant difference compared to the NBS reference sediment exposure (P≤0.001).
Figure 4.5 A-B. Distribution of CYP1A protein (burgundy staining) in larval tissues at ≤ 24 h post hatch exposed throughout development to sediments collected from an unoiled reference location in North Bay Sansbois in 2011 (NBS 2011), from a mildly oiled location in Wilkinson Bay in 2011 (WB 2011), and a heavily oiled location at Grande Terre Island in 2010 and 2011 (GT 2011 and 2010). Figure depicts representative oiled sediment-exposed larvae in (A) to provide anatomical reference for (B) (see below). (A) Representative image of larvae at <24 hours post hatch that were exposed to WB sediment throughout embryonic development. CYP1A was mostly found localized in gill (G), intestine (I), head kidney (HK), liver (L), heart (H) endothelium, vascular endothelial cells (arrows), and in epithelial cells lining the buccopharyngeal cavity (asterisks) in fish exposed to oiled sediments (i.e. WB and GT sediments). Fish exposed to unoiled sediment from NBS showed staining for CYP1A in the heart endothelium and kidney tubules (See supplementary data, S2). Larvae were sectioned at 4 µm. Figure depicts a montage of 12 images captured using a 20X objective. (B) Tissue sections from larvae at <24 hours post hatch. Gill tissues from fish exposed to WB 2011, GT 2011, and GT 2010 sediments had CYP1A-positive vascular endothelial cells and increased CYP1A in epithelial and pillar cells in the gill filaments and lamellae. Epithelial cells lining the buccopharyngeal cavity also showed increased CYP1A in these fish. Intestine showed light staining for CYP1A protein in the epithelial cells and endothelial cells in the submucosa in these fish. Head kidneys tubules and vascular endothelial cells in fish exposed to oiled sediment had higher CYP1A staining compared to fish exposed to unoiled NBS 2011 sediment. Hepatocytes of liver tissue of fish exposed to oiled sediments showed increased CYP1A expression compared to those of fish exposed to unoiled NBS sediment. Images captured through a 40X objective. Arrows = vascular endothelial cells, chevrons = gill lamellae, asterisks = buccopharyngeal cavity, arrow heads = kidney tubules. All tissues from A and B were sectioned at 4µm. Scale bar = 50µm. All slides were counterstained with hematoxylin (blue).
In Whitehead et al., 2012 [34], adult Gulf killifish collected at GT in 2010 exhibited molecular and protein-level responses that are diagnostic of exposure to crude oil. In that study, though PAHs were low in tissues and water samples, PAHs were elevated in GT sediments [34] relative
to reference sites. The methods used in the experiments reported here were designed assess the potential for field-collected sediments from oiled sites to elicit lethal or sub-lethal effects. By utilizing field-collected sediments as opposed to laboratory-spiked sediments or water, we attempted to characterize the developmental potential of Gulf killifish at locations that received oil from the DWOS. The results suggest that sediments were a persistent source of biologically-available toxicants oiled sites for over one year following the landfall of oil. Furthermore, since PAHs in field-collected water and tissues of resident animals have tended to be below the detection limits of analytical chemistry in areas that received contaminating oil, biological responses appear to be more sensitive indicators of contamination [34, 121].

4.4 IMPLICATIONS
Integration of diverse endpoints spanning multiple levels of biological organization in both laboratory and field studies with indigenous organisms as site-specific indications of ecosystem health enriches the understanding of the effects of environmental change. In seeking to characterize the effects of the DWOS on at-risk fish, a field study examining the health of populations of Gulf killifish was initiated prior to the landfall of oil, and is ongoing. Here we presented evidence of exposure to PAHs in adult fish coincident with the oil contamination from the DWOS. Genome expression profiling indicates significant divergence in genomic responses of fish from an oiled location compared to reference sites. These responses are diagnostic of exposure to the toxic components of oil, offer insight into shared and organ-specific impacts, and are highly correlated with CYP1A protein expression responses in the gills, liver, head kidney, and intestine of adult and larval fish. Exposure to sediments from oiled locations caused cardiovascular defects in embryonic fish, delayed hatching, and reduced overall hatching success. Those larvae that do hatch are smaller and have yolk-sac and pericardial edema. These
data include results that encompass two breeding seasons and indicate that contaminating oil from the DWOS impacts organismal fitness, which may translate into longer-term effects at the population level for Gulf killifish and other biota that live or spawn in similar habitats.
CHAPTER FIVE – CONCLUSION

The preceding chapters illustrate the effects of exogenous stressors on fish physiology and suggest the need for combining laboratory and field experimentation to more accurately predict the *in situ* condition. We see new insights into the interaction between the fish and glochidia that will foster new methods for artificial propagation of mussel larvae, and aid in the understanding of how fish respond to glochidial infection in a controlled environment. Using the laboratory studies to determine how fish respond to infection along with field-based studies that examine these phenomena in the natural environment will enable future investigators to forecast how mussels and fish interact *in situ*. This stresses a coupling of laboratory and field-based experimentation to best understand the environmental relevance of the findings. In the studies that follow this work, a holistic approach was used by incorporating both laboratory and field-methodologies to understand how anthropogenic stressors may affect fish in the natural environment, and by doing so found that these effects may be predictive of population-level effects.

