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Multigeneration Effects of Chronic Exposure to Polycyclic Aromatic Hydrocarbons in Gulf Killifish (Fundulus grandis)

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MULTIGENERATION EFFECTS OF CHRONIC EXPOSURE TO POLYCYCLIC AROMATIC HYDROCARBONS IN GULF KILLIFISH
(Fundulus grandis)

A Thesis

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 Master of Science

in

The School of Renewable Natural Resources

by

Andrea Yammine
B.S., University of North Carolina Wilmington, 2015
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ...................................................................................................................... ii

LIST OF TABLES....................................................................................................................................... v

LIST OF FIGURES................................................................................................................................. vi

ABSTRACT................................................................................................................................................ viii

CHAPTER 1: INTRODUCTION .................................................................................................................. 1

CHAPTER 2: USE OF SURFACE MUCUS AS A BIOMARKER FOR ENDOCRINE DISRUPTION IN GULF KILLIFISH ....................................................................................................................... 5
  2.1 Introduction........................................................................................................................................ 5
  2.2 Materials and Methods ...................................................................................................................... 9
    2.2.1 Validation..................................................................................................................................... 9
    2.2.2 Laboratory WAF exposure ....................................................................................................... 11
    2.2.3 Statistical analysis ..................................................................................................................... 13
  2.3 Results............................................................................................................................................... 14
    2.3.1 Validation..................................................................................................................................... 14
    2.3.2 Laboratory WAF exposure ....................................................................................................... 16
  2.4 Discussion.......................................................................................................................................... 19

CHAPTER 3: MULTIGENERATION EFFECTS OF THE POLYCYCLIC AROMATIC HYDROCARBON NAPHTHALENE ON GULF KILLIFISH Fundulus grandis ........................................................................ 25
  3.1 Introduction....................................................................................................................................... 25
  3.2 Materials and Methods .................................................................................................................... 30
    3.2.1 Parental (F₀) collection and production of F₁ generation .......................................................... 30
    3.2.2 F₁ generation WAF exposure .................................................................................................. 31
    3.2.3 F₁ generation reproductive parameters from WAF exposure ................................................. 32
    3.2.4 F₂ generation larval health ..................................................................................................... 33
    3.2.5 F₂ generation rearing .............................................................................................................. 34
    3.2.6 F₂ generation naphthalene exposure ....................................................................................... 34
    3.2.7 Biomarkers of naphthalene exposure in F₂ generation ............................................................ 35
    3.2.8 Statistical analysis ................................................................................................................... 37
  3.3 Results............................................................................................................................................... 38
    3.3.1 F₁ generation WAF exposure and reproduction ..................................................................... 38
    3.3.2 F₂ generation larval health ..................................................................................................... 42
    3.3.3 F₂ generation naphthalene exposure ....................................................................................... 43
LIST OF TABLES

Table 3.1: Mean (+SEM) number of total embryos collected, and mean (+SEM) number of hatched and unhatched embryos in control and exposed groups (n= 8 clutches). No significant difference was found between the WAF and control groups (Hatched $p = 0.14$; unhatched $p = 0.13$).
LIST OF FIGURES

Figure 2.1: Linear regression describing the relationship between (A) 11-ketotestosterone (11-KT) concentrations in plasma (pg/ml) and mucus (pg/ml) samples of n = 17 male Gulf killfish, Fundulus grandis, and (B) Vitellogenin (VTG) concentrations in plasma (pg/ml) and mucus (pg/µg of protein) samples of n = 13 female Gulf killfish, Fundulus grandis. Both relationships are significant (p < 0.05)………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………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Figure 3.7: Optical density (OD) of lysozyme in mucus of the parental exposed, and parental control, across naphthalene exposure (40 days) and post-naphthalene exposure (40 days). These two groups were compared to one another and the linear relation of time × treatment × parental-history was significant ($p < 0.05$)
ABSTRACT

The goal of this study was to examine potential adaptation within two generations of Gulf killifish exposed to polycyclic aromatic hydrocarbons (PAH) while also investigating the use of mucus as an effective and non-lethal alternative to determine concentrations of 11-ketotestosterone (11-KT) and vitellogenin (VTG) of *F. grandis*. Multiple biomarkers were used to assess adaptation within these fish, such as lysozyme (enzyme critical in innate immunity), and 11-ketotestosterone and vitellogenin (endocrine hormones often affected by PAHs).

Mucal concentrations of 11-KT and VTG were determined and evaluated for effectiveness through comparative assays with blood plasma. This method was then applied to evaluate potential endocrine disruptions in *F. grandis* undergoing chronic exposure to a water accommodated fraction (WAF) of crude oil. Both 11-KT and VTG showed significant positive relationships between their respective concentrations in the plasma and mucus. Four populations of *F. grandis* with different exposure histories were exposed to WAF for 60 days, and followed 40 additional days after WAF treatments. Concentrations of 11-KT in mucus decreased throughout the 40 day WAF exposure period when compared to responses within control treatment males. Exposure history of the populations also had a significant effect on observed 11-KT concentrations in mucus; exposed Gangs Bayou males (reference site) had a decrease in 11-KT concentrations, while concentrations of 11-KT of exposed males from Vince Bayou (superfund site) increased. Exposed females showed near null concentrations of mucosal VTG when exposed to WAF regardless of exposure history while control groups did not exhibit decreases in mucus VTG concentrations.
The second portion of this thesis details the exposures of two successive generations of Gulf killifish first to WAF and the second to naphthalene. Sperm motility and oocyte cell wall thickness was significantly lower in adults exposed to WAF, while egg viability and survival activity index were not. For the resulting progeny, lysozyme assessed within the surface mucus of Gulf killifish with naphthalene exposure significantly decreasing the relative concentration of lysozyme regardless of parental history. Changes in concentrations of lysozyme were also significant with the linear relationship of time, treatment and parental history with individuals from exposed parents showing overall higher concentrations than individuals from control parents. EROD induction was significantly higher in fish exposed to naphthalene regardless of parental history. Results show potential alteration to PAH metabolism and immunology within two generations in Gulf killifish.
CHAPTER 1: INTRODUCTION

On April 20, 2010, the Deepwater Horizon oil platform exploded and released over 80 million liters of Macondo 252 (MC 252) crude oil into the Gulf of Mexico. Sublethal effects of the Deepwater Horizon oil spill in the Gulf of Mexico have been studied extensively since then; however, potential multigenerational, developmental, and endocrine effects following exposure to polycyclic aromatic hydrocarbons (PAH) are poorly understood and in need of further exploration. Additionally, population-level differences in sensitivities throughout the oiled range following this event have not been widely examined. Population-based data is vital when assessing impacts of environmental disasters affecting a large geographic range due to potential of localized health and reproductive effects to future generations.

This study focused on Gulf killifish (*Fundulus grandis*) as an appropriate model, because they are native residents of the exposed estuaries, have high site fidelity, and are dependent on marsh grasses for spawning (Lotrich et al., 1975; Greely et al., 1983). Gulf killifish are amenable to laboratory and field studies, and are preferred for testing chemical stressors due to their ability to tolerate a wide range of environmental conditions (Burnett et al., 2007). Chemical effects on these relatively tolerant species could translate to similar or more severe effects in less tolerant species. These fish are also established models for human development (Burnett et al., 2007) and share common mechanisms of oil toxicity with all vertebrates making it possible to establish links with long-term effects and chronic oil exposure.

Oil exposure has led to negative physiological impacts in fish in both the laboratory and in the wild (Bender et al., 2016; Mager et al., 2014). When considering the sublethal effects of oil, it is important to address the issue of reproductive success which is essential to maintaining healthy population levels. Modeling the reproductive and survival impacts from an initial
sublethal toxicant exposure on a Chinnok salmon population has demonstrated that even a 10% decrease in reduction in spawning success can lead to an approximate 40% decrease in population abundance over a 20 year period (Spromberg and Meador in 2005). Negative effects on teleost reproduction have resulted from changes in steroidogenic enzyme expression and hormone concentrations by destabilizing the hypothalamo-pituitary-gonad (HPG) axis. Chronic exposure to PAHs is known to disrupt endocrine function, and potential disruptions in the concentrations of steroids such as 17β-estradiol and testosterone could inhibit normal gonadal and gamete development (Bugel et al., 2011).

Reproductive steroid production is initiated by peptide hormones, stimulating the pituitary gland to produce gonadotropin hormones. These pituitary hormones then initiate a cascade of events leading to the conversion of cholesterol into steroids like testosterone and estradiol which act to facilitate gonad maturation and investment (Booc et al., 2014). Cytochrome P450 aromatase is the key steroidogenic enzyme responsible for conversion of androgens to estrogens. Often times, endocrine disrupting chemicals mimic androgens and estrogens, causing a disruption in normal enzyme function (Patel et al. 2006). Most species show relatively low levels of CYP1 constitutively expressed, and can be easily induced by exposure to naphthalene and other PAHs. In fish, the exposure causes time and dose dependent induction of EROD, CYP1A1 mRNA, biliary BaP metabolites, and DNA adduct formation (Willett et al., 1995).

Crude oil is partitioned into different fractions in the marine environment. The water accommodated fraction (WAF) is the portion of crude oil that is soluble in water after an allotted mixing period. WAF has the highest concentration of dissolved petroleum hydrocarbons (Faksness et al., 2015) and consequently, WAF contains the most acutely toxic substances in oil
due to its increased bioavailability to marine organisms (Neff, 1981). WAF is made up of low molecular weight aliphatic compounds, aromatic hydrocarbons and polycyclic aromatic compounds (PAH). PAH exposure is acutely toxic and has been known to cause increased embryo mortality, alterations in time to hatch, growth reduction, spinal deformities, and yolk sac edema in many different larval teleosts (Carls and Thedinga, 2010; Barron et al., 2004; Hawkins et al., 2002). PAH exposure has also resulted in disruptions to endocrine functions, and this thesis will evaluate potential endocrine disruption in relation to WAF exposure period, as well as in relationship of exposure history of populations from historically oiled sites and populations from oil-free sites in Texas and Louisiana and the sensitivity and tolerance to WAF exposure. This comparative study begins to demonstrate if genetic variation among populations contributes to their endocrine function in the context of WAF exposure, thus potentially affecting reproductive success.

