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Benzo[a]pyrene effects on Fundulus heteroclitus reproductive endpoints

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BENZO[a]PYRENE EFFECTS ON *FUNDULUS HETEROCLOTUS* REPRODUCTIVE ENDPOINTS

A Thesis
presented in partial fulfillment of requirements
for the degree of Master of Science
in the Environmental Toxicology Graduate Program
The University of Mississippi

by
FRANK T. BOOC

July 2013
ABSTRACT

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) that modulates aromatase enzyme function and, thus, potentially interrupts normal reproductive function. The aim of this study was to use a fish model, Fundulus heteroclitus, to assess whether BaP exposure adversely impacted reproduction. Adult fish were exposed to waterborne BaP concentrations of (0, 1 or 10 µg/L) for 28 days. Males and females were combined for the second half of the exposure (days 14-28) in order to quantitate egg production and fertilization success. Egg fertilization and subsequent hatching success of F1 embryos was significantly decreased by 10 µg/L BaP. In males, both gonadosomatic index (GSI) and plasma testosterone concentrations were significantly reduced compared to controls by 10 µg/L BaP. An increase in empty follicles and interstitial fibrosis was observed by histopathological examination of testes. Other biomarkers including male liver somatic index (LSI), liver vitellogenin (vtg) mRNA expression and sperm concentrations were not significantly affected. In females, estradiol concentrations were also significantly reduced after BaP exposure, but egg production, GSI, LSI, vtg expression and oocyte maturation were not altered. Steroid concentrations in Fundulus larvae from exposed parents at 1 and 3 weeks post hatch were not significantly changed. BaP exposure at these environmentally relevant concentrations caused negative alterations to both biochemical and phenotypic biomarkers associated with reproduction and multigenerational embryo survival.
DEDICATION

This work is dedicated to everyone who has been there for me throughout the highs and lows of life.
LIST OF ABBREVIATIONS AND SYMBOLS

11-ketotestosterone (11-KT)

17α-ethinylestradiol (EE2)

17β-estradiol (E2)

Aryl hydrocarbon receptor (AhR)

Benzo[a]pyrene (BaP)

BaP-7,8-dihydrodiol-9,10 epoxide (BPDE)

Coefficient of variance (CV)

Comprehensive Environmental Response, Compensation & Liability Act (CERCLA)

Cytochrome P450 (CYP)

Cytochrome P450 aromatase (CYP19)

Early life-stage (ELS)

Elizabeth River (ER)

Endocrine disrupting chemicals (EDC)
Epoxide hydrolase (EH)

Follicle stimulating hormone (FSH)

Gonadosomatic index (GSI)

Gonadotropin-releasing hormone (GnRH)

Gonadotropin receptor (GTR)

Hypothalamus pituitary gonad (HPG)

Institutional Animal Care and Use Committee (IACUC)

Lethal concentration (LC)

Liver somatic index (LSI)

Luteinizing hormone (LH)

Manganese superoxide dismutase (MnSOD)

Median tolerance levels (TLM)

New Bedford Harbor (NBH)

Octanol water partitioning coefficient (Kow)

Organization for Economic Co-operation and Development (OECD)
Polychlorinated biphenyls (PCBs)

Polycyclic aromatic hydrocarbon (PAH)

Reactive oxygen species (ROS)

Scorton Creek (SC)

Side-chain cleavage enzyme (SCC)

Solid phase extraction (SPE)

Steroid acute regulatory protein (StAR)

Testosterone (T)

Tributyltin (TBT)

Vitellogenin (vtg)
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CHAPTER 1: INTRODUCTION