Following the explosion of the Deepwater Horizon oil platform, the landfall of crude oil provided a natural experiment (i.e. experimental conditions were set by factors that were not under our control). It was thought that the animals within areas that received crude oil from the Deepwater Horizon oil spill would undergo stress due to toxic exposure, thereby causing observable population-level impacts over time, as seen following the Exxon Valdez oil spill. In order to test this hypothesis, an extensive field study was initiated prior to landfall of oil to gain a baseline physiological profile, then after oiling to begin long-term evaluations of local fish health in order to characterize the effects of this anthropogenic stressor over time.
Field collections of fish and subsequent analysis of physiological endpoints of exposure and indications of adverse effects were conducted for over one year following the landfall of oil. These data provided evidence of exposure to crude oil coincident with the timing and location of oiling. In order to understand how exposure to oil might ultimately affect Gulf killifish populations, it was necessary to gauge the reproductive potential of these fish. This was done by exposing Gulf killifish embryos to oiled sediments and water under laboratory conditions to determine if there was an effect on development that would indicate a possible reduction in the likelihood of survival. In an attempt to create a replicable in vitro experiment that closely resembled the in situ condition, exposures were done in a common-garden fashion, where the sediment source was the only variable that differed. Under laboratory conditions, temperature, salinity, water availability, and lighting were kept constant, but nitrogenous wastes and animal predation kept absent. This eliminated a number of extraneous variables that could impact survival if these experiments were conducted in the field. However, field-collected water and sediments likely contained numerous additional variables such as bacterial colonies, small predatory organisms, and background contaminants in addition to any crude oil, or lack thereof. By allowing these variables to remain, interpretations of the results allow for a more accurate extrapolation of the environmental reality. This tradeoff between controlling for some environmental variables, while allowing others to remain increases the predictability of the results to reflect the environmental condition. Future studies are warranted to study how these and other environmental variables could influence the toxic effects of oil. The data produced from this experiment combined with the evidence of exposure in adult fish together suggest that the fecundity of these fish in oiled locations is affected after exposure throughout two successive breeding seasons. Furthermore, this work highlights the importance of combining laboratory and
field-based experiments to enhance the environmental relevance of experimental data, and to predict the effects of natural and anthropogenic stressors on exposed biota.
REFERENCES


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APPENDIX 1 – GENOMIC AND PHYSIOLOGICAL FOOTPRINT OF THE DEEPWATER HORIZON OIL SPILL ON RESIDENT MARSH FISHES1 [1].

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A1.1 INTRODUCTION
Following the Deepwater Horizon (DWH) drilling disaster on April 20, 2011 in the Gulf of Mexico, acute oiling and the resulting mortality of marine wildlife were evident. In contrast, the sub-lethal effects, critically important for predicting long-term population-level impacts of oil pollution [2], have not been well described following the DWH disaster. Here we report the results of a four month field study monitoring the biological effects of oil exposure on fish resident in Gulf of Mexico coastal marsh habitats.

Gulf killifish (Fundulus grandis) was used as our model species because they are among the most abundant vertebrate animals in Gulf of Mexico-exposed marshes [3-5]. Furthermore, the Atlantic-distributed sister species to F. grandis (F. heteroclitus) has a narrow home range and high site fidelity especially during the summer [6, 7], and among fishes is relatively sensitive to the toxic effects of organic pollutants [8]. Though home range and toxicology studies are lacking for F. grandis, we infer that F. grandis is also relatively sensitive to pollutants and exhibits high site fidelity, such that their biology is likely affected primarily by their local environment, given their recent shared ancestry with F. heteroclitus [9] and similar physiology,

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life history, and habitat [10-14]. We sampled from populations resident in Gulf-exposed marshes before oil landfall (05/01 to 05/09/2010), during the peak of landfall (06/28 to 06/30/2010), and after much of the surface oil was no longer apparent two months later (08/30 to 09/01/2010) at six field sites from Barataria Bay, LA, east to Mobile Bay, AL (Figure A1.1, Table S1 in supporting online material).

Figure A1.1. Location of field study sites and incidence of oil contamination. A) Location of field sampling sites. Color coding is consistent with other figures. Red star indicates DWH spill site. B) Photograph (by A. Whitehead) of the GT field site on 06/28/2010, showing contaminating oil and minnow traps in the marsh. C) Proximity of nearest surface oil to each field site determined by Synthetic Aperture Radar, where rows are field sites and columns are days and color of cells represent no data (light gray), black (nearest surface oil > 4 km), and increasing intensity of red indicates closer proximity of oil. Three field sampling trips are highlighted (blue boxes).