Multigenerational studies can characterize deleterious impacts at an ecosystem level, while single generation studies reflect the only the organismal level (Corrales et al., 2015). Corrales et al. (2015) found that parental dietary exposure to benzo(a)pyrene (BaP) in zebrafish (Danio rerio) led to deformities in offspring and observations of this adverse effect persisted across generations. The authors established multigenerational phenotypes, allowing future studies to focus and test the molecular pathways altered by multigenerational exposure to PAHs. Populations chronically exposed to stressors in the environment may have the ability to adapt to those stressors (Meyer and DiGuilio, 2003). In fact, a population of mummichog (Fundulus heteroclitus) in an acutely toxic site has been found to be reproductively successful with offspring appearing to be more resistant in acute toxicity tests and the developmental abnormalities less frequent compared when compared to previous generations (Wills et al.,
The killifish at this site also exhibited chronic effects of creosote contaminated sediment exposure but were relatively resistant to its acute effects (Bacanskas et al., 2004). These fish had reduced levels of EROD and CYP1A1 induction, and there was evidence that the fish stored the BaP in adipose tissue instead of bio transforming the chemical potentially illustrating a relationship between exposure history and the ability to biotransform BaP. They also found that the fish with an exposure history metabolized BaP slower, and these fish appear to have developed protection from carcinogenesis through the reduction in PAH elimination.

The overall objective of this study is to assess the potential transgenerational effects of chronic exposure to PAHs within the scope of reproductive success in adult killifish. The specific objectives of this work are: (1) validate the use of mucal swabs as an effective and non-lethal alternative to measure 11-KT and VTG levels by comparing concentrations in mucus and blood plasma; (2) use mucal swabbing as a minimally invasive method to evaluate endocrine disruptions in killifish undergoing chronic exposure to crude oil. Finally, (3) assess reproductive and biochemical markers from two generations of Gulf killifish exposed to PAHs.
CHAPTER 2: USE OF SURFACE MUCUS AS A BIOMARKER FOR ENDOCRINE DISRUPTION IN GULF KILLIFISH

2.1 Introduction

Biologically based tools such as histopathology, bioassays, and morphology are increasingly used to complement environmental toxicology monitoring efforts. Biomarkers define organismal effects of potential toxicants in addition to quantifying exposure histories (Bartell 1990). For example, the use of vitellogenin as a biomarker within the aquatic environment has yielded insights into the mechanism of endocrine disruption from persistent compounds such as dioxins, polycyclic aromatic hydrocarbons (PAH), and halogenated aromatic hydrocarbons (Rice and Yang, 2000; Bugel et. al, 2011). There are a variety of other biomarkers that allow investigators to examine reproductive, biotransformative, and neurological endpoints using conventional laboratory methods as well as sample collections and evaluations relying on histology, blood and tissues such as gills, brain, and liver to determine various effects of potential pollutants (Ownby et. al, 2002; De la Terre et. al, 2002; Kennedy and Smyth, 2015; Feist et. al, 2015). Although biological materials such as blood and tissues are extremely informative in toxicological assessments, their collection is often lethal for study organisms prompting non-lethal collection alternatives to progressively become more common.

Lethal sampling of test organisms can be particularly difficult or impossible when working with rare or limited populations, or repeated sampling of individuals over time. Sacrificing endangered fish such as the razorback sucker (Xyrauchen texanus) is not desirable and in most cases not permitted, and alternative non-lethal sampling methods were created. A muscle biopsy via a ‘plug’ using a biopsy punch allowed for the determination of selenium concentrations in muscle (Waddell and May, 1994). Caudal fin clips and dorsolateral scales have
been used to compare muscle tissue mercury concentrations among marine fish (Piraino and Taylor, 2013); a minimally invasive gill biopsy method has been demonstrated as able to measure Na⁺, K⁺ -ATPase activity in salmonids (McCormick, 1993). Similarly, the collection of surface mucus offers a less invasive biomarker with the potential to assess a number of biological functions within the fish (Shephard, 1994; Schultz et al, 2005).

Measurable components in mucus are linked to homeostatic processes because mucus is vital for respiration, excretion, protection as well as disease and pathogen resistance (Shephard, 1994; Beck and Peatman, 2015). Mucus has also been linked to early stress conditions in fishes. Free hemoglobin was detected in skin mucus samples after being exposed to a handling stressor for 3 to 4 minutes (Smith and Ramos, 1976). This non-lethal technique allowed for the detection of early stress conditions permitting culturists to avoid stressful situations that encourage the development of diseases (Smith and Ramos, 1976). There is also evidence that the biochemistry of mucus correlates, in part, with that of the blood. A correlation between 11-ketotestosterone concentrations in blood and mucus was established while looking at temporal patterns in koi (Cirinus carpio) to assess reproductive investment throughout the spawning season (Schultz et al., 2007). A significant correlation between blood plasma and mucus concentrations of vitellogenin was also established in White Bass (Morone chrysops) during their annual spawning season (Barkowski and Haukenes, 2014). This allowed for the use of mucus to correctly identify the sex of this fish 90% of the time (Barkowski and Haukenes, 2014). These studies demonstrated that endocrine health and reproductive investment can be assessed using assays for compounds such as 11-ketotestosterone (11-KT) and vitellogenin (VTG) using surface mucus as a minimally invasive alternative to plasma. Detection of metabolites within mucus has allowed for the formation of a fish mucus metabolome for the fathead minnow (Pimephales promelas).
which indicated 72 out of 204 metabolites were significantly linked to sex (Ekman et. al, 2015). This liquid chromatography–mass spectrometry method was also able to detect significant changes in the mucus metabolome from sublethal exposures to bisphenol A within laboratory conditions. The continued collection mucus from the same individuals within a sample site or a controlled laboratory setting could increase connectivity to individual-specific endocrine responses via repeated sampling.

The HPG axis is responsible for regulating activities leading to successful reproduction and endocrine disrupting chemicals (EDCs) can destabilize the HPG axis of organisms residing in aquatic habitats. Within male teleost fishes, 11-KT is a major sex steroid responsible for spermatozoa development, with concentrations peaking at the height of the spawning season (Schultz et. al, 2007). Synthesis of this androgen is initiated by the release of gonadotropins which stimulate the production of testosterone in the testis. Within females, VTG is a phospholipoglycoprotein and represents a precursor of egg yolk produced by hepatocytes and regulated by estrogen (Nicolas, 1998). The actions of a variety of EDCs have been characterized. For example, chronic naphthalene exposures of 0.5 to 1ppm resulted in a decline of plasma concentrations of estradiol and testosterone within females as well as decreased the responsiveness of ovarian tissue to in vitro hormonal stimulation (Thomas and Budiantara, 1995). Another example is the administration of a single intraperitoneal injection of benzo[a]pyrene on rainbow trout (Oncorhynchus mykiss) resulting in a severe decline of circulating estradiol and testosterone concentrations (Kennedy and Smyth, 2015) blocking the release of estradiol and testosterone in fish treated with gonadotropin releasing hormone. It is assumed that the estrogenic nature of PAHs result in their binding to estrogen receptors acting as an antagonist by inhibiting the action of estrogens or potentially serving as agonists by producing
a downstream action at the receptor site (Sumpter and Jobling, 1995; Arukwe et al., 1997; Thomas et al., 2009). Many studies have shown that the activation of the P450 enzyme corresponds with changes in reproductive success of fish; the anti-estrogenic actions of P450-inducing compounds disrupt vitellogenesis and egg production (Thomas, 1990; Nicolas, 1999; Meucci and Arukwe, 2006). Induction of the CYP1A/P450 enzyme is also believed to contribute to lower circulating estradiol and testosterone concentrations due to enhanced clearance since the same enzyme is responsible for estradiol elimination and biotransformation (Spink et al., 1992; Hammond et al., 1997).

Gulf killifish (*Fundulus grandis*) are native residents of coastal estuaries in the Gulf of Mexico, have high site fidelity, and are dependent on marsh grasses for spawning (Lotrich et al., 1975; Greely et al., 1983). *Fundulus* has been a significant historical and biological model complex. Research on this genus has increased fundamental knowledge on the mechanisms used to adapt to environmental changes leading to a better understanding on selected integrated systems such as reproduction and responses to toxicants (Burnett et al., 2007). Gulf killifish are amenable to laboratory and field studies. *Fundulus spp.* were recognized early as a useful laboratory and representative ecological model due to their ecological importance and their well-characterized and observable embryonic development (Eisler, 1986; Taylor, 1999). The sensitivity of *Fundulus* to toxins has allowed demonstration of mechanisms responsible for reproductive effects and recovery of reproductive function following toxicant exposure (Rice and Xiang, 2000; Ownby et al., 2002; Brown et al., 2011; Oziolor et al., 2014). Gulf killifish and its sister species, the Mummichog (*Fundulus heteroclitus*), are sexually dimorphic and fractional spawners with a protracted spawning season lasting up to 8 months (Taylor, 1999; Brown et al., 2011). *Fundulus grandis* spawning corresponds with lunar phases, whereby egg production
peaks occur between full and new moon phases, a period associated with continual egg production (Green et al., 2010). Reproductive hormones and PAH metabolism are intrinsically linked and would be expected to alter the reproductive output within this continually spawning estuarine fish (Nicolas, 1999).

Populations of organisms exposed chronically to stressors in the environment may have the ability to adapt to those stressors. Recent studies have found naturally occurring ‘resistant populations’ of fish, potentially identified as pollution-driven population adaptation via multiple generations of chronic exposure (Ownby et al., 2002; Oziolor et al., 2014). When exposed to toxic compounds such as PAHs and PCBs Atlantic killifish (Wills et al., 2010) and Gulf killifish (Oziolor et al., 2016) have developed resistance to the otherwise toxic levels of such contaminants, and developed alternate traits in order to minimize the negative impact these compounds had on their reproduction (Oziolor et al., 2016; Bickman et al., 2011).

This study was designed to: (1) validate and confirm the use of mucal swabs as an effective and minimally invasive alternative to blood sampling by comparing mucal concentrations of 11-KT and VTG from *F. grandis* to that observed in blood plasma; (2) use this minimally invasive method to evaluate potential endocrine disruption in *F. grandis* under chronic exposure to a water accommodated fraction (WAF) of crude oil; and (3) assess potential differences in endocrine disruption among populations with different exposure histories.