1.0 Fundulus heteroclitus as a model organism

Many toxicological experiments require the use of in vivo studies with vertebrate animal models. Xenobiotic/contaminant alterations to normal homeostatic functions often involve complex organ systems. In vitro methods may better provide mechanistic data, but are unable to give a broad picture of physiological effects. Attempts at recreating these complexities through ex vivo techniques are technically demanding, expensive, and still cannot account for the whole animal. The following study utilizes a marine fish model Fundulus heteroclitus in order to assess the suspected reproductive and developmental effects of a potential endocrine disruptor. In general, fish models provide several advantages over rodent/mice models in terms of bioassay length and cost (Ankley and Johnson, 2004). In most fish embryonic development is also transparent, and thus, easier to visualize and assess. Negative effects from toxicants are often conserved between vertebrate species. Dioxins, like 2,3,7,8-tetrachlorodibenzodioxin, are very potent toxicants resulting as by-products of industry practice and manufacturing. In both humans and fish, a primary mechanism of toxicity is through aryl hydrocarbon receptor (AhR) activation (Bugel et al., 2013; Corton, 1996). AhR-mediated toxicity includes reproductive and developmental alterations including lowered spawning success, changes in ovarian morphology, and influences on immune
system development (Halden et al., 2011; Nagayama et al., 2007). In addition to the assessment of environmental toxicants, fish models are being widely used to study the mechanisms behind a variety of human diseases including Alzheimer’s and Parkinson’s as well as blood, kidney, and cardiovascular disorders (Gama Sosa et al., 2012; Howe et al., 2013; Dooley and Zon, 2000).

1.1 Habitat and general characteristics

*Fundulus heteroclitus* (Teleostei: Cyprinodontidae), also known as killifish or mummichog, typically inhabit brackish waters and coastal marshes along the US-Canada east coast ranging from Florida all the way up to the coast of St. Lawrence (Bigelow et al., 1953; Armstrong and Child, 1965). This species has a very small home range of around 35 meters, and therefore, does not migrate during either warm or cold seasons (Lotrich, 1975). Mature *Fundulus* will typically measure between 7 to 10 cm sometimes reaching up to 15 cm. The males are easily delineated by the series of spots located towards the tail with yellow bellies during the mating season. The females lack the distinct spotting and are more grayish in appearance (Armstrong and Child, 1965). *Fundulus* spawn during the summer months where longer periods of light and warmer climates are involved. During this season, *Fundulus* living north of the 41° latitudinal line spawn according to water temperature, while more southern populations exhibit spawning cycles that correlate with the lunar cycle during high tides (Taylor et al., 1979; McMullin et al., 2009). Females may carry several hundred viable eggs at one time with almost 100% fertilization success (Armstrong and Child, 1965). An additional advantage for toxicology laboratory studies is that *Fundulus* embryos are hardy. They can tolerate over a dozen degree range of temperatures from 12° to 27° F and properly
develop in different salinities from distilled water to seawater (Solberg, 1938). Hatching occurs approximately at ~17 dpf. The larvae will take 9-12 months to become reproductively mature, and they have an average lifespan of 4 to 5 years (Kneib and Stiven, 1978; Meyer et al., 2002).

1.2 Tolerance to environmental changes

The characteristic of having a small home range has necessitated the *Fundulus* to become very tolerant to environmental changes and stressors. They have the ability to withstand broad ranges of temperature, pH, and salinity (Doudoroff, 1945; Gonzalez et al., 1989; Marshall et al., 1999). In addition, there are multiple examples of tolerance to pollutants present in their habitats. *Fundulus* exposed to large concentrations of polychlorinated biphenyls (PCBs) at the New Bedford Harbor (NBH) Superfund site continue to survive due to their ability to adapt (Bello et al., 2001). PCB’s toxicity is mediated through the AhR pathway, and cytochrome P450 (CYP) 1A1 is typically induced by PCB exposure, yet in NBH-adapted fish, compared to fish from the relatively pristine Scorton Creek (SC) site, the CYP1A1 gene was significantly down-regulated. In another example, *Fundulus* larvae from a site affected by metal contamination showed resistance to methylmercury exposure. Compared with the pristine population, the contaminant adapted larvae showed a significant reduction in skeletal and cardiovascular alterations although total egg production of the parental generation was reduced as well (Weis et al., 1981). The Elizabeth River (ER) is another Superfund site and highly industrialized waterway with high concentrations of polycyclic aromatic hydrocarbon (PAH) contamination (Bieri et al., 1986). A primary mechanism of PAH
toxicity is due to their biotransformation into toxic metabolites by the CYP1 family of P450 enzymes (Tuvikene, 1995). Killifish have several adaptations that account for their resistance to PAH toxicity. First, ER fish exhibited significantly decreased CYP1A and CYP1B1 enzyme activity (Wills et al., 2010). Second, they also had significantly increased glutathione concentrations and manganese superoxide dismutase (MnSOD) activity (Meyer et al., 2003a). Both antioxidants are involved in the defense against reactive oxygen species (ROS) and free radicals that are the typical PAH metabolites. Lastly, two forms of AHR have been described in Fundulus, AHR1 and AHR2 (Karchner et al., 1999). Resistant populations exhibited increased expression of AHR1 in multiple tissues including the liver, kidney, and gills as well as reduced inducibility of AHR2 (Powell et al., 2000; Meyer et al., 2003b). Upon knockdown of AHR2, larvae exhibited a significant reduction in deformities associated with PAH mediated teratogenicity suggesting that the AHR2 pathway is responsible for potentiating PAH toxicity (Clark et al., 2010).