A1.2 RESULTS AND DISCUSSION

Remote sensing and analytical chemistry were used to characterize exposure to DWH oil, where remote sensing data are spatially and temporally comprehensive but of low resolution, and
chemistry data are of high resolution but patchy in space and time. Ocean surface oil was remotely detected through the analysis of images from Synthetic Aperture Radar (SAR) [15]. Proximity of the nearest oil slick to each field site (e.g., Figure S1 in supporting online material) was measured for each day that SAR data were available, from 05/11 through 08/13/2010, to approximate the location, timing, and duration of coastal oiling (Figure A1.1 C). Though surface oil came close to many of our field sites in mid-June, only the GT site was directly oiled (Figure A1.1B and A1.11C). Though the GT site had been clearly contaminated with crude oil for several weeks prior to our sampling (Figure A1.1C, Figure S2 in supporting online material), and retained much oil in sediments (Table S2 in supporting online material), only trace concentrations of oil components were detected in sub-surface water samples collected from the GT site on June 28, 2010, and tissues did not carry abnormally high burdens of oil constituents at any site or time-point (Table S2 in supporting online material). Despite a low chemical signal for oil in the water column and tissues at the time of sampling, we detect significant biological effects associated with the GT site post-oil.

We sampled multiple tissues from adult Gulf killifish (average weight = 3.5 g) from each of six field sites for each of three time-points (only the first two time-points for site MB) spanning the first four months of the spill event (Figure A1.1C). We compared biological responses across time (before, at the peak, and after oiling) and across space (oiled and un-oiled sites) and integrated responses at the molecular level using genome expression profiling with complimentary protein expression and tissue morphology. Genome expression profiles, using microarrays and RNAseq, were characterized for livers because the organ is internal and integrates xenobiotic effects from multiple routes of entry (gill, intestine, epithelial), and because liver is the primary tissue for metabolism of toxic oil constituents. Tissue morphology and
expression of CYP1A protein, a common biomarker for exposure to select PAHs, was characterized for gills, the organ that provides the greatest surface area in direct contact with the surrounding aquatic environment. In addition, we exposed developing embryos to field-collected water samples to document bioavailability and bioactivity of oil contaminants for this sensitive early life stage.

The oiling of the GT site at the end of June 2010 is associated with a clear functional genomic footprint. Of the 3296 genes included in our analysis, expression of 1600 and 1257 genes varied among field sites and throughout the time-course, respectively (p<0.01) (Dataset S1 in supporting online material). For the 646 genes that varied in expression only among sites (no significant time effect or site-by-time interaction), site variation followed a pattern of population isolation-by-distance, which is consistent with neutral evolutionary divergence (Figure A1.2A) and consistent with population genetic expectations [16]. Most importantly, 1500 genes indicated a pattern of site-dependent time-course expression (significant interaction; FDR<0.01), where the trajectory of genome expression through time was divergent at the GT site compared to all other sites (Figure A1.2B and A1.2C), particularly at the second time-point which coincides with oil contamination (Figure A1.1C).