### 2.2 Materials and Methods

#### 2.2.1 Validation

*Fish collection and holding:* Fish originated from a captive population housed at the Aquaculture Research Station, Louisiana State University Agricultural Center (Baton Rouge,
LA). The source population was originally collected near Golden Meadow, LA prior to the Deepwater Horizon (DWH) oil spill. Fish were held in recirculating systems supplied with water at 20°C and salinity of 12 ‰.

**Mucus and plasma sampling:** The methodology developed for mucus collection and processing was described in Schultz et al. (2005). Individual fish were collected from tanks and immediately swabbed on both sides using a 1 cm² section of polyurethane sponge (Buf-Puf gentle facial sponge, 3M, St. Paul, MN). Swabbing consisted of three non-overlapping and unidirectional anterior to posterior motions on each lateral side beginning posterior of the operculum and terminating anterior to the caudal peduncle. The sponge was then placed in a 3 ml syringe. Ice-cold 20 mM Tris-buffered saline and Halt™ protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL) was added to the cylinder. The sponge was compressed in the barrel of the syringe with a plunger and fluid passed through an 18-gauge needle and collected in a 1.5 ml centrifuge tube. Fish were euthanized with tricaine methanesulfonate (Tricaine-S, Western Chemical, Inc., Ferndale, WA). The caudal peduncle was severed and blood collected via a heparinized capillary tube and placed in a heparinized microcentrifuge tube, centrifuged at 4°C (×16,000g for 15 minutes) and the plasma transferred into microcentrifuge tubes. Plasma and mucus samples were immediately stored at -80°C prior to use. Protein concentration was determined via a Bradford-Coomasie assay (Pierce Biotechnology, Rockford, IL) and the samples diluted to a target protein concentration of 40 µg/ml for both mucus and plasma. Samples from 21 males and 8 females were used to assess 11-KT concentration (11-Keto Testosterone EIA kit, Cayman Chemical, Ann Arbor, MI) while 19 females and 8 males were used to assess VTG (Semi-quantitative biomarkers Vitellogenin, Biosense Laboratories AS, Norway). All samples were analyzed in triplicate. Interplate variation was determined by
analyzing the same sample in triplicate on 3 to 4 plates. Intraplate variation was determined by testing one sample, two times in triplicate on the same plate. The standard deviation surrounding the mean concentrations recorded for intraplate and interplate variation sample was used to calculate a coefficient of variation for each type of sample.

To confirm the efficacy of our antibody, and create aliquots of a VTG standard for the VTG ELISA assay, 10 males were injected with 5 mg/kg 17α-ethynlestradiol (Sigma-Aldrich, dosage of 5mg/kg). After 2 injections approximately 72 hours apart and an additional 24 hour waiting period; plasma and mucus were collected as previously described. Plasma samples were sent on dry ice to the University of Florida, Center for Environmental and Human Toxicology for VTG isolation and quantification via methods described in Folmar et al. (2000).

2.2.2 Laboratory WAF exposure

*Fish collection and holding:* Three different Gulf killifish populations were collected from sites that have been historically contaminated, as well as historically ‘clean’ or ‘reference’ sites. The contaminated site included Vince Bayou, TX (29°43’10” N; 95°13’ 13” W) (U.S. Superfund site with a long history of organic contamination) (Dubansky et al., 2013; Oziolor et al., 2014). The reference sites included Gangs Bayou, TX (29° 15’ 30.4” N, 94° 54’ 45.9” W) and individuals from a captive population at the Aquaculture Research Station, LSU Agricultural Center. Each population was maintained in quadruplicate tanks, each tank housing 6 or 9 females and 3 or 5 males, respectively, to maintain a stocking density of 2 females to every male.

Twenty-four 60-L tanks were used for the exposure portion of this study, 12 tanks for WAF treatment and 12 tanks for control treatments.

Siporax biofilter media were submerged in MC-252 surrogate crude oil for 48 hours for the WAF treatment and left clean for the control treatment. The media were then transferred to a
10 cm x 25 cm PTFE (polytetrafluorethylene) lined polyvinyl chloride chamber within one of two upwelling systems adapted from previous studies (Carls, 2000; Kennedy, 2005). These upwellers generated treatment water by continuously pumping water though a system of piping, across the beads and into a 1000 L PTFE-lined fiberglass tank. This provided the constant mixing needed to solubilize a portion of the crude oil. After a 24 hour mixing period in an upwelling system, WAF and control water was drawn off from the bottom of the systems into separate 40-L tanks. Drawing water from the bottom of the upwellers prevents the oil slick at the surface of the WAF upwell from contaminating WAF water and other surfaces that would come in direct contact with the fish. Every four days 50% of the water in the fish exposure tanks was removed, and 30 L of WAF or control water was added accordingly to each of the 24 tanks.

Water samples were collected at days 4, 20, 32, 36, and 60 and from a representative WAF and control tank for PAH analysis. Samples were collected within one hour after the addition of treatment or control water from the upwellers to tanks with fish. Seventy-five PAHs included associated alkylated homologues were extracted and analyzed from each 1 L sample of treatment water by ALS Environmental (Kelso, WA, USA) with U.S. Environmental Protection Agency methods 3510C and 8270D, respectively (EPA, 1996; EPA, 1998). Method detection limits were < 0.03 µg/L. Results of the chemical analyses were used to create “target PAH” (TPH) and is the sum of all 75 analytes.

During the exposure period, mucus was collected from 6 females and 4 males from each population and exposure group as previously described. Sampling occurred on day 20, 40 and 60 during WAF exposures. Swabs were also taken 80 and 100 days after the start of the exposures and considered a recovery period since WAF exposures were terminated at 60 days. Plasma was collected from 5 females in each population and treatment group on day 60. Mucus was then
analyzed for 11-KT and VTG concentrations using enzyme-linked immunosorbent assay kits (11-Keto Testosterone EIA kit, Cayman Chemical, Ann Arbor, MI; Semi-quantitative biomarkers Vitellogenin, Biosense Laboratories AS, Norway).

2.2.3 Statistical analysis

A linear regression was performed to characterize the relationship between plasma and mucus concentrations for both 11-KT and VTG for the initial validation portion. Mucal samples were normalized by protein content to correct for potential differences in mucosal content due to swabbing techniques or fish size and sex for both the validation and WAF laboratory exposure studies. In the laboratory WAF exposure study, a general linear model with a mixed model analysis of covariance was performed for the curved-linear relationship of treatment, population and time for 11-KT mucus concentrations. The general linear model for female VTG mucus concentrations during the laboratory WAF exposures did not meet model assumptions of linearity and normality, due to multiple zero values within the data set. Therefore, for females, a generalized linear mixed model with a negative binomial probability distribution and log link transformation was used for VTG mucus concentrations. Source of fish was treated as a random effect in this model to enhance the precision of estimation. Finally, a zero-inflation generalized linear model was also used to account for high number of zero values due to treatment for the analysis of VTG concentrations in plasma. Statistical analyses were performed using Statistical Analysis Software, Version 9.4 of the SAS System for Windows (SAS Institute Inc., Cary, NC). A threshold of significance was set at $\alpha = 0.05$. 
2.3 Results

2.3.1 Validation

A significant positive linear relationship between 11-KT concentrations in plasma and mucus samples was detected for males ($R^2 = 0.27$, $n = 17$, $p < 0.05$; Fig. 2.1A). Concentrations of 11-KT ranged from 194.5 to 8703 pg/ml in plasma and from 97 to 822 pg/ml in mucus samples.

A significant positive linear relationship between VTG concentrations in plasma and mucus samples was detected for females ($R^2 = 0.46$, $n = 13$, $p < 0.05$; Figure 2.1B). Concentrations of VTG ranged from 3.84 to 14.28 pg/ml in plasma and from 0.003 to 0.006 pg/μg of protein in mucus samples. Detectable amounts of VTG were measured in plasma and mucus of males injected with estradiol. Concentrations of VTG for these males ranged from 16.87 and 129.5 pg/ml in plasma and from 0.0031 and 0.0038 pg/μg of protein in mucus samples.

The coefficient of variation (CV) for mucus samples was 29.5% ($n = 4$) for interplate and 14.3% ($n = 4$) for intraplate variation for 11-KT. The CV for mucus samples was 29.26% ($n = 3$) for interplate and 18.73% ($n = 3$) for intraplate variation for VTG. Plasma CV calculations for 11-KT and VTG were not able to be determined due to a low volume of individual samples.
Figure 2.1: Linear regression describing the relationship between (A) 11-ketotestosterone (11-KT) concentrations in plasma (pg/ml) and mucus (pg/ml) samples of n = 17 male Gulf killifish, *Fundulus grandis*, and (B) Vitellogenin (VTG) concentrations in plasma (pg/ml) and mucus (pg/µg of protein) samples of n = 13 female Gulf killifish, *Fundulus grandis*. Both relationships are significant (p < 0.05).
2.3.2 Laboratory WAF exposure

Gulf killifish exposed to WAF had a geometric mean TPH concentration of 151 µg/L (106.2 -223.6) throughout the 60 day exposure period. Mean TPH values for the control tanks were 2.5 µg/L (2.0 -3.29). Mucal concentrations of 11-KT in control treated adult males decreased statistically significantly throughout time ($p < 0.05$), while WAF treated reference and superfund populations displayed patterns over time. For WAF treated adult males, the linear relationship of the interaction time × treatment and time × treatment × population for Vince Bayou (superfund site) and Gangs Bayou (reference site) populations were statistically significant with respect to their linear interaction of time × treatment ($p < 0.05$) where that the effect of the treatment over time of the experiment differed between the populations with WAF exposed males of Vince Bayou (superfund site) expressing higher concentrations of mucosal 11-KT and over time the difference diminished. Exposure Gangs Bayou males (reference site) had a statistically significant decrease in mucosal 11-KT concentrations when compared to mucosal 11-KT concentrations of exposed males from contaminated sites but were not significantly different from their respective control treatments (Fig. 2.2) ($p < 0.05$). WAF exposed females showed near null concentrations of mucosal VTG regardless of exposure history (Fig. 2.3).