1.3 Short term bioassay and endocrine disruption

Historically, toxicity experiments using fish models started out only lasting one to four days with the focus on acute toxicity endpoints such as median tolerance levels or lethal concentrations (TLm/LC50s) of chemicals required to kill 50 percent of the exposed target species (Henderson et al., 1959). As concern grew for sub-lethal toxicant effects, more studies incorporated additional endpoints including survival, growth, fecundity, and hatching into whole life-cycle bioassays (Mount and Stephan, 1969). Identifying the more subtle consequences from toxicant exposure did have one key disadvantage which dealt with the drastic increase in required time to complete the
studies. For fathead minnows, a life-cycle experiment dealing with exposure to the insecticide chlorpyrifos, lasted over six months (Jarvinen et al., 1983). The switch from four to 200 day long exposures were also more expensive, so an effort was made to assess which of the endpoints associated with chronic testing would be most sensitive to toxicant exposure. Data from 32 full life-cycle tests with fathead minnows were analyzed (McKim, 1977), and it was concluded that the embryonic and juvenile life-stages were most susceptible. Early life-stage (ELS) testing was recommended for establishing water quality parameters and has more recently been validated by the Organization for Economic Cooperation and Development (OECD), and they have provided extensive guidelines for short term embryo toxicity screens (OECD, 1992). Important aspects of the OECD protocol included an exposure duration of 96 hours with mortality recordings at the end of each 24 hour period, less than a 10% mortality in the controls, and having a sample size of seven fish per concentration of test chemical used. The main drawback to this type of focus was the absence of reproductive biomarkers.

During the 1990’s, several studies observed that certain chemicals in the environment were interfering with wildlife endocrine systems. Tributyltin (TBT) was used as an anti-fouling agent in paints for the underside of floating vessels. Upon leaching into the aquatic environment, female molluscs, such as the sea snail (*Littorina littorea*) became masculinized due to morphological malformations in the oviduct and changes in prostate length (Bauer et al., 1995). Synthetic estrogens, such as 17α-ethinylestradiol (EE2) used in oral contraceptives, made their way into aquatic habitats due to the inability of sewage treatment plants to filter out these types of hormones (Larsson et al.,
1999). Newly hatched fish such as the chinook salmon later developed into 100% females after two hours of treatment with a high concentration of EE2 (400 μg/L) (Piferrer and Donaldson, 1992). Zebrfish exposed to much lower concentrations (5-25 ng/L) of EE2 did not result in severely skewed gender percentages but still exhibited a dose-dependent reduction in the number of females capable of spawning as well as a significant reduction in ovary size (Van den Belt et al., 2001). Recognition of endocrine active contaminants in the environment lead to amendments to the Safe Drinking Water Act so that the EPA could implement additional screening procedures to detect if certain chemicals could mimic the actions of hormones. They also defined endocrine disrupting chemicals (EDCs) as any toxicant/xenobiotic “that interferes with the synthesis, secretion, transport, binding action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior” (Kavlock et al., 1996). Due to the increased scope required for toxicant screening, fish bioassays with suspect EDCs needed to incorporate reproduction and development in a manner that assayed the most sensitive and relevant endpoints for the shortest exposure duration possible.