Previous studies have identified genes that are transcriptionally responsive to planar polychlorinated biphenyl (PCB) exposures in killifish [17]. Planar PCBs, dioxins, and polycyclic aromatic hydrocarbons (PAHs: the primary toxic constituents in crude oil) are all mechanistically related insofar as they exert biological effects, in whole or in part, through aryl-hydrocarbon receptor (AHR) signaling pathways; indeed, morpholino knockdown of the AHR is protective of the toxic effects of PAHs and PCBs in killifish [18] and exposures to PCBs and PAHs induce common genome expression responses in flounder [19]. Of the genes that were transcriptionally
responsive to PCB exposures (from [17]), 380 were included in the current analysis. Expression of this subset of genes is predictive of transcriptional divergence in fish from the GT site coincident with oil contamination compared to other field sites (Figure S3 in supporting online material), especially for the top 10% of PCB-responsive genes (Figure A1.2D). Transcriptional activation of these planar PCB-responsive genes in developing killifish embryos is predictive of induction of developmental abnormalities, decreased hatching success, and decreased embryonic and larval survival [17, 20]. This set of genes includes members of the canonical battery of genes that are transcriptionally-induced by ligand-activated AHR signaling, such as cytochrome P450s, cytochrome B5, and UDP-glucuronosyltransferase (Figure A1.2F set 1), for which increased transcription is particularly diagnostic of exposure to select hydrocarbons [21]. Indeed, many genes which are transcriptionally induced or repressed by AHR activators (dioxins, PCBs, PAHs) show induction or repression at the GT site coincident with crude oil contamination (Figure A1.2F set 1). An independent measure of genome expression, RNAseq, also indicates AHR activation in GT fish from June 28th, 2010, compared to reference RNA (e.g., up-regulation of cytochrome P450s, UGT, and AHR itself; Figure A1.2E). In parallel, up-regulation of CYP1A protein was detected in gills from GT fish sampled post-oil and in early-life stage fish upon controlled exposures to GT waters (Figure A1.3). These data appear diagnostic of exposure to the toxic constituents in contaminating oil (PAHs) at sufficient concentration and duration to induce biological responses in resident fish. Sustained activation of the CYP1A gene (as in Figure A1.2F and Figure A1.3) was predictive of persistent exposure to sub-lethal concentrations of crude oil components and negative population-level impacts in fish, sea otters, and harlequin ducks following the Exxon Valdez oil spill (reviewed in [2]), though PAH toxicity may be mediated through AHR-independent pathways as well [22].
Figure A1.2 A-F. Genome expression between field sites and across time. (A-D) Principle component analysis of divergently expressed genes, etc. (E) RNA-Seq data (F) Heat maps of genes from 1. AhR ligand regulated genes 2. Genes associated with proteolysis, 3. Cardiovascular function, and 4. Ion transport. GT was the only site to be directly oiled, which occurred between the 1st and 2nd sampling times (Fig. A1.1, Table S2 in supporting online material). A) For genes that vary only among sites (no expression change with time or interaction), pairwise site-specific transcriptome divergence along principal component (PC) 1, as a function of pairwise geographic distance, shows a pattern consistent with isolation-by-distance. B) Trajectory of genome expression responses through time for each of six field sites from the pre-oil sample time (dot at base of arrow) through the peak-oil (middle dot) to latest post-event sample times (dot at head of arrow) following PC analysis of genes showing statistically significant main effects (“site”, “time”) and interaction terms. Divergence along PC1 is isolated in (C) where bars for each site from left to right represent sampling times earliest to latest. D) Expression divergence along PC1 for the subset of genes that is dose-responsive to PCB exposure (top 10% of PCB-responsive genes). E) RNAseq data showing genes up and down-regulated (X-axis positive and negative, respectively) in fish from GT sample time 2 (coincident with oil) compared to reference RNA, where select genes are identified. Inset shows genes dramatically down-regulated at GT. See Dataset S2 for detailed RNAseq data in supporting online material. F) Expression levels for specific genes (rows) and treatments (columns), where cell color indicates up (yellow) or down (blue) regulation scaled according to site-specific expression level at the pre-oil sample time, for genes with divergent expression at the GT site. Genes are grouped into functional categories and scale bars indicate N-fold up or down regulation.
Transcriptional responses in other sets of co-expressed genes offer insights into the potential biological consequences of contaminating oil exposure at the GT site. Several gene ontology (GO) categories were enriched in the subset of genes that showed GT-specific expression.
divergence coincident with site and time-specific oil contamination (Table S3 in supporting online material). GO enrichment indicates activation of the ubiquitin-proteasome system (Figure A1.2F set 2), which among diverse functions, is important for cellular responses to stress, cell cycle regulation, regulation of DNA repair, apoptosis, and immune responses [23]. The AHR protein itself plays a role as a novel ligand-dependent E3 ubiquitin ligase that targets sex steroid (estrogen and androgen) receptor proteins for proteosomal destruction, thereby impairing normal cellular responses to sex hormones in reproductive tissues, and this response can be activated by planar PAHs [24]. Significant down-regulation of transcripts for egg envelope proteins zona pellucida (ZP3, ZP4) and choriogenin (ChgHm, ChgH) that we detect at the GT site coincident with oil exposure (Figure A1.2F set 1) may be linked with this AHR-dependent proteolytic pathway, since their transcription is estrogen-dependent [25, 26] and is down-regulated by exposure to PAHs in fish [26-28]. In corroboration, RNAseq detects ZP, ChgH, and vitellogenin (VTG) transcripts dramatically down-regulated in GT fish (Figure A1.2E). Though the transcriptional response that we detect is in male fish, these proteins are synthesized in male livers (reviewed in [26, 28]) and down-regulation is consistent with anti-estrogenic effects from exposure to PAHs [29]. Possible impacts on reproduction merit attention because water only from the GT site induced CYP1A protein in the gills of developing killifish (Figure A1.3), at low concentrations of total aromatics and alkanes (Table S2 in supporting online material), and more than two months after initial oiling, indicating persistent bioavailability of PAHs. Marsh contamination with DWH oil coincided with the spawning season for many marsh animals including killifish [30], and reproductive effects are predictive of long-term population-level impacts from oil spills [2].
Controlled exposures of developing killifish to water collected from GT on June 28 and August 30 (2010) induced CYP1A protein expression in larval gills relative to fish exposed to GT water pre-oil and exposed to un-oiled BLB site water (Figure A1.3). This response is consistent with the location and timing of oil contamination, and indicates that the remaining oil constituents dissolved at very low concentrations at GT post-landfall (Table S2 in supporting online material) were bioavailable and bioactive to developing fish. Although exposures to PAHs stereotypically induce cardiovascular system abnormalities in developing fish at relatively high concentrations (e.g., [22]), none were observed in these animals. However, even very low-concentration exposures during development, insufficient to induce cardiovascular abnormalities in embryos, can impair cardiac performance in adulthood [31]. The adult fish sampled in situ from the oil-contaminated GT site showed divergent regulation of several genes involved in blood vessel morphogenesis and heme metabolism coincident with oil contamination (Figure A1.2F set 3). Multi-generation field studies are necessary to confirm cardiovascular effects from DWH oil contamination of marshes that coincided with spawning.
Figure A1.3. CYP1A protein expression (dark red staining) in larval killifish gills (24 days post-fertilization) exposed to waters collected from Grande Terre (GT; oiled) and Bayou La Batre (BLB; not oiled) during development (40x magnification, bars = 10 µm). CYP1A expression is elevated in the lamellae of larvae exposed during development to waters collected from GT post-oil (trips 2 and 3) compared to background levels of CYP1A expression in larvae exposed to GT water pre-oil (trip 1), compared to CYP1A in fish exposed to waters collected from BLB which was not directly oiled, and compared to CYP1A in fish reared in laboratory control water. Nuclei were stained using hematoxylin (blue). Analytical chemistry of exposure waters is reported in Table S2 in the supporting online material.