Among females, changes in concentrations of VTG were significant with the linear relationship of time, treatment, and population (each main effect $p < 0.05$) where over time, female mucosal VTG concentration decreased. Between treatments, exposed females had decreased mucosal VTG concentrations. Between populations, females of Vince Bayou (superfund site) expressed lower concentrations of mucosal VTG, however, unlike males, there was no interaction between the effect of time, treatment, or population. Mortality for individuals in the control group was 0%, while mortality of individuals in WAF conditions regardless of population was 7%
throughout the exposure and recovery period. Analyses of day 60 plasma from control and WAF exposed females from reference sites, Gangs Bayou (GB), and Aquaculture Research Station (ARS) and superfund site, Vince Bayou (VB) showed that control fish had statistically significant more circulating vitellogenin than WAF females (Fig. 2.4) ($F = 7.8, p < 0.05$).

Figure 2.2: Concentrations of 11-ketotestosterone (11-KT) in mucus of males of the superfund site (Vince Bayou) and reference site (Gangs Bayou) throughout WAF exposure (60 days) and non-WAF exposure (40 days). These two populations were compared to one another and the linear relation of time x treatment x population was significant ($p < 0.05$).
Figure 2.3: Concentrations of vitellogenin (VTG) in mucus of females of the superfund site (Vince Bayou), as well the reference site (Gangs Bayou) throughout WAF exposure (60 days) and non-WAF exposure (40 days). Changes in concentrations of VTG were significant with the linear relationship of time, treatment and population ($p < 0.05$).
Figure 2.4: Mean (±SE) plasma vitellogenin (VTG) in pg/ml from WAF exposed and Control females of Gulf killifish from WAF exposure day 60 (n = 5) representing populations from Gangs Bayou (GB), Vince Bayou (VB) and Aquaculture Research Station (ARS). Statistical difference was determined between the two treatment groups (p < 0.05)

2.4 Discussion

Surface mucus concentrations of VTG and 11-KT within this study align in a linear relationship with plasma values and demonstrate the potential utility of this biomarker. Mucus and plasma concentrations of VTG have been measured in several other species including Shorthead Redhorse *Moxostoma macrolepidotum*, the endangered Copper Redhorse *M. hubbsi* (Maltais and Roy, 2009), Atlantic Salmon *Salmo salar* (Meucci and Arukwe, 2005), and *Cichlasoma dimerus* (Moncaut et al. 2003) but many of these investigations did not produce direct linkages between VTG concentrations in these biofuilids. The relationship between VTG concentrations in mucus and plasma samples in the current study was higher than the relationship between VTG optical density in mucus and plasma previously reported in White Bass ($r^2 = 0.32$)
(Barkowski and Haukenes, 2014). Previous investigators have quantitative VTG using Western blots where the current study produced a correlation coefficient with a direct quantification of species specific VTG from the mucus and plasma using an isolated standard within an EIA (Van Veld et al. 2005). In the current study, the correlation coefficient for the relationship between 11-KT plasma and mucus concentrations was lower than that reported by Schultz et al. (2005) who determined a relationship in koi ($r^2 = 0.83$), but was similar to that reported by Barkowski and Haukenes (2014) in White Bass ($r^2 = 0.22$). Schultz et al. (2005) postulated that their correlation coefficient resulted from a protracted period of gonadal development resulting in an increased range of 11-KT concentrations producing reduced residuals. For a fractional spawner like the Gulf killifish there are multiple spawns within a protracted period of time leading to several peaks in testosterone concentrations throughout the spawning period (Lamba et al., 1983). Such variation could be another cause for the increased residuals observed in the current study.

The current study used purified vitellogenin from *F. grandis* and antibodies specific to *F. heteroclitus*, allowing for increased species-specific sensitivity. Though the antibodies were not specific to *F. grandis*, it is effective given the detection of VTG in estradiol injected males. Alternative methods for VTG quantification include Pro-Q phosphoprotein detection, and offers an advantage over immunoassays since they do not require antibodies to be developed for each species of interest (Van Veld et al. 2005). Interplate variation for the validation study between plasma and mucus VTG concentrations were close to the acceptable ranges published by the manufacture for the assay and similar investigations have attributed elevated interplate variation as a result of repeated freezing and thawing of samples (Barkowski and Haukenes 2014). The methods employed in the current study reduced the risk of VTG breakdown with only one freeze/thaw step in the assessment of the mucus sample. Vitellogenin is known to be an unstable
compound, due to the easily cleaved R-groups of the peptide (Goodwin et al. 1992) and repeated freezing of the same sample likely alters the measured concentrations of VTG (Brodeur et al. 2006). In an effort to enhance the precise quantification of VTG, a stable VTG fragment was developed which could serve as a standard for immunoassays for goldfish (*Carassius auratus*), and potentially be developed for Gulf killifish or other relevant model fishes in the future (Wang et al., 2015).

Females from the validation portion of the current study were not sampled during the spawning season, while individuals from the laboratory exposure portion were actively spawning. This offset sampling period resulted in plasma VTG concentrations ranging from 3.84 to 14.28 pg/ml and from 0 to 2919.15 pg/ml in the validation and laboratory exposure, respectively. It is possible that a greater range in VTG concentrations within the validation portion of the current study could have resulted in decreased residuals between plasma and mucus VTG, and the validation portion should not be extrapolated beyond the scope of the correlation. Similar variation in reproductive investment was seen in Schultz et al. (2007) which served to strengthen the correlation coefficient for the relationship between plasma and mucus 11-KT.

Females exposed to PAHs exhibited reduced to near null concentrations of VTG in both mucus and plasma. Gangs Bayou (reference site) males in the current study also exhibited decreases in concentrations of 11-KT in the mucus. Similar decreases in testosterone were found in male Atlantic croaker *Micropogonias undulates* exposed to oiled sediments for 5 to 8 weeks (Thomas and Budiantara, 1995). Many PAHs act as an antagonist to normal reproductive hormone production, as they mimic androgens and estrogens through receptor binding causing a disruption in normal enzyme and HPG function (Patel et al. 2006). Cytochrome P450 aromatase
is the key steroidogenic enzyme responsible for conversion of androgens to estrogens (Kennedy and Smyth, 2015). Most species show relatively low levels of CYP1 enzyme, and can be easily induced by exposure to PAHs. These compounds act on multiple sites of the HPG axis and disrupt reproductive function, causing decreased circulating concentrations of gonadal steroids (Truscott, 1992). In general, exposure to contaminants such as PAHs lead to decreases in plasma estradiol and subsequent reduced likelihood of entering vitellogenesis, as demonstrated in English sole exposed to oil contaminated sediments (Casillas et al., 1991; Johnson et al., 1993). The disruption to the HPG axis is potentially due to membrane damage or interference with hormone membrane receptors on target tissues and could explain the decreased responsiveness of gonadal tissue to stimulation from gonadotropin releasing hormone (Kennedy and Smyth, 2015).

There appears to be differences between the reference and superfund populations in the current study with respect the ability of these individuals to resume pre-exposure conditions after the 60-d WAF treatment. Specifically, WAF exposed reference site males had slight increases in 11-KT concentrations at the end of the treatment, demonstrating that the fish were able to return to pre-exposure conditions. Demonstrating the actions of CYP1A, killifish fed a diet containing EDCs had increased levels of microsomal CYP1A, and decreased further upon initiation of reproductive cycles (Rice and Yang, 2000). The increased reproductive steroids released during the spawning cycle could explain the reduced or inhibited CYP1A induction, further suggesting the possibility of the interconnection of reproductive functions and PAH metabolism. Alterations in the CYP1A enzyme strongly coincide with changes to the reproductive success of fish (Meucci and Arukwe, 2006); where PAHs decrease androstenedione and estrogen synthesis through the inhibition of CYP1A.
Males in the current study appeared to show adaptations to PAH exposure resulting in different patterns over time. The WAF exposed males from Vince Bayou (superfund site) responded to WAF with increased 11-KT concentrations while Gangs Bayou exposed males (reference site) had a decrease in 11-KT concentrations. The control males from both populations had very similar fluctuations in 11-KT concentrations throughout the exposure and recovery period. This is not the first case documenting pollution-driven adaptations of *F. grandis* (Ownby et al., 2002; Wills et al., 2010; Oziolor et al., 2014). A resistant population of *F. heteroclitus* from a superfund site on the Elizabeth River in Portsmouth Virginia exhibit a recalcitrant AHR pathway, which gives rise to protection from contaminant induced cardiac deformities (Clark and Di Giulio, 2012). Oziolor et al. (2014) determined that *F. grandis* from sampling sites similar to the ones of the current study had slower elimination of PAHs, an adaptive protection against carcinogenesis and negative reproductive consequences. Females within the current study observed decreased mucus VTG concentrations for both reference and superfund sites when exposed to WAF compared to their respective controls. Furthermore, model analysis indicated the role of population, however, responses to WAF exposures within these laboratory exposures were different between males and females. This indicates the presence of potential alterations in responses to PAH exposure over multiple generations, which may blunt negative effects on reproductive endocrine signaling pathways by WAF leading to less of an impact on reproductive success.

This study provides insights for the use of mucal swabbing as a means of monitoring potential endocrine disruption within investigations of environmental toxicology. In the current study, repeated sampling proved to be effective in characterizing differences in a chronic exposure study. The high survival rate observed in this study illustrates the potential to be more
widely adopted for use in fishes, especially with rare or endangered populations when blood sampling and excessive handling can be problematic and in instances when carrying out a repeated sampling regime. Repeated sampling was especially useful in characterizing impact in this study as it allowed for increased statistical power by conducting a general linear model with a mixed model analysis of covariance to study the interaction of treatment, population and time. The opportunity to carry out repeated sampling on the same individuals in a toxicological study without collecting blood is valuable, considering the lethality of blood collection in model fishes such as zebrafish, fathead minnows and Fundulus spp. Mucus is continuously secreted and replaced, allowing the swabbed fish in the current study to recover or replace mucus lost from swabbing (Awad et al., 2015). However, as a consideration and caution to the application of this technique, continual removal of the mucus could potentially decrease the immune health of the fish since the mucus is the first line of defense against infection through the epidermis (Raj et al., 2011). Throughout the WAF exposure and subsequent recovery period within the current study, mucus swabs were performed on fish over four times with no recorded skin infections or other observable negative effects. Other methods of non-lethal sampling exist such as the collection of a tissue biopsy, however, post collection survival has not been extensively studied and may require greater amounts of handling (Hamilton, 1994). The utility of mucal swabbing also includes an ease of collection making it possible to characterize seasonal reproductive maturation, assess potential endocrine disruption in populations residing within polluted sites, crowdsourcing data collection via ‘citizen-science’, or increase statistical power by increasing samples collected.
CHAPTER 3: MULTIGENERATION EFFECTS OF THE POLYCYCLIC AROMATIC HYDROCARBON NAPHTHALENE ON GULF KILLIFISH Fundulus grandis

3.1 Introduction

Major catastrophes such as the Deepwater Horizon oil spill have increased our understanding of the impacts of crude oil toxicity on fish health, development, and reproduction. The DWH oil spill originated at a depth and under high energy, thus facilitating the dissolution of PAHs by increasing mixing time and energy facilitating the distribution of oil into the water column (Reddy et al., 2012; Ryerson et al., 2012). Functional studies have further investigated the toxicity of PAHs on fish development and identified specific PAHs responsible for specific impacts. For example, PAHs with a lower molecular weight such as 2-ring compounds such as naphthalene have been shown to target the nervous system, and reproductive investment (Pollino et al., 2009; Bender et al., 2016; Kennedy et al., 2015). Higher ring compounds (3- to 4-ring compounds) are credited with causing anatomical malformations such as faulty cardiac development associated with bradycardia and arrhythmia (Mager et al, 2014; Hicken et al. 2011; Incardona et al., 2006; Incardona et al., 2004). PAHs are the primary toxic compound in crude oil, but components such as alkanes, cycloalkanes and various organic compounds are part of this complex mixture making it difficult identify all potential effects of chronic sublethal oil exposure (Bender et al., 2016; Bagi et al., 2014).