While some biomarkers of EDC exposure are more obvious in their importance for assessing changes to normal endocrine function and reproduction, clarification on the role and importance of vitellogenin (vtg) is necessary. Vtg is an egg yolk precursor protein that is normally synthesized in the liver of a sexually mature female fish. The primary factor inducing vtg production is estrogen. From the liver, it is carried in the bloodstream to the ovary and subsequently deposited into the oocytes (Wahli et al., 1981). This particular endpoint is used because both the male and female fish have the
molecular machinery to synthesize vtg, but only females have enough circulating estrogen concentrations to initiate and start vtg production. Measuring vtg in the males is, therefore, a great way to screen for potential estrogenic contaminants in the environment. Vtg induction also stunts male fish growth and spermatogenesis due to the diversion of metabolic resources (Herman and Kincaid, 1988) while abnormal increases of vtg in female fish have been associated with a significant reduction in eggs produced (Laenge et al., 1997).

Another important endpoint associated with EDC exposure is the quantitation of steroid concentrations. Altered steroid homeostasis is one of the most direct ways to confirm consequences of endocrine disrupters (Peters et al., 2007). Changes in downstream or upstream steroid hormones can also help investigators identify which steroid biosynthesis enzymes to focus on as potential toxicant targets. For example, ketoconazole is a fungicide that is a steroidogenesis inhibitor. This particular xenobiotic causes significant reductions in testosterone (T) and 17β-estradiol (E2) in fathead minnows (Villeneuve et al., 2007). Investigators determined that an important upstream cholesterol side-chain cleavage enzyme (P450SCC) was being significantly down-regulated in exposed fish and, thus, provided a plausible mechanism for the reduction in both androgens and estrogens (Ankley et al., 2009).

In order to more efficiently screen for EDCs, fish bioassays with reproductive endpoints were conducted in much shorter time-frames compared to traditional life-cycle experiments. One particular study using fathead minnows (Ankley et al., 2001) involved a 2-3 week pre-exposure phase followed by a short-term reproduction test that spanned 21 days. Four replicate tanks were used with four females and two males per
tank. There were two environmentally relevant concentrations of methoxychlor and methyltestosterone used with a 5-10 dilution factor between high and low doses. The primary endpoints studied were egg production, gonadosomatic index (GSI), gonad histology, sex steroids, vtg, hatching success, as well as larval development and morphology. Significant reductions in fecundity, plasma concentrations of T, 11-ketotestosterone (11-KT), and E2 occurred, but these effects frequently did not appear in a dose dependent manner as variability was present with observations at the highest exposure concentrations. A drawback of the study design was the lack of a power analysis to check if a sample size of four tanks would be sufficient to limit the chance of having a type II (β) statistical error. Type II error represents the likelihood of failing to observe significant toxicant effects when present. The assay proposed was quick and cost efficient, but it had limited sensitivity to detect toxic changes.

A similar short-term endocrine bioassay using Fundulus was also developed (MacLatchy et al., 2003). Exposures only lasted for 1-2 weeks and also used four replicate aquaria per treatment group, but problems with the experimental design were again noted. There was pseudo-replication because all fish present in one tank shared the same living conditions and should not have been counted as separate samples. A second problem was that there was again no determination of type II error. Lastly, the endpoints measured only dealt with steroid and vtg concentrations, but failed to incorporate egg production. In subsequent studies (Peters et al., 2007), several fecundity endpoints including egg production, spawning rates, and percent fertilization were added to the experimental design. Furthermore, the exposure duration was increased to a total of 28 days in order to better assess chronic toxicity and to determine
endocrine biomarker changes when males and females were separate and together although statistics were still conducted by fish.

To measure the power to detect significant differences in egg production, a meta-analysis was conducted on over 62 different reproductive studies using fish as the model organism (Bosker et al., 2009b). The average sample size was 5.7 with a mean coefficient of variance (CV) of 43.8%. The study indicated that the lack of replicates, absence of a pre-selection phase for breeding tanks, and short collection periods in typical bioassays resulted in a low power of around 10% with only the ability to detect approximately a 70% reduction in egg production (Bosker et al., 2009). Decreased fecundity is a critical indicator to predict impacts of EDC exposure at a population level. One particular modeling experiment using fathead minnows indicated that a 40% loss in fecundity could cause almost a 100% reduction of the wild population after a simulated 20 years of exposure to 17-β-trenbolone (Miller and Ankley, 2004).