Coastal salt-marsh habitats are dynamic and stressful, where changes in environmental parameters such as temperature, hypoxia, and salinity, can continuously challenge resident
wildlife. Regulation of ion transport in fish is particularly important for facilitating homeostasis in response to salinity fluctuations that are common in estuaries. We found altered regulation of multiple ion transport genes in fish from the GT site coincident with oil contamination (Figure A1.2G, set 4). For example, V-type proton ATPases are up-regulated, and Na\(^{+}\),K\(^{+}\)-ATPase subunits and tight-junction proteins are down-regulated, coincident with oiling at the GT site, in the absence of substantial changes in environmental salinity (Table S2 in supporting online material). Other genes important for osmotic regulation in killifish [32] are also divergently down-regulated at GT including type II iodothyronine deiodinase (DIO2), transcription factor jun-B (JUNB), and arginase 2 (ARG2). In corroboration, RNAseq data show down-regulation of DIO2, JUNB, and ARG2 in GT fish compared to reference fish (Figure A1.2E). Though the physiological consequences of oil exposures are typically studied in isolation, it is reasonable to predict that exposure to oil may compromise the ability of resident organisms to physiologically adjust to natural stressors.

Induction of CYP1A protein expression is a hallmark of AHR signaling pathway activation, making it a sensitive biomarker of exposure to select planar PAHs and other hydrocarbons [21]. Though liver is the key organ for CYP1A-mediated metabolism of these substrates, gills tissues represent the most proximate site of exposure to PAHs. Due to direct contact with the environment and the nature of the gill as a transport epithelia, gill may be a more sensitive indicator of exposure to contaminants than the liver [33]. CYP1A protein was markedly elevated in GT fish post-oiling compared to GT fish pre-oil and compared to fish from other field sites that were not directly oiled (Figure A1.4). CYP1A induction was localized predominantly to pillar cells of the gill lamellae and within undifferentiated cells underlying the interlamellar region, which may have contributed to the filamental and lamellar hyperplasia observed during
trips 2 and 3, and the gross proliferation of the interlamellar region observed during trip 2 in GT fish (Figure A1.4). These effects imply a decrease in the effective surface area of the gill, a tissue that supports critical physiological functions such as ion homeostasis, respiratory gas exchange, systemic acid-base regulation, and nitrogenous waste excretion [34]. Currently, it is unclear the degree to which oil-induced effects may interact with commonly-encountered challenges, such as fluctuations in hypoxia and salinity, to compromise physiological resilience.

By integrating remote sensing and in situ chemical measures of exposure, and linking these with integrated measures of biological effect (genome expression and tissue morphology), we provide evidence that links biological impacts with exposure to contaminating oil from the DWH spill within coastal marsh habitats. Though body burdens of toxins are not high, consistent with reports indicating that Gulf of Mexico seafood is safe for consumption [35], this does not mean that negative biological impacts are absent. Our data reveal biologically-relevant sub-lethal exposures causing alterations in genome expression and tissue morphology suggestive of physiological impairment persisting for over two months after initial exposures. Sub-lethal effects were predictive of deleterious population-level impacts that persisted over long periods of time in aquatic species following the Exxon Valdez spill [2], and must be a focus of long-term research in the Gulf of Mexico, especially since high concentrations of hydrocarbons in sediments (Table S2 in supporting online material) may provide a persistent source of exposures to organisms resident in Louisiana marshes.
Figure A1.4. CYP1A protein expression in adult killifish gills (dark red staining) sampled in situ from all sampling times (columns) and locations (rows) (40x magnification, bars = 10 µm). The MB site was only sampled on trips 1 and 2, and gills from trip 1 at the BLB site were not available for processing. Fish gills from the GT site during trips 2 and 3 showed high CYP1A expression and elevated incidence of hyperplasia of the lamellae (L) and interlamellar space (IL) on the gill filaments (F) coincident with oil contamination. CYP1A protein was elevated at the GT site post-oiling (trips 2 and 3) compared to GT pre-oil (trip 1), and compared to other field sites, none of which were directly oiled. Nuclei were stained using hematoxylin (blue). Exact site locations and sampling dates are in Table S1 in supporting online material.
A1.3 METHODS

The locations (latitude, longitude) of our field sampling sites and dates for sampling at each site are summarized in Table S1 in supporting online material. Gulf killifish (*Fundulus grandis*) were caught by minnow trap and tissues excised immediately. Liver was preserved in RNAlater for genome expression (microarray and RNAseq) analysis. Gill tissues were fixed in situ in buffered zinc-based formalin Z-Fix (Anatech LTD). Succinct methods follow, and more detailed methods are available online.