Endocrine disrupting chemicals such as PAHs can destabilize the hypothalamo-pituitary-gonadal (HPG) axis of organisms residing in aquatic habitats. Chronic exposure to PAHs such as naphthalene decreases circulating concentrations of estradiol and testosterone, and also decreases the responsiveness of ovarian tissue to in vitro hormonal stimulation (Thomas and Budiantara, 1995). Administration of a single intraperitoneal injection of benzo[a]pyrene on rainbow trout
(Oncorhynchus mykiss) resulted in a severe decline of circulating estradiol and testosterone concentrations (Kennedy and Smyth, 2015). When the same fish were given gonadotropin releasing hormone to initiate spawning, control individuals had an increase in estradiol and testosterone while benzo[a]pyrene treated fish did not. It is postulated that the estrogenic nature of PAHs cause binding to estrogen receptors acting as an antagonist by inhibiting the action of estrogens or potentially serving as agonists by producing a downstream action at the receptor site (Sumpter and Jobling, 1995; Arukwe et al., 1997; Thomas et al., 2009). Many studies have shown that the activation of the P450 enzyme corresponds with changes in reproductive success of fish; the anti-estrogenic actions of P450-inducing compounds are known to disrupt vitellogenesis and egg production (Thomas, 1990; Nicolas, 1999; Meucci and Arukwe, 2006). Induction of the CYP1A/P450 enzyme is also believed to contribute to lower circulating estradiol and testosterone concentrations due to enhanced clearance, because the same enzyme is responsible for estradiol elimination and biotransformation (Spink et al., 1992; Hammond et al., 1997). Measurement of ethoxyresorufin-O-deethylase (EROD) induction is a highly sensitive indicator of contaminant uptake in fishes, providing evidence of receptor mediated induction of the CYP1A enzyme by xenobiotic chemicals (Willet et al., 2000). This assay provides a fingerprint of the presence of Ahr-active compounds in fish.

The most significant concern with exposure to PAHs and other environmental disrupting chemicals is the impact they have on populations with exposure to PAHs potentially resulting in developmental deformities several generations after the initial exposure. In a study conducted by Corrales et al. (2014) a first generation of zebrafish (F0) were exposed to different concentrations of dietary benzo[a]pyrene, and then maintained within a PAH free environment for 4 generations (F1, F2, F3, and F4). Deformities such as body shape, tail shape, and heart
development were observed. The researchers determined that parental dietary exposure to benzo[a]pyrene had adverse effects on the immediate offspring, and some adverse effects persisted across all subsequent generations. Models have been developed to examine the impact of low-level toxicant responses on the various life history parameters that control population growth rate and stable age distribution (Spromberg and Meador, 2005). Examining populations of coho salmon (Oncorhynchus kisutch), sockeye salmon (Oncorhynchus nerka), and ocean-type chinook salmon (Oncorhynchus tshawytscha) inhabiting the polluted Puget Sound projected that a 10% change in reproductive fecundity parameters could lead up to a 64% decline in baseline population percentages after a simulated 20 years of toxic impact (Spromberg and Meador, 2005). A more recent study conducted by Booc et al. in 2014 on mummichog (Fundulus herteroclitus) suggested that males’ fertility was the most impacted. Investigators determined that it was fertilization success and not egg production that had decreased significantly as a results of a 28 days aqueous exposure to 0, 1, or 10 µg/L of benzo[a]pyrene. Male gonad weight and plasma testosterone concentrations were decreased by 38% and 86%, respectively while females’ estradiol concentrations decreased significantly but gonad weight and egg production remained unaffected.

Gulf killifish (Fundulus grandis) provide an ideal species for the study of reproductive assessment across multiple generations, as they are native to coastal estuaries in the Gulf of Mexico and due to their protracted spawning season, abundant egg production and clear embryos (Brown et al., 2011; Green et al., 2010). Research on the Fundulus genus has increased our fundamental knowledge on the mechanisms used to adapt to environmental stressors leading to a better understanding on selected integrated systems such as reproduction and responses to toxicants (Burnett et al., 2007). Many toxicological studies have been conducted on wild
populations of *Fundulus*, and most have established that after multiple generations of chronic exposure to PAHs and PCBs alterations and possibly adaptations occur at a cost to their fitness (Meyer and Giulio, 2003; Fort et al., 2014; Ownby et al., 2002; Bugel et al., 2011; Oziolor et al., 2014; Wills et al., 20110; Patel et al., 2006).

Surface mucus is the biochemical interface between the fish and the aqueous external environment, and is continuously exposed to microbes and stressors. The composition of mucus is complex and includes multiple biologically active substances that function as a humoral innate immune system by inhibiting the entry of pathogens to the epithelial cells (Sanchooli et al., 2012). One such humoral factor is lysozyme, produced mainly by monocyte macrophages and neutrophils and whose function is to catalyze the linkages between N-acetylmuramic acid and the N-acetyl-D-glucosamine in both gram positive and gram negative bacteria (Beck and Peatman, 2015). Lysozyme acts as an opsonin which binds and enhances the process of phagocytosis and activates the complement system. Modulation of lysozyme levels in plasma have been found in response to environmental (pH, temperature, toxins) and intrinsic properties (gonadal growth) (Subramanian et al., 2007; Saurabh et al., 2008; Ghafoori et al., 2014).

Swabbing for surface mucus offers a less invasive and non-lethal biomarker than blood sampling with the potential to assess a number of biological functions within the fish such as 11-ketotestosterone, vitellogenin, and lysozyme (Schultz et al., 2007, Ekman et al. 2015). Lysozyme in particular is abundant in surface mucus and it is among the constituents contributing as a first line of defense again pathogens (Ghafoori et al., 2014).

Exposure to PAHs over multiple generations has given rise to multiple populations that have adapted to handle the stress associated with these xenobiotics (Oziolor et al., 2014; Ownby et al., 2002). In the current study, naphthalene was selected as an exposure compound with a
second generation of Gulf killifish previously exposed to Macondo-252 crude oil over benzo[a]pyrene due to its abundance in Louisiana Crude oil comparatively to benzo[a]pyrene, thus ensuring that both generations of this study were exposed to the same prototypical PAH (supplemental data from Mager et al, 2015; Kuhl et al 2013). A single PAH exposure was selected over a WAF for the second generation to avoid large variation in the concentrations of tPAH the fish were exposed to. The main objective of this study is to examine reproductive and biochemical markers from two generations of Gulf killifish exposed to PAHs (Figure 3.1). The current study seeks to (1) assess reproductive parameters of the parents (F₁) while undergoing a 40-day static renewal exposure to WAF of crude oil; (2) examine larval parameters to determine quality of resulting larvae (F₂); and (3) measure multiple biomarkers within the F₂ while undergoing a 40-day static renewal exposure to aqueous naphthalene.

**Figure 3.1:** Flow-chart detailing exposure regimes and objectives for two generations of Gulf killifish.
3.2 Materials and Methods

3.2.1 Parental (F₀) collection and production of F₁ generation

Wild caught fish were spawned in captivity to produce a cohort of individuals, thus removing potential environmental or selective effects of the source population. Gulf killifish were collected near the Florida State University’s Coastal and Marine Laboratory at St. Teresa, Florida and returned to laboratory facilities at the LSU AgCenter’s Aquaculture Research Station in Baton Rouge, Louisiana. These wild caught fish were designated as the F₀ generation and spawned within the laboratory by increasing day length from 10L : 14D to a final photoperiod of 14L : 10D over a six week period. Water temperature was increased from 15 °C to 27 °C during this six week period with immersion heaters at a rate of 2 °C per week. Spawning substrate consisted of a 10 × 13 cm rectangle of Spawntex (Blocksom and Co., Michigan City, IN, USA) placed within a 10 cm diameter PVC tube, approximately 15 cm in length. The PVC tubes were placed at the bottom of triplicate 50-L tank and embryos were collected from spawning substrate every 3 days with resulting larvae hatched in a separate recirculation system.

Progeny of the wild caught fish are referred to as the F₁ generation within the current study. They were stocked within three identical 250-L circular tanks at an approximate salinity between 12‰ and 16‰ and density of 2 fish per liter. The total system consisted of 7000-L recirculation system with biological filtration and UV-sterilization. The fish were fed a commercial feed (32% protein, 9% lipid; Cargill Inc., Wayzata, MN, USA) five days per week to apparent satiation two times per day. Male and female fish reach mean weights of 12.2 ± 0.5 g and 9.9 ± 0.4 g, respectively, prior to stocking into WAF exposure tanks.
3.2.2 F₁ generation WAF exposure

A constant supply of WAF water was produced in a large upwelling system to allow for large-volume replacement exposures. Pre-weighed Siporax biofilter media (Sera, Heisenberg, Germany) were submerged in MC-252 surrogate crude oil for 48 hours. This oil laden media was weighted and then transferred to a 10 cm x 25 cm PTFE (polytetrafluorethylene) lined polyvinyl chloride chamber within one of two upwelling systems adapted from previous studies (Carls, 2000; Kennedy, 2005). These upwellers generated WAF treatment water at a premixed salinity of 18‰ by continuously circulating water though a system of PTFE piping across the Siporax beads and into a 1000-L PTFE-lined fiberglass tank. This provided constant mixing at a rate of 20 L/minute needed to generate a water accommodated fraction of the crude oil.