Bosker and colleagues continued to optimize the *Fundulus* reproductive bioassay including significant improvements to increase power to detect significant changes in egg production from 11.2 to 85.7% with an effect size of 25% (Bosker et al., 2010). One of the refinements dealt with increasing the sample size from four to eight tanks, although the largest improvement occurred as a result of an extensive pre-selection phase in which 48 20-L aquariums were stocked with three males and three females each. Eggs were collected twice a week for two weeks. In order to achieve around 80% power at a 25% effect size, any tank that averaged 30% below the total egg production or was outside the range of one standard deviation was not used for the experiment. In the same study, an additional refinement included an extension of the egg collection
phase from 1 to 4 weeks which alone increased the power to detect fecundity alterations from 36 to 62%. The efforts of several of these short-term improvements acted as the guiding framework for the following toxicological investigation into benzo[a]pyrene (BaP) described in this thesis.

2.0 Steroid biosynthesis pathway

Steroids are synthesized in a variety of organs including the brain, adrenal gland, and placenta. The major androgens and estrogens are primarily produced in the leydig cells of the testis and the granulosa and theca cells in the ovary (Rempel and Schlenk, 2008). The major androgens in fish are T and 11-KT while the major estrogen is E_2. In female fish, estrogen has prominent roles in regulating sexual differentiation, reproductive behavior, and germ cell development (Callard et al., 2001; Guiguen et al., 2010; Bhandari et al., 2005). Spermatogenesis in males is primarily regulated by T, while 11-KT is responsible for the final maturation of spermatids into spermatozoa during spermiogenesis (Cochran, 1992). The initial signal for steroid production comes from luteinizing and follicle stimulating hormones (LH/FSH) produced from the pituitary gland. Upon binding with the gonadotropin receptors (GTH-R1,R2), free cholesterol is released in the target tissue (Rempel and Schlenk, 2008). Cholesterol is the starting organic molecule in any steroid network whether the vertebrate be mouse, fish, or human (Figure 1). Cholesterol is transported from the outer to inner mitochondrial membrane by the steroid acute regulatory (StAR) protein where it is subsequently converted to pregnenolone by P450scc (Jefcoate et al., 1992; Arukwe, 2008). Pregnenolone is the obligate steroid precursor hormone that can further be converted to sex steroids. There are two other types of downstream steroids which include cortisol and aldosterone.
These steroids play roles in stress response and blood pressure regulation, respectively, but the focus on endocrine disruption remains primarily on the major gonadal steroids.

![Steroid Biosynthesis Pathway](image)

**Figure 1. Steroid Biosynthesis Pathway**

2.1 Cytochrome P450 aromatase (CYP19)

Downstream in the biosynthesis pathway, cytochrome P450 aromatase (CYP19) is the primary enzyme responsible for the conversion of androgens into estrogens. In almost all mammalian models except for the pig, there is only one form of the gene that is constitutively expressed in several different tissues including placenta, adipose, gonad, and brain (Conley and Hinshelwood, 2001). Fish however, have two distinct forms of the CYP19 gene which include CYP19A1 (gonad form) and CYP19A2 (brain form) (Dong and Willett, 2008). The brain form is involved in sexual behavior,
morphology, and neuronal differentiation while the gonad form is involved in oocyte
development and sexual differentiation (Callard et al., 2001; Kwon et al., 2001; Melo and
Ramsdell, 2001). Alterations to aromatase from EDCs can have negative consequences
on endocrine related functions. Clotrimazole is a fungicide that inhibits both forms of
aromatase activity in fish (Noaksson et al., 2003; Shilling et al., 1999) and is implicated
in changes to gonad physiology (Baudiffier et al., 2013). Fadrozole is another known
aromatase inhibitor that significantly decreases the number of mature oocytes in
fathead minnows (Ankley et al., 2002).