Satellite imagery (Synthetic Aperture Radar) was analyzed to provide estimation of the timing, location, and duration of coastal oil contamination. The calculated distance from each field sampling site to the nearest oil slick was calculated from the “straight line” distance from the GPS position of the station (Table S1 in supporting online material) to that of the observed oil across any and all intervening geographical barriers (e.g., Figure S1 in supporting online material).

Analytical chemistry of water, tissue, and sediment samples was performed to offer detailed characterization of exposure to contaminating oil (data reported in Table S2 in supporting online material). Sample dates and locations are summarized in Table S1 in supporting online material. All sample extracts were analyzed using gas chromatography interfaced to a mass spectrometry detector system. Spectral data were processed by Chemstation™ Software and analyte concentrations calculated based on the internal standard method.

Genome expression across sites and time was characterized using custom oligonucleotide microarrays. Genome expression was measured in liver tissues from five replicate individual male fish per site-time treatment (five biological replicates) hybridized in a loop design including a dye swap. Data were lowess-normalized then mixed model normalized using linear mixed
models to account for fixed (dye) effects and random (array) effects. Normalized data were then analyzed using mixed model analysis of variance, with “site” (GT, BSL, BFP, BLB, MB, and FMA) and “sampling time” (sampling trips 1, 2, and 3) (see Table S1 in supporting online material) as main effects, including an interaction (site-by-time) term. False discovery rate was estimated using Q-value [36]. Principal components analysis was performed using MeV [37]. Gene ontology enrichment was tested using DAVID [38].

For RNAseq, transcript abundance was compared between liver mRNA from three replicate fish (RNA was not pooled) from the GT site from June 28th (2010) and mRNA from two control samples. All RNA samples were sequenced on the Illumina GA platform (Expression Analysis, Inc., Durham, NC). Following quality control filtering, quantitative transcript abundance analysis was performed by mapping sequence reads to target sequences (6810 unique F. heteroclitus target EST sequences – see Dataset S2 in supporting online material) using the Bowtie short read alignment software [39]. A custom Perl script determined the number of fragments mapped to each target sequence. The Bioconductor package DESeq (version 2.8) [40] was used to determine statistical significance of each differentially expressed target using a negative binomial method with P-values adjusted by the Benjamini-Hochberg procedure.

Gill tissues were sampled from all field sites for morphological analysis and immunohistochemical analysis of CYP1A protein expression. Gill tissues from the first and second gill arch were sectioned along the longitudinal axis at 4 µm thickness and probed with mAb C10-7 against fish CYP1A [41]. Sections were counter-probed using the Vectastain® ABC immunoperoxidase system, using ImmPACT™ Nova RED™ peroxidase substrate kit to visualize the CYP1A protein in red. Tissue sections were counter-stained with Vector® Hematoxylin QS.
F. grandis embryos obtained from parents not exposed to oil (collected from Cocodrie, LA) were exposed to water samples from Grande Terre (LA) and Bayou La Batre (AL) collected subsurface on the dates indicated in Table S1 in supporting online material. Following fertilization, 20 embryos were randomly transferred in triplicate to one of the six field-collected waters (two field sites x three time points) at 3h post-fertilization. Embryos were also exposed to a laboratory control consisting of artificial 17ppt water. Larvae were sampled at 24 days post-fertilization (DPF) and fixed in z-fix solution (Anatech LTD.). Sectioning and staining was as described in the previous section.

A1.4 REFERENCES


### A1.5 ACKNOWLEDGEMENTS

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A1.6 SUPPORTING ONLINE MATERIAL
Supporting online material available online at:

http://www.pnas.org/content/early/2011/09/21/1109545108/suppl/DCSupplemental
### APPENDIX 2 – SUPPLEMENTAL MATERIAL FROM CHAPTER FOUR