Six 60-L static tanks were used for the first exposure portion of this study, three tanks for WAF treatment and three tanks for control treatments with aeration provided with atmospheric air using a glass tube each stocked with 6 male and 6 female Gulf killifish. After a 24 hour mixing period in the upwelling system, WAF and control water was drawn off from the bottom of the systems into 25-L PTFE lined carboys for distribution to treatment tanks. Drawing water from the bottom of the upwellers prevents the oil slick at the surface of the WAF upweller from contaminating WAF water and other surfaces that would come in direct contact with the fish. Every four days, 50% of the water in the tanks was removed, and 30 L of WAF or control water was added accordingly to each of the six tanks. Water samples were collected every four days during the 40 day exposure and sent to ALS Environmental (Kelso, WA, USA) for analysis as described in Kuhl et al. (2013). Seventy-five PAHs including associated alkylated homologues were analyzed with the sum of these analytes creating a “target PAH” (TPH) value.
3.2.3 F1 generation reproductive parameters from WAF exposure

Ovaries from six females were collected on days 10, 20 and 40 from WAF exposure and control treatments. Gonad samples were preserved in 10% neutral buffered formalin and sent for processing to the LSU School of Veterinary Medicine, Histology Department for embedding, sectioning, and hematoxylin and eosin staining. The resulting gonad histology slides were assessed to determine presence of maturation phase oocytes for morphometric analysis (Blazer et al., 2002; Greely et al., 1988). These oocytes displayed a fused yolk and a thickened vitelline envelope. Oocytes were photographed using a digital camera for measurements of envelope and chorion thickness using the program ImageTool (Version 3.0, University of Texas Health Science Center, San Antonio, TX).

The testes of three males were sampled on day 10, and 20. Testes were then crushed into 500 µL of Hanks balanced salt solution (300 mOsmol/kg), then further diluted 1:9 and analyzed for quality and quantity using Computer-Assisted Sperm Analyzer (CASA) (Hamilton Thorne Inc., Beverly, MA, USA) as described in Yang and Tiersch (2011). Total sperm concentration and percent motility were averaged from 3 separate videos taken for each individual sample at 200× magnification using a dark field microscope interfaced to the CASA program. Sperm were considered mobile if they progressed in a forward direction, while sperm vibrating in place were considered immobile and were excluded from analysis. Progressive sperm motility only includes those cells activity moving forward and was calculated with the CASA following the settings of Tiersch and Yang (2012) for Gulf killifish.

After the 40-day WAF exposure, fish were transferred to a recirculating system with 60-L tanks to eliminate PAH exposure and present spawning substrate. Spawning was induced by maintaining appropriate photoperiod and temperature conditions for the spawning season (14
light: 10 dark, 24°C (Green et al., 2010). Spawning substrate consisting of Spawntex (Blocksom and Co., Michigan City, IN, USA) were placed in the tanks, and eggs were collected every 4 days for a total of 8 clutches from each treatment. Collected embryos were incubated at 25°C within 10 ml petri dishes with 18‰ salinity water. Water was exchanged in the dishes every 48 hours. The number of embryos collected from the spawning substrate, number of embryos which display neurulation (viability), and number of embryos that hatch (hatchability) were assessed visually during incubation and at hatch of each WAF treatment and control collection.

3.2.4 F2 generation larval health

Upon hatch, larvae were evaluated by calculating a survival activity index (SAI) for each clutch collected every 4 days after WAF exposure. SAI is a measure of starvation tolerance used to evaluate larval health (Matsuo et al., 2006). Twenty-four larvae from each treatment group and each egg collection (clutches 2-8) were individually placed in 6-well plates at a salinity of 18‰ with 24 hour water replacements. Mortality was recorded each day until complete mortality was reached and the data transformed into SAI values for each egg collection and treatment according to the following equation:

\[
SAI = \frac{1}{N} \sum_{i=1}^{k} N - h_i *
\]

Where \( N \) was the total number of larvae, \( h_i \) was the cumulative mortality by the \( i \)th day, and \( k \) was the number of days elapsed until all larvae are dead (Matsuo et al., 2006).
3.2.5 F₂ generation rearing

Embryos produced in clutches 2-8 by the F₁ generation following a 40-day WAF exposure were reared to sexual maturity within a common garden environment to provide F₂ individuals with different parental exposure histories. These fish were divided into two groups based on parental exposure history, whereby parental exposed fish represent F₂ individuals whose F₁ parents were exposed to WAF for 40 days prior to spawning and parental control represent F₂ individuals whose F₁ parents were not exposed to WAF prior to spawning. Similar to the rearing of the F₁ generation, these larvae were stocked within identical 50-L tanks at an approximate salinity between 12‰ and 16‰ and density of 2 fish per liter. The total system consisted of 600-L recirculation system with biological filtration and UV-sterilization. Fish were fed a commercial feed (32% protein, 9% lipid; Cargill Inc., Wayzata, MN, USA) five days per week to apparent satiation two times per day. Parental exposed male and female fish reached mean weights of 11.3 ± 0.9 g and 9.3 ± 0.7 g, respectively, prior to naphthalene exposure experiments. Parental control male and female fish reached mean weights of 9.3 ± 0.7 g and 8.2 ± 0.5 g, respectively, prior to naphthalene exposure experiments.

3.2.6 F₂ generation naphthalene exposure

Parental exposed and parental control F₂ generation Gulf killifish were exposed to an aqueous solution of naphthalene (targeted at 100µg/L) or control treatments for 40 days. Each group and treatment was separated into 3 to 4 static tanks, each tank housing 4 males and 3 females. Fourteen 40-L tanks filled with 25 L of water were used for the exposure portion of this study, 8 tanks for naphthalene treatments and 6 for control treatments. Each treatment or control tank was stocked with 4 males and 3 females. An initial target concentration of 100 µg/L of
aqueous naphthalene was achieved by mixing 2.5mL of a 1mg/mL stock solution formed with ethanol with 25 L of water. Salinity was maintained at 12‰ with Crystal Sea MarineMix (Marine Enterprises International, Baltimore, MD) and municipal water in an upweller for 24 hours. Every four days 60% of the water in the 14 tanks was removed, and 15 L of water was added and re-dosed with naphthalene accordingly. Water samples were collected every four days during the 40 day exposure and sent to ALS Environmental for analysis of naphthalene, 1-methylnaphthalene, and 2-methylnaphthalene. The geometric mean concentration of naphthalene throughout these exposures was determined to be 39.8 ± 7.2 µg/L PAH. After 40 days fish were moved to new tanks for a 40 day period without Napthelene exposure.

3.2.7 Biomarkers of naphthalene exposure in F2 generation

Livers were collected and immediately frozen at -80 °C from naphthalene exposed and control individuals representing different parental exposure histories. Collections were made from 5 males after 40 days of Naphthalene exposure and 40 days after a recovery period in the absence of naphthalene. CYP1A activity was determined using an ethoxyresorufin-O-deethylase (EROD) assay with liver microsomes. Assay methodology was modified and adapted to a plate-reader as described in Willet et al. 2000. Protein concentrations of the microsomal suspension were determined via a Bradford-Coomasie assay (Pierce Biotechnology, Rockford, IL) and diluted to a target protein concentration of 250 µg/ml. Samples were assayed in triplicate and consisted of 20 µl of microsomal suspension with 180 µl of cofactor solution (0.1M HEPES, 120 µM NADPH, 5mM magnesium sulfate; pH 8.0). Standards were created with increasing concentrations of resorufin (5 - 500 pmol/200 µl), and bovine serum albumin (5 µg/ml). Energetic kinetics were monitored every minute for 15 minutes via fluorescence with emissions
and excitation set to 530 and 590 nm, respectively. Specific enzyme activities were expressed as the amount of substrate converted per minute and normalized for protein content.

Mucus was collected from 6 males and 6 females from each treatment and parental history group to determine lysozyme activity between Naphthalene treatments with different parental exposure histories. Sampling occurred on day 0, 20, and 40 during naphthalene exposures. Swabs were also taken 60 and 80 days after the start of the exposures and considered a recovery period since naphthalene exposures were terminated at 40 days. Mucus collection and processing was developed as described in Schultz et al (2005). Individual fish were collected from tanks and immediately swabbed on both sides using a 1 cm² section of polyurethane sponge (Buf-Puf gentle facial sponge, 3M, St. Paul, MN). Swabbing consisted of three non-overlapping and unidirectional anterior to posterior motions on each lateral side beginning posterior of the operculum and terminating anterior to the caudal peduncle. The sponge was then placed in a 3-ml syringe. Ice-cold 20 mM Tris-buffered saline and Halt™ protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL) was added to the cylinder. The sponge was compressed in the barrel of the syringe with a plunger and fluid passed through an 18-gauge needle and collected in a 1.5 ml centrifuge tube.

Protein concentrations of the mucus were determined via a Bradford-Coomasie assay (Pierce Biotechnology, Rockford, IL) and diluted to a target protein concentration of 70 µg/ml. Lysozyme concentration in the mucus was assessed with a quantitative ELISA using a primary antibody M242 (5 ug/mL) developed by Charles Rice at Clemson University. The secondary antibody was anti-mouse IgG conjugated to alkaline phosphatase and diluted 1:1000 (Thermo Scientific, Rockford, IL). Developing solution consisted of p-nitrophenolphosphate diluted to 2 mg/ml in alkaline phosphatase buffer. Samples were run in duplicate with a standard. To confirm
that the assay detected the lysozyme protein, a western blot was conducted at the LSU AgCenter’s Biotechnology Laboratory using the same antibody and mucus with at a protein concentration of 300 µg/ml. The secondary antibody for the western blot was anti-mouse horseradish peroxidase diluted to 1:5000, and developing buffer was Pierce ECL Wester Blotting substrate (Thermo Scientific, Rockford, IL).

3.2.8 Statistical analysis

Statistical analyses were performed using Statistical Analysis Software, Version 9.4 of the SAS System for Windows (SAS Institute Inc, Cary, NC). All statistical tests were performed at a significance level of α = 0.05. Data were reported as mean ± standard error of the mean (SEM) unless otherwise stated.