2.2 Relationship with the hypothalamus-pituitary-gonad (HPG) axis

The initiation of steroid biosynthesis is largely under the regulation of the
complex feedback loop associated with the HPG axis. First, the hypothalamus in
response to stimuli from the central nervous system, releases gonadotropin-releasing
hormone (GnRH) that further stimulates the pituitary gland to release LH/FSH (Naor and
Catt, 1981). LH in males is responsible for initiating testosterone production in the testis
while FSH has a role in spermatogenesis and vtg production (Rempel and Schlenk,
2008). Steroid hormones have the ability to up or down-regulate the transcription of
precursor peptide hormones due to the presence of nuclear receptors in the pituitary
and hypothalamus (Spencer et al., 1991; Clayton et al., 1980). Due to the complexity of
the axis, any disruption to homeostatic function in one component would likely produce
effects in to all three parts and ultimately affect reproductive success.

3.0 PAHs/BaP
Polycyclic aromatic hydrocarbons (PAHs) are a class of environmental contaminants that are ubiquitous and persistent in the environment due to their low water solubility (Cerniglia, 1993). The simplest PAH is naphthalene and contains only two rings. BaP is considered a high molecular weight PAH with five fused rings and an octanol water partitioning coefficient ($\text{LogK}_{\text{ow}}$) of 6.06 (Evans and Landrum, 1989). As the number of rings increases, so does the hydrophobicity, but solubility and volatility are reduced making BaP even more persistent in the sediment.

3.1 Sources of PAH/BaP contamination and effects on humans

PAH exposure occurs through a number of inhalation routes from both man-made and natural sources such as the incomplete combustion of fossil fuels, tobacco smoke, forest fires, and volcanic eruptions (Baek et al., 1991; Miller et al., 2004). PAHs are also consumed from burnt foods and contaminated fruits and vegetables (Larsson et al., 1983; Rojo Camargo and Toledo, 2003). The primary routes for PAH contamination in the aquatic environment result from atmospheric deposition, surface runoff, industrial activity, as well as uncontained leaks. Pregnant women exposed to PAHs are reported to have increased difficulty in conceiving, increased rates of miscarriage, and developmental abnormalities in their offspring (Choi et al., 2006; Wu et al., 2010a). The mechanisms behind reproductive difficulty following PAH exposure are not well known. Tobacco smoke carries a wide array of PAHs and other chemicals (Gmeiner et al., 1997), and women exposed to cigarette smoke also have been observed to have a significant increase of BaP in their follicular fluid (Neal et al., 2008).
3.2 PAH effects on aquatic vertebrae

Fish have exhibited several forms of toxicity resulting from PAH exposure. Zebrafish embryos exposed to phenanthrene (3-ringed PAH) experienced defects to cardiac conduction and morphology including edema and abnormalities to craniofacial structures, spine, and jaw size (Incardona et al., 2004). The same PAH also caused direct cytotoxicity to rainbow trout gill cells (Schirmer et al., 1998). Higher molecular weight PAHs such as BaP are implicated in the formation of DNA adducts and liver tumors in several species of fish including Japanese medaka, guppy, feral eels, and killifish (Hawkins et al., 1990; van der Oost et al., 1994; Willett et al., 1995; Wang et al., 2010). BaP’s role in tumor induction stems from its biotransformation into BaP-7,8-dihydrodiol-9,10 epoxide (BPDE) metabolite which is often called the “ultimate carcinogen” (Lehr et al., 1985). Upon metabolism by the CYP1 family of P450s, an initial epoxide is formed between the 7-8 positions on the molecule (Fig. 2). Epoxide hydrolase (EH) subsequently converts the epoxide into a dihydrodiol which can further be metabolized and conjugated through phase II metabolism and excreted. However, in BaP’s case, the BaP-7,8-diol can again be metabolized by P450 creating an epoxide between the 9-10 position. Due to the presence of a bay region between the 10-11 positions, the area is considered too obstructed for EH to detoxify the epoxide. BPDE can then covalently bind to DNA and cause oxidative stress and tumor induction (Shimada and Fujii Kuriyama, 2004).
Figure 2. BaP Metabolism (adapted from Shimada and Kuriyama, 2004)