**Table A2.1. Aromatic analytes.** Aromatic compounds (PAHs) found in sediment samples used in embryonic exposures.

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<th>Abbreviation</th>
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<th>WB 2011</th>
<th>GT 2011</th>
<th>GT 2010</th>
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<td>2764.767</td>
<td>9493.694</td>
</tr>
<tr>
<td>C1-Chrysenes</td>
<td>C1</td>
<td>4.835</td>
<td>3627.253</td>
<td>3491.640</td>
<td>14549.279</td>
</tr>
<tr>
<td>C2-Chrysenes</td>
<td>C2</td>
<td>7.244</td>
<td>3126.278</td>
<td>3104.345</td>
<td>11892.472</td>
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<td>C3-Chrysenes</td>
<td>C3</td>
<td>5.040</td>
<td>1707.957</td>
<td>2108.935</td>
<td>6804.801</td>
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<td>C4-Chrysenes</td>
<td>C4</td>
<td>0.000</td>
<td>1262.721</td>
<td>1147.546</td>
<td>2689.117</td>
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<td>Benzo (b) Fluoranthene</td>
<td>BBF</td>
<td>3.093</td>
<td>53.657</td>
<td>75.214</td>
<td>582.591</td>
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<td>Benzo (k) Fluoranthene</td>
<td>BKF</td>
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<td>49.565</td>
<td>87.059</td>
<td>412.712</td>
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<tr>
<td>Benzo (c) Pyrene</td>
<td>BEP</td>
<td>2.797</td>
<td>692.688</td>
<td>705.788</td>
<td>1698.497</td>
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<tr>
<td>Benzo (a) Pyrene</td>
<td>BAP</td>
<td>2.859</td>
<td>73.962</td>
<td>94.902</td>
<td>263.524</td>
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<tr>
<td>Perylene</td>
<td>PER</td>
<td>11.092</td>
<td>44.934</td>
<td>102.674</td>
<td>339.451</td>
</tr>
<tr>
<td>Indeno (1,2,3 - cd) Pyrene</td>
<td>ICP</td>
<td>3.374</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
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<tr>
<td>Dibenz(a,h) anthracene</td>
<td>DBA</td>
<td>0.986</td>
<td>0.000</td>
<td>0.000</td>
<td>153.117</td>
</tr>
<tr>
<td>Benzo (g,h,i) perylene</td>
<td>BZP</td>
<td>3.901</td>
<td>175.938</td>
<td>133.284</td>
<td>273.718</td>
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</table>
**Table A2.2.** Alkane analytes. Alkane compounds found in sediment samples used in embryonic exposures.