*F*<sub>1</sub> generation WAF exposures

Oocyte histology morphometrics, sperm motility and concentration, and embryo production and hatch metrics were compared between WAF and control treatments and exposure time with two-way mixed model analysis of variance (ANOVA). Larval SAI was calculated as per Matsua et al. (2006) and compared between WAF and control exposure treatments and clutches (2-8) with two-way mixed model ANOVA.

*F*<sub>2</sub> generation Naphthalene exposures

Liver microsome samples were normalized by protein content to correct for potential differences in size and extraction efficiency. EROD activity was shown as a fold change over basal activity in the liver of the F2 generation. Expression was shown as the multiple of the control (fold change) normalized to the unexposed parental control group. For lysozyme assessment, mucal samples were normalized by protein content to correct for potential
differences in mucosal content due to swabbing techniques or fish size and sex. Normality of EROD activity and mucus lysozyme optical density (OD) was tested with the Kolmogorov-Smirnov-test and viewed via residual probability plots with PROC UNIVARIATE. Differences in EROD activities by parental exposure history and time was determined with two-way mixed model ANOVA. Lysozyme mucus OD data were log transformed prior to analysis. The log transformed lysozyme mucus OD response to time, treatment, and parental exposure history served as a baseline Lysozyme mucus OD for each treatment and parental exposure history since mucus lysozyme OD was determined for each treatment and parental exposure history at day 0. A general linear model with a mixed model analysis of covariance was performed using PROC GLM for the curved-linear relationship of treatment, parental exposure history, and time for lysozyme mucus optical density (OD). Interpretation of significance for this model was viewed using Type I Sum of Squares as initial day 0 lysozyme mucus OD functioned as a covariable.

3.3 Results

3.3.1 F₁ generation WAF exposure and reproduction

Water samples collected from the WAF treatment group for TPH analysis were damaged for sample days 4 and 8. The geometric mean for TPH concentration throughout the exposure period was $16.7 \pm 5.1 \mu g/L$ with the highest and lowest TPH concentrations of 39.1 and 6.9 $\mu g/L$ occurring on days 28 and 8, respectively (Figure 3.2).
Figure 3.2: Mean target polycyclic aromatic hydrocarbons (TPH) concentrations (µg/L) within 40-day WAF exposure treatments of F₁ generation Gulf killifish.

WAF treatment had a significant overall effect on thickness of the vitelline envelope, resulting in WAF females having oocytes with thinner vitelline envelopes ($p < 0.05$). There was no significant changes to envelope thickness in oocytes across time, nor for the interaction of time × treatment. Average thickness for control females across day 10, 20 and 40 was $59.4 ± 3.6$ µm, while average cell wall thickness for WAF females was $49.8 ± 2.6$ µm (Fig 3.3).
Spermatozoa concentration was significantly lower in WAF-treated males at 10 and 20 days of WAF exposure ($p < 0.05$) (Fig. 3.4). Motility of spermatozoa also differed between the WAF exposed males and the control males ($p < 0.05$), control males had a mean motile sperm concentration of $92.05 \times 10^7$/ml while WAF males had a mean motile sperm concentration of $18.95 \times 10^7$/ml. Sperm concentration and motility in WAF and control treated males decreased significantly throughout time ($p < 0.05$). WAF treated males had a statistically significant decrease in sperm concentration and motility when compared to sperm concentration and motility of control individuals with respect to the interaction of time × treatment ($p < 0.05$).
Figure 3.4: Mean (±SEM) total sperm concentration ($10^7$/mL) in control and exposed males at day 10 and 20 (n = 3). A significant difference was found between the WAF and control groups ($p < 0.05$). Asterisks indicate significant differences between exposure groups at each time point (ANOVA, two way, $p < 0.05$)

There were similar numbers of total eggs produced by WAF and control females over all 8 clutches (Control = 1385, WAF = 1123), and no significant difference were detected between unhatched embryos and viable embryos of exposed and control females (Table 3.1). WAF exposed females produced on average more unhatched embryos than control females (Control = 19 ±7; WAF = 33 ±4, $p = 0.13$).

Table 3.1: Mean (±SEM) number of total embryos collected, and mean (±SEM) number of hatched and unhatched embryos in control and exposed groups (n= 8 clutches). No significant difference was found between the WAF and control groups (Hatched $p = 0.14$; unhatched $p = 0.13$).
3.3.2 F₂ generation larval health

Both WAF and control treatments produced embryos that were collected from spawning substrate and collected every 4 days forming a series of clutches. The first clutch was not included in the analysis as embryos were not collected from either treatments 4 days after the end of the WAF exposure. Indices of larval tolerance to starvation varied significantly across time with clutches produced further away from the 40-day exposure treatment surviving longer \((p < 0.05)\). No significant differences were found between larvae from exposed parents and larvae from control parents, nor was the interaction of time \(\times\) treatment significant \((SAI_{Control} = 165.6 \pm 9, SAI_{WAF} = 134.2 \pm 10)\) (Fig. 3.5). Larvae from control females survived an average of 17.35 days when starved, while larvae from WAF females survived 15.27 days on average under the same conditions.

![Figure 3.5 Survival activity index (SAI) values for larvae produced by females from control and exposed treatment groups over 7 clutches (n=10 per treatment/clutch). The higher the clutch number, the further away from exposure to WAF. No significant difference was found across treatment (p > 0.05).](image-url)
3.3.3 F₂ generation naphthalene exposure

Fold EROD induction was significantly increased in groups of killifish that underwent exposure to naphthalene compared to their control counterparts ($p < 0.05$). There were no significant interactions among the effects of time, treatment, or parental history (Fig 3.6).

![Graph a)](image)

**Figure 3.6:** Mean fold EROD induction (± SEM) for F₂ generation on a) day 40 of naphthalene exposure (n=6), and b) 40 days after cessation of naphthalene exposure (n=12).
Mucus lysozyme OD in naphthalene and control treated adults decreased significantly throughout time ($p < 0.05$). There was a significant curvilinear response of lysozyme mucus OD across time by both parental exposure and treatment (time $\times$ time $\times$ parental exposure $\times$ treatment, $p < 0.05$). Naphthalene exposed individuals from control parents were significantly different in mucosal lysozyme OD when compared to mucosal lysozyme of naphthalene exposed individuals with WAF exposed parents but were not significantly different from their respective control treatments (Fig. 3.7) ($p < 0.05$).

Figure 3.7: Optical density (OD) of lysozyme in mucus of the parental exposed, and parental control, across naphthalene exposure (40 days) and post-naphthalene exposure (40 days). These two groups were compared to one another and the linear relation of time $\times$ treatment $\times$ parental history was significant ($p < 0.05$).
3.4 Discussion

In the current study sperm concentration and motility decreased significantly with exposure to crude oil within the F1 generation males. Similar decreases in sperm concentrations were found in polar cod (*Boreogadus saida*) exposed to dietary petroleum at varying doses for 31 weeks (Bender et al., 2016). Low motility of sperm could potentially affect fertilization success, and has been shown to be an indicative factor in fertilization in Atlantic cod (Rudolfsen et al., 2008). Polar cod exposed to water produced as a bi-product of oil and gas production for 28 days resulted in reduced spermatogenesis and increased prevalence melano-macrophages within the testes when compared to control fish (Geraudie et al., 2014). This reduction in sperm motility and concentration could be due to the disruption of endocrine mediated effects of PAHs on 11-ketotestosterone levels which could interfere with spermatogenesis. The Gulf killifish females from the current study exposed to crude oil did not show any changes in spawning productivity as measured by eggs collected at regular intervals from spawning substrate. Similar studies show varying results, as polar cod demonstrate no changes in circulating steroid concentrations or alterations in female germ cell development (Bender et al., 2016). Female mummichog from the highly contaminated Newark Bay, NJ (USA) exhibited an inhibition of oocyte development due to deficient 17β-estradiol and decreased AHR sensitivity of the VTG pathway to 17β-estradiol (Bugel et al., 2011). Females from reference sites in New Jersey produced an average of 140 eggs per female while Newark Bay females produced an average of 11 eggs per female with the embryos from the contaminated sites exhibiting 34% greater mortality and a 28% reduction hatch when compared to their control counterparts. A separate study examining reproduction in mummichog and fathead minnow (*Pimephales promelas*) after exposure to PAH contaminated sediments in a flow through system for 21 days found decreased
reproductive fecundity, lower male body weight, altered endocrine hormone concentrations, and decreased estradiol, VTG and aromatase activity in females (Fort et al., 2015). In the current study, the F1 generation exposed to WAF produced reduced numbers of viable embryos throughout 8 egg collection periods resulting in 42% embryos while control parents produced 55% viable embryos.

Tolerance to starvation was most affected by time from exposure with clutches produced further away from the end of exposures surviving longer. Traditionally SAI is a nutritional biomarker (Takeuchi, 2001; Russo et al., 2017), but for the current study it was used as a biomarker for maternal condition providing an indirect link between maternal reproductive investment and embryo quality. This metric could potentially serve as a biomarker comparing two or more generations and to our knowledge represents the first use of this metric within an ecotoxicological study. Though no significant difference was detected between larvae from exposed parents and larvae from control parents, larvae from control parents survived on average 17.35 days while larvae from WAF females survived an average of 15.27 days. SAI is an indirect measure of yolk volume, and presumably WAF larvae would not survive as long as control larvae due to their reduced endogenous yolk reserves. Similar decreases in egg yolk volume were found in female mummichog from the superfund site in Newark Bay, which could potentially lead to negative ontogenetic consequences as developing larvae transition from endogeneous to exogeneous feeding prior to acquisition of feeding morphology (Bugel et al., 2011). In the current study, the vitelline envelope of oocytes from WAF females was significantly thinner than the envelope on oocytes from control females. Exposure to PAHs causes changes in circulating estradiol and testosterone concentrations which may interfere with the vitellogenesis pathway and alter yolk investment. The AHR protein acts as a unique ligand-dependent E3 ubiquitin
ligase that targets estrogen and androgen receptors when exposed to PAHs. This response can cause significant down-regulation of transcripts for egg envelope proteins (zona pellucida and choriogenin) resulting in thinner vitelline envelope (Whitehead et al., 2012). Fort et al. (2015) demonstrated that mummichog exposed to a flow through exposure to sediment PAH for 21 days exhibited decreased reproductive fecundity, altered endocrine function (decreased E2 and T), and decreased VTG and aromatase activity in females. This indicates potential negative effects on reproductive endocrine signaling pathways which could alter offspring survival and health.