Extensive work has assessed PAH effects on the fish immune system. Winter flounder and Atlantic cod exposed to sediment contaminated with petroleum hydrocarbons showed signs of immunosuppression due to increased susceptibility to infection from a blood protozoan, *Trypanosoma murmanensis* (Khan, 1987). Both infected species exhibited increased rates of mucus production, mortality, and deficiencies in gonad development. With regards to acquired immunity, PAH exposure reduced the proliferation of T-lymphocytes in spot croaker and Japanese medaka (Faisal and Huggett, 1993; Carlson et al., 2002). Lysozymes and phagocytes associated with non-specific immunity were depressed in fish injected with PAH containing extracts of diesel-oil mud and BaP (Tahir and Secombes, 1995; Carlson et al., 2004).
Specific to PAH-induced reproductive toxicity, American flagfish exhibited significant reductions in egg production after exposure to oil-water emulsions in static and flow-through conditions (Hedtke and Puglisi, 1980). A six month exposure to petroleum hydrocarbons significantly reduced the testis somatic index in cunner fish (Payne et al., 1978) while Atlantic cod chronically exposed to PAHs experienced delays in spermatogenesis, spermiation, and reduced food consumption (Kiceniuk and Khan, 1987; Khan and Kiceniuk, 1984). English sole collected from a highly industrialized waterway (Puget Sound, WA) exhibited lower spawning success and depressed plasma vtg and estradiol concentrations (Casillas et al., 1991). A more recent study with Nile tilapia inhabiting a PAH contaminated lake system with over 100 active oil wells resulted in vtg induction and increased steroid concentrations (Rodas-Ortiz et al., 2008). Often, fish are exposed to complex mixtures of PAHs in the environment and elucidating the mechanisms responsible for reproductive and developmental alterations can prove to be a difficult task. As a result, the following investigation focuses on BaP as a model hydrocarbon in order to analyze reproductive deficits associated with PAH exposure.

3.3 BaP as an endocrine disruptor

BaP is ranked as the eighth most hazardous substance by Comprehensive Environmental Response, Compensation & Liability Act (CERCLA) standards (http://www.atsdr.cdc.gov/SPL/index.html). In addition to being a carcinogen and immuno/developmental toxicant, BaP modulates aromatase in fish, but with variable effects. Killifish exposed to environmentally relevant waterborne concentrations of 10 µg/L BaP for 15 days did not exhibit mRNA alterations to either CYP19 form, although enzyme activities for female ovarian aromatase were significantly depressed during
both summer and winter BaP exposures while female brain aromatase activity significantly increased in the winter (Patel et al., 2006b). Zebrafish larvae exposed to BaP for three days showed a significant increase in CYP19A2 mRNA expression while a chronic 56 day exposure with zebrafish adults resulted in reduced female ovary size, decreased egg production, and a significant increase in brain aromatase expression (Hoffmann and Oris, 2006). Japanese medaka embryos exposed to similar concentrations of BaP (0.01-10 µg/L) caused delays in hatching time and reductions in body weight (Chikae et al., 2004). The conflicts observed with aromatase alterations potentially resulted from the use of whole tissue or larvae extracts in PCR analysis. A more recent study with killifish observed the cellular expression of aromatase in larvae, juveniles, and reproductively mature adults through the use of in situ hybridization (Dong et al., 2008). At both 10 and 100 µg/L concentrations, BaP caused a significant reduction in CYP19A2 expression during all observed stages of development, but CYP19A1 was only reduced during the juvenile phase. Even though the data is not uniform, significant alterations occurred with an important steroid enzyme and with reproductive biomarkers. Our primary aim is to incorporate Fundulus heteroclitus in a short term reproductive fish bioassay to further evaluate BaP’s effects on reproduction and development.