<table>
<thead>
<tr>
<th>Alkane analyte</th>
<th>Abbreviation</th>
<th>NBS 2011</th>
<th>WB 2011</th>
<th>GT 2011</th>
<th>GT 2010</th>
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</thead>
<tbody>
<tr>
<td>nC-10 Decane</td>
<td>nC-10</td>
<td>0.005</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
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<td>nC-11 Undecane</td>
<td>nC-11</td>
<td>0.002</td>
<td>0.190</td>
<td>0.162</td>
<td>0.253</td>
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<tr>
<td>nC-12 Dodecane</td>
<td>nC-12</td>
<td>0.003</td>
<td>0.247</td>
<td>0.176</td>
<td>0.261</td>
</tr>
<tr>
<td>nC-13 Tridecane</td>
<td>nC-13</td>
<td>0.005</td>
<td>0.298</td>
<td>0.222</td>
<td>1.125</td>
</tr>
<tr>
<td>nC-14 Tetradecane</td>
<td>nC-14</td>
<td>0.009</td>
<td>0.382</td>
<td>0.339</td>
<td>15.240</td>
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<tr>
<td>nC-15 Pentadecane</td>
<td>nC-15</td>
<td>0.030</td>
<td>1.061</td>
<td>1.699</td>
<td>80.041</td>
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<tr>
<td>nC-16 Hexadecane</td>
<td>nC-16</td>
<td>0.074</td>
<td>3.205</td>
<td>10.125</td>
<td>196.679</td>
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<tr>
<td>nC-17 Heptadecane</td>
<td>nC-17</td>
<td>0.302</td>
<td>8.608</td>
<td>31.577</td>
<td>336.989</td>
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<tr>
<td>Pristane</td>
<td>Pr</td>
<td>0.152</td>
<td>46.781</td>
<td>57.100</td>
<td>213.886</td>
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<tr>
<td>nC-18 Octadecane</td>
<td>nC-18</td>
<td>0.730</td>
<td>14.456</td>
<td>52.705</td>
<td>421.749</td>
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<tr>
<td>Phytane</td>
<td>Ph</td>
<td>0.224</td>
<td>61.431</td>
<td>71.001</td>
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<tr>
<td>nC-19 Nonadecane</td>
<td>nC-19</td>
<td>0.604</td>
<td>18.076</td>
<td>70.280</td>
<td>470.108</td>
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<tr>
<td>nC-20 Eicosane</td>
<td>nC-20</td>
<td>0.230</td>
<td>22.782</td>
<td>93.038</td>
<td>556.688</td>
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<tr>
<td>nC-21 Henecicosane</td>
<td>nC-21</td>
<td>0.253</td>
<td>27.642</td>
<td>83.092</td>
<td>515.843</td>
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<tr>
<td>nC-22 Docosane</td>
<td>nC-22</td>
<td>0.275</td>
<td>29.058</td>
<td>74.272</td>
<td>492.299</td>
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<tr>
<td>nC-23 Tricosane</td>
<td>nC-23</td>
<td>0.362</td>
<td>27.471</td>
<td>62.550</td>
<td>473.583</td>
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<tr>
<td>nC-24 Tetracosane</td>
<td>nC-24</td>
<td>0.266</td>
<td>27.932</td>
<td>50.090</td>
<td>461.284</td>
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<tr>
<td>nC-25 Pentacosane</td>
<td>nC-25</td>
<td>0.311</td>
<td>35.423</td>
<td>54.726</td>
<td>266.837</td>
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<tr>
<td>nC-26 Hexacosane</td>
<td>nC-26</td>
<td>0.143</td>
<td>24.087</td>
<td>39.739</td>
<td>249.737</td>
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<tr>
<td>nC-27 Heptacosane</td>
<td>nC-27</td>
<td>0.298</td>
<td>23.071</td>
<td>32.594</td>
<td>218.873</td>
</tr>
<tr>
<td>nC-28 Octacosane</td>
<td>nC-28</td>
<td>0.098</td>
<td>21.002</td>
<td>30.689</td>
<td>203.434</td>
</tr>
<tr>
<td>nC-29 Nonacosane</td>
<td>nC-29</td>
<td>0.327</td>
<td>20.072</td>
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<tr>
<td>nC-30 Triacosane</td>
<td>nC-30</td>
<td>0.086</td>
<td>19.406</td>
<td>25.086</td>
<td>193.176</td>
</tr>
<tr>
<td>nC-31 Henriacosane</td>
<td>nC-31</td>
<td>0.287</td>
<td>23.075</td>
<td>38.591</td>
<td>179.615</td>
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<tr>
<td>nC-32 Dotriacosane</td>
<td>nC-32</td>
<td>0.127</td>
<td>22.960</td>
<td>47.009</td>
<td>188.716</td>
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<tr>
<td>nC-33 Triatriacosane</td>
<td>nC-33</td>
<td>0.192</td>
<td>33.067</td>
<td>48.175</td>
<td>179.252</td>
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<tr>
<td>nC-34 Tetraatriacosane</td>
<td>nC-34</td>
<td>0.089</td>
<td>32.729</td>
<td>43.847</td>
<td>174.453</td>
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<tr>
<td>nC-35 Pentatriacosane</td>
<td>nC-35</td>
<td>0.172</td>
<td>40.425</td>
<td>58.316</td>
<td>187.350</td>
</tr>
</tbody>
</table>
Figure A2.1 A-B. (A) Total PAHs (tPAH) and (B) total $n$-alkanes in sediments in August, 2011 collected from North Bay Sansbois (NBS), Wilkinson Bayou (WB), and Grande Terre (GT).
Figure A2.2. Concentration of polycyclic aromatic hydrocarbons (PAHs) in sediments collected from an unoiled reference location in North Bay Sansbois in 2011 (NBS 2011), a lightly oiled location in Wilkinson Bay in 2011 (WB 2011), and from a heavily oiled location at Grande Terre Island in 2011 and 2010 (GT 2011 and GT 2010).
Figure A2.3. Concentration of \( n \)-alkanes \((C_{10} - C_{35})\), pristine (Pr) and phytane (Ph) in sediments collected from an unoiled reference location in North Bay Sansbois in 2011 (NBS 2011), a lightly oiled location in Wilkinson Bay in 2011 (WB 2011), and from a heavily oiled location at Grande Terre Island in 2011 and 2010 (GT 2011 and GT 2010).
Figure A2.4. Distribution of CYP1A protein (burgundy staining) in larval Gulf killifish at ≤ 24 h post hatch exposed throughout development to sediments collected from an unoiled reference location in North Bay Sansbois in 2011 (NBS 2011), from a mildly oiled location in Wilkinson Bay in 2011 (WB 2011), and a heavily oiled Grande Terre Island in 2011 and 2010 (GT 2011 and GT 2010). Refer to WB 2011 for anatomical reference (Reproduced from Figure 5A). CYP1A was mostly found localized in gill (G), intestine (I), head kidney (HK), liver (L), heart (H) endothelium, vascular endothelial cells (arrows), and in epithelial cells lining the buccopharyngeal cavity (asterisks) in fish exposed to oiled sediments (i.e. WB and GT sediments). Larvae exposed to all oiled sediments had CYP1A-positive vascular endothelial cells. Only larvae exposed to the heavily oiled GT sediments (GT 2011 and GT 2010) showed CYP1A protein in the external epithelia. Fish exposed to unoiled sediment from NBS (NBS 2011) showed staining for CYP1A in the heart endothelium and kidney tubules only. Larvae were sectioned at 4 µm. Each image is a montage of up to 24 images captured using a 20X objective. Images were individually sized to fit figure dimensions and do not reflect larval length. Scale bars approximately 100µm.
Figure A2.4.
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