**New Proposal:  COLLABORATIVE RESEARCH:  Variation in PAH metabolism in Gulf killifish (*Fundulus grandis*) and selection for PAH-resistant genotypes along the Gulf Coast.**

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Cluster: Understand

Approximate Budget: $160,000

**Significance:** The Cyp1a1 and Cyp1b1 proteins metabolize polycyclic aromatic hydrocarbons (≥ 4 ring PAHs). Evidence indicates that Cyp1 proteins are polymorphic in outbred populations and that underlying genetic differences are in part responsible for differences in metabolism and sensitivity to PAHs. Recent research suggests that genetic and non-genetic factors account for selection of PAH-resistant phenotypes in both the Atlantic tomcod (*Microgadus tomcod*) and the Atlantic killifish (*Fundulus heteroclitus*). In addition there appears to be a positive relationship between intraspecific sensitivity differences in these species, Cyp1 variants, and Cyp1 expression {Wirgin, 2004 #567;Yuan, 2001 #566;Wills, 2010 #556;Wills, 2009 #558}. PAHs in weathered crude oil are probable long-term contaminants in oiled Gulf coastal areas including estuaries. These PAHs also bioaccumulate in and are toxic to marine organisms {Meador, 1995 #564;Hylland, 2006 #565}. PAHs are potent inducers of Cyp1 activity in marine fishes, and Cyp1 induction is considered a sensitive biomarker of exposure to weathered crude oil {Lee, 2005 #562}. One key species that will encounter these PAHs is the Gulf killifish (*Fundulus grandis*). A recent study suggests that on average the Gulf killifish exhibits limited acute sensitivity to Alaska North Slope crude oil (ANSC) or dispersed ANSC oil, but for a small fraction of individuals, exposure is toxic {Liu, 2006 #563}. This indicates that there may be underlying genetic susceptibility factors influencing such acute sensitivity such as polymorphisms in Cyp1 proteins. Furthermore, the hydrocarbon content, biochemical and physical processing, and the sheer volume of oil resulting from the Gulf oil leak creates a substantially different situation than the spill in the Prince William Sound. We propose to establish time-sensitive population genetic information for *Cyp1a1* and *Cyp1b1* in the Gulf killifish. This data is critical to our hypothesis that long-term exposure to crude oil PAHs in Gulf coastal estuaries provides a selective gradient favoring PAH-resistant phenotypes in the Gulf killifish. Selection on existing genetic variants encoding the polymorphic enzymes affording resistance necessitates collection of such baseline data. We propose to conduct controlled exposures to characterize the biological mechanisms underlying resistance, sensitivity, and the selective potency of long-term, multigenerational exposure to crude oil PAHs in the Gulf killifish. We will initially collect samples from 1 site in southeastern LA (Barataria Bay) and 1 site in MS (Biloxi Bay). This research will set the foundation for continued study of genetic changes and selective conditions resulting from the Gulf oil leak in the Gulf killifish.

**Objectives:**

* Determine the polymorphic nature of the expressed *Cyp1a1* and *Cyp1b1* genes in coastal populations of *F. grandis*. Establish the current genetic composition of selected populations in these two genes impacted by the Gulf oil leak.
* Conduct controlled laboratory exposures to oil to characterize variation in Cyp1 induction and activity in the Gulf killifish. Examine EROD induction (benzo[a]pyrene, beta napthoflavone as positive controls) and hepatic genotoxicity and characterize varation in exposure and effect biomarkers for future testing of gene-environment interactions.

**Experimental Design:**

*Aim 1:*

Gulf killifish collected from 2 locations will be placed into aerated buckets and returned to the lab. The 2 locations that will serve as collection sites are Barataria Bay in southeastern Louisiana, and Biloxi Bay in Mississippi. We will collect a minimum of 1000 fish from each site. Two hundred randomly selected fish from each site will be used for sequence analysis and identification of polymorphisms in the *Cyp1a1* and *Cyp1b1* genes. This number is sufficient for detecting polymorphisms at frequencies ≥ 5% and possibly ≥ 2.5%. Considerable molecular data is available on Cyp1a1 and Cyp1b1 for the closely related species *F*. *heteroclitus* {Morrison, 1998 #560;Powell, 2004 #559;Wills, 2010 #556;Wills, 2009 #558;Zanette, 2009 #557}. Degenerate primer sets for both *Cyp1a1* and *Cyp1b1* along with a set of published primers (Cyp1a) designed from closely related and distantly related teleosts will be used to amplify by the PCR full length amplicons representing entire transcripts of *Cyp1a1* and *Cyp1b1*. Amplicons will be purified and DNA sequences for each expressed gene will be generated using a Beckman Coulter CEQ8000. The BLAST program will be used to identify homologs to *Cyp1a1* and *Cyp1b1* obtained from the Gulf killifish to ensure proper gene targeting. Clustal X will be used to align DNA sequences and amino acid sequences to identify polymorphisms in the sampled populations. Prosite will be used to identify functional motifs in proximity to identified polymorphisms. The programs Polyphen and SIFT will be used to predict impacts to the encoded protein that polymorphisms are likely to have functional impacts on the encoded proteins. This will facilitate the development of polymorphism-specific genotyping methods and help to prioritize the testing of functional differences in controlled exposure experiments.

*Aim 2: Variation in Cyp1 activity in Gulf killifish exposed to dispersed oil.*

To assess induction of Cyp1a and Cyp1b-like proteins and bioactivation of PAHs for polymorphic variants, we will expose Gulf killifish collected from the two sites to dispersed oil under controlled laboratory settings. Each exposure will consist of Control (no exposure), crude oil (concentration to be determined), and a positive control for cyp induction (benzo[a]pyrene, 10 ug/L) for 96 hours. At the conclusion of the exposure, all fish will be removed from each tank, euthanized and livers excised and flash frozen in liquid nitrogen. A subsample of liver tissue will be removed for concurrent genotoxicity assessment (Aim 3). Cyp1 activity will be quantified using the well-established ethoxyresorufin-o-dethylase (EROD) assay. The remaining tissues will be flash frozen and archived at -80°C in anticipation of polymorphism-specific genotyping planned for assessing activity-genotype interactions.

*Aim 3: Variation in hepatic genotoxicity in Gulf killifish exposed to dispersed oil*

The alkaline Comet assay will be conducted using liver tissue from fish that are concurrently exposed to dispersed oil and controls as in Aim 2. Liver tissue will be harvested immediately following 28 days of exposure, flash frozen in liquid nitrogen, and archived at -80°C prior to analysis. Liver tissue will be homogenized in cold phosphate-buffered saline without magnesium or calcium to yield a suspension of single cells. We will use materials and the procedure described by Trevigen Inc. (Gaithersburg, MD). DNA damage will be assessed using fluorescence microscropy and automated image analysis. Tail moment, olive moment, and tail length will be used to characterize DNA damage. A minimum of 100 nuclei will be examined per sample. The remaining tissues will be flash frozen and archived at -80°C in anticipation of polymorphism-specific genotyping planned for assessing DNA damage-genotype interactions.

**Expected Results:** The results of the experiments outlined in this proposal will determine 1) the polymorphic nature of the *Cyp1a1* and *Cyp1b1* genes in wild Gulf killifish along the Gulf of Mexico coastline, 2) variation in Cyp1 activity in Gulf killifish exposed to dispersed oil, 3) variation in hepatic genotoxicity in Gulf killifish exposed to dispersed oil, and 4) banked tissues for developing polymorphism-specific genotyping assays and specifically defining the interaction between genetic polymorphisms, Cyp1 activity, and hepatic genotoxicity.