3.4 Study Hypotheses

Due to BaP’s role in reducing aromatase expression in Fundulus and documented adverse outcomes on fertility following PAH exposure, we expect BaP to cause negative alterations to reproductive success and development. Additional
molecular endpoints such as circulating testosterone and estradiol concentrations, vtg, and histopathology will also be altered by BaP.
CHAPTER 2: BENZO[a]PYRENE EFFECTS IN *FUNDULUS HETEROCLOTUS* USING A SHORT-TERM REPRODUCTIVE BIOASSAY
Introduction

The steroid biosynthesis pathway and its involvement with the hypothalamus-pituitary-gonad (HPG) axis is one of the key factors involved in maintaining proper function of reproduction and development. The multitude of enzymes working in concert is very similar between vertebrate animals (Payne and Hales, 2004; Lohr and Hammerschmidt, 2011). Upstream peptide hormones are responsible for stimulating steroid production (Zohar et al., 2010), and gonadotropins are synthesized in the hypothalamus and stimulate the pituitary gland to produce luteinizing and follicle stimulating hormones (LH/FSH) (Shimizu et al., 2008). They subsequently initiate a cascade of events that leads to cholesterol being converted into downstream steroids that include testosterone and estradiol. Aromatase is one of the primary downstream enzymes responsible for converting androgens into estrogens (Simpson, 2003). In fish, two different genes encoding aromatase CYP19A1 and CYP19A2 are localized in the gonads and brain, respectively (Callard and Tchoudakova, 1997).

Negative effects on teleost reproduction have resulted from changes in steroidogenic enzyme expression and hormone concentrations (Munkittrick et al., 1991; Rempel and Schlenk, 2008b). Environmental endocrine disrupting chemicals (EDCs) are a primary culprit in destabilizing the HPG axis of organisms residing in aquatic habitats. One such class of EDCs includes polycyclic aromatic hydrocarbons (PAHs) that result from the incomplete combustion of carbon. PAHs have many natural and anthropogenic sources which include forest fires, volcanic eruptions, tobacco smoke, and car exhaust (Maisto et al., 2006; Neal et al., 2008; Olivella et al., 2006; van Metre et al., 2000).
Benzo[a]pyrene (BaP) is a PAH that is known for causing cancer and is an aryl hydrocarbon receptor ligand that stimulates the expression of the CYP1 family of P450 monooxygenases. The same enzymes also biotransform BaP into several metabolites (Scornaienchi et al., 2010), one of which is considered the ultimate carcinogen, due to the presence of a stable epoxide (Van et al., 1985). More recently, BaP has been recognized as a reproductive and developmental toxicant. Reproductive impairment has occurred in wild populations of fish, including Fundulus, residing in PAH contaminated environments (Pait and Nelson, 2009; Collier et al., 1998; Nicolas, 1999). In BaP-injected croaker (Microgonias undulates) ovarian growth and steroidogenesis were decreased (Thomas, 1990). BaP also affects fish aromatase (CYP19) expression, although the reported effects vary depending on study design (Patel et al., 2006; Hoffman and Oris, 2006). Through the use of in situ hybridization, cellular expression of CYP19A2 in the brain was significantly reduced in both Fundulus larvae and adults by BaP (Dong et al., 2008).

The following study utilized Fundulus heteroclitus in a short term waterborne exposure in order to assess if BaP’s published effects on aromatase modulation would translate into reproductive deficits. The experiment was adapted from previously described studies (Bosker et al., 2010; MacLatchy et al., 2003; Peters et al., 2007) in order to quickly observe potential impacts to reproductive and steroidogenic endpoints of BaP exposure.
Materials and methods

Fish Care

A parental population of *Fundulus heteroclitus* was collected from an uncontaminated site at the Newport River near Beaufort Inlet, NC. They were raised under the University Institutional Animal Care and Use Committee (IACUC) approved conditions. Sexually mature fish were bred and kept in salt water (20-25 ppt). The temperature was maintained at 20-25 °C with a 14:10 light-dark cycle in the summer versus a 10:14 light-dark cycle in the winter. Adult fish were fed twice daily with tropical flake fish food (Tetramin, Tetra Werke, Germany) and live brine shrimp. First generation offspring from wild parents were used for the studies described here.

Adult BaP Exposure

For 14 or 28 days adult male and female *Fundulus* were exposed to the following treatments: control (300 µL ethanol = solvent carrier), BaP 1µg/L or BaP 10 µg/L. For the first 14 days, males and females were kept in separate 30-L tanks with 6 fish per tank and 5 tanks per treatment group. Half of the fish were dissected on day 14 for the liver, gonad