In the current study, surface lysozyme activity decreased with aqueous exposure to naphthalene. Lysozyme in an important constituent of innate immunity and surface mucus lysozyme appears to potentially serve as a biomarker for PAH exposure. Toxicants have complex effects on surface mucus and the skin of fishes, and can vary based on the amount, release, physical state, and composition of the toxicant (Bols et al., 2001). Waterborne exposure to 10 µg/L of methylmercury of gilthead seabream (Sparus aurata) increased serum lysozyme after 30 days of exposure (Guardiola et al., 2016). Lysozyme activity decreased with sub-lethal concentrations (300 µg/L) of mercury exposed over a period of 7 days in plaice (Pleuronectes platessa L.) (Fletcher, 1986). Although previous research has indicated that lysozyme activity is similar between plasma and mucus (Guardiola et al., 2014), other studies have indicated that lysozyme production by epidermal cells creates greater mucus lysozyme activity when compared to plasma activity and can sometimes be localized to specific tissues (Grinde et al. 1988; Fast et al., 2002). The mucus lysozyme activities in the current study were monitored over 80 days, with mucus lysozyme OD decreasing throughout time. Dab (Limanda limanda) exposed to 4, 8, 12 and 16% oil-based drilling mud for 4 weeks displayed decreases in plasma lysozyme activity, while individuals caught at a recently contaminated site (oil tanker accident) also exhibited
similar decreases in lysozyme activity (Tahir et al., 1993; Secombes et al., 1991). Lysozyme is a convenient biomarker for innate immunity, due to the ease of collection, ability to freeze samples and is a relatively inexpensive ELISA assay which allows for long term monitoring of toxicological effects of toxicants. The lysozyme assay with surface mucus also proved to be very sensitive, allowing detection of disruption at the level of treatment, as well as previous parental exposure.

EROD induction in the current study was affected by exposure to naphthalene. Adult *F. grandis* exposed to 10, 1, 1, and 10 ppm of Arcolor 1254, tributyltin, 3 methylcholanthrene and nonyl-phenol respectively over a 16-week period showed suppressed GSH responses, as well as altered CYP1A (Rice and Xiang, 2000). The breakdown of naphthalene by the cytochrome P450 enzyme in the cells causes the toxicity of this compound. Naphthalene is metabolized into Naphthalene-1,2-oxide and causes initiation of cancers via its activation and interaction of 1,2-naphthaquinone with DNA to form the depurinating adducts (Saeed et al., 2007). The breakdown of naphthalene and a number of other aromatic compounds is similar to the metabolic activation of carcinogenic natural estrogens (D'Arcy Doherty et al., 1985). Mummichog larvae from a superfund river in Virginia and a reference site were exposed to two 24-hour aqueous exposures of BaP (0-400 µg/L), and contaminated larvae showed lowered EROD induction compared to 'clean' larvae (Wills et al., 2010). The reference site larvae had greater whole body concentration decreases of BaP over time, increased DNA lesions, and more hepatic focus of cellular alteration (Wills et al., 2010). The breakdown of xenobiotic substances suggests that differences between a previously exposed population and reference population possibly originates in their ability to bio transform PAHs either by activation or elimination of the parent compound. In the current study, parental control fish treated with naphthalene has a fold EROD induction of 2.5 while parental
exposed fish treated with naphthalene had a fold EROD induction of 1.5 on day 40 of the exposures.

The results of this study suggest that within the F₁ generation there is some degree of reproductive impairment in Gulf killifish undergoing a chronic WAF exposure to crude oil. For the F₂ generation, there is evidence of limited alteration to PAH tolerance within two generations of exposure to PAHs as demonstrated by both hepatic EROD induction and lysozyme activity within the mucus. Given sufficient time, many fish populations could evolve a life history strategy whereby a set of trade-offs in allocation of energy and resources between growth, current reproduction, future reproduction and survival. Previous models have determined that the longer the lifespan of a fish, the more at a disadvantage it is with dealing with impacts (Spromberg and Birge, 2005). Evidence of such pollution mediated adaptation has been previously documented in the _Fundulus_ genus (Owbny et al., 2002; Wills et al., 2010; Oziolor et al., 2014). Oziolor et al. (2014) compared resistance to PCB126 and coal tar in Gulf killifish embryos from a control site and embryos from a superfund site in Texas and found that the superfund embryos had a reduction in basal induction of CYP1A affording them resistance to toxicant induced cardiovascular teratogenesis and negative reproductive consequences. A resistant population of mummichog from a superfund site in Portsmouth Virginia exhibited a recalcitrant AHR pathways, which gave rise to protection from contaminant induced cardiac deformities (Clark and DiGiulio, 2012). However this tolerance to PAHs carried a cost, and the offspring of this Virginia superfund population were more susceptible to anthropogenic and natural stressors (Meyer and Di Giulio, 2003). The F₂ generation in the current study showed increased lysozyme expression and reduced EROD induction in the individuals with parental exposure. This could indicate the potential development of resistance to PAH exposure over two
generations, which would allow for reduced negative effects on innate immune function and reproductive endocrine signaling pathways.
CHAPTER 4: SUMMARY AND CONCLUSIONS

The effects of PAHs over multiple generations of vertebrates have yet to be truly understood, but it is increasingly vital to know due to increasing exposure to PAHs and other potentially harmful compounds in nature.

Creating non-lethal alternative tests for multiple biomarkers will become ever more vital as environmental toxicology studies increase in frequency, and to allow for repeat testing on small or endangered fishes. The current study developed and tested the efficiency of one such non-lethal alternative; the use of surface mucus rather than blood to test for VTG and 11-KT in Gulf killifish. Using surface mucus to test for these reproductive biomarker has been done before with other larger species of fishes such as white bass, and koi (Barkowski and Haukenes, 2014; Schultz et al., 2007). A validation of this method was conducted by paired sampling of mucus and blood, and both 11-KT and VTG showed significant positive relationships between their respective concentrations in the plasma and mucus. Mucus was not an ideal biomarker for predicting plasma concentrations of 11-KT and VTG demonstrated by the low $R^2$ values; despite the low $R^2$, mucus can be used to look at temporal patterns and give an idea whether individuals are sexually mature and ready to spawn. Though greater resolution in VTG and 11-KT concentrations were achieved with plasma, there is a clear trade-off in terms of utility when compared to mucus.

Mucal swabbing was then applied to a laboratory experiment to evaluate potential endocrine disruptions in F. grandis undergoing a chronic exposure to a water accommodated fraction of crude oil. Three populations of F. grandis with different exposure histories were treated with WAF for 60 days, followed by an additional 40 days recovery in clean conditions. The mucal swabbing proved to be sensitive enough to pick up not only endocrine disruption, but
also differences in PAH metabolism illustrated by endocrine function among different populations. Concentrations of 11-KT and VTG changed with exposure to PAHs. Males from control sites had more severe declines in 11-KT concentrations than males from previously exposed sites. Exposed females showed near null concentrations of mucosal VTG when exposed to WAF regardless of exposure history while control groups did not exhibit decreases in mucus VTG concentrations. These data demonstrate the utility of mucus sampling as a less invasive tool to assess endocrine disruption in fish exposed to PAHs in laboratory and field settings, as well as showing potential multigenerational adaptation to PAH exposure and metabolism.

The multigenerational portion exposed two successive generations of Gulf killifish to PAH to determine if any tolerance had developed within a generation of exposure. The first generation was a population from Florida that had never been exposed to any contaminants. This population was exposed for 40 days to a water accommodated fraction of crude oil containing 40 µg total PAH / L, then allowed to recover and spawn for an additional 40 days immediately following the exposure. Males’ reproductive output was found to be the most affected by WAF treatment, for exposed males had a mean motile sperm concentration of $18.95 \times 10^7$/ml while control males had a mean motile sperm concentration of $92.05 \times 10^7$/ml. Reference site males exposed to WAF in the Chapter 2 had lower concentrations of mucosal 11-KT, which could explain the lowered sperm motility and concentrations seen in WAF males from the Chapter 3. Female reproductive output was not significantly different, however mean thickness of the vitelline envelope was significantly lower in exposed females. This study utilized an index of larval quality normally used in nutrition studies to draw a link between health of the F1 and F2 generations. Larval quality was evaluated using the survival activity index (SAI), a measure of starvation tolerance. Though no significant differences were found in SAI between the larvae
from WAF treated adults and larvae from control adults, control larvae survived on average 17.35 days when starved while WAF larvae survived only 15.27 days on average under the same conditions. Females exposed to WAF have lower concentrations of VTG as was demonstrated with the females exposed to WAF in chapter 2, which would lead to lower levels of egg yolk protein and resulting in lower SAI for larvae which exposed parents.

The offspring of these fish were also exposed to naphthalene. The mature F₂ generation was exposed to aqueous naphthalene for 40 days, and monitored for an additional 40 days after naphthalene treatments. The mucal swabbing technique developed previously was used to measure lysozyme concentrations. Surface mucus was again demonstrated as an effective biomarker, in this case for the innate immune system. Lysozyme was detected in the mucus of Gulf killifish and the technique distinguished groups of Gulf killifish with different parental exposure history upon exposure to naphthalene. EROD induction was determined using liver microsomes from individuals collected on day 40 and day 80, and fold induction was significantly increased in groups of killifish that underwent exposure to naphthalene compared to their control counterparts. These data demonstrate that tolerance to PAHs could potentially be developing within two generations of exposure. Evidence of PAH tolerance was also demonstrated in the previous study when males from the superfund site had increased concentrations of 11-KT when exposed to WAF as opposed to their reference site counterparts that showed decreased concentrations of 11-KT under the same conditions.

This thesis demonstrated (1) the utility of mucus to test for endocrine disruption, and (2) potential multigenerational adaptation and tolerance to PAHs. These results may be applied to future ecotoxicological studies, allowing for repeated sampling on small or endangered fishes. PAHs such as naphthalene and BaP have been classified as carcinogens to humans and other
vertebrates. This study and others have shown that PAHs adversely affect reproductive potential. This study will permit for better prediction of possible adverse effects of PAHs and specifically naphthalene at the population level in both diagnostic and predictive risk assessment.


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62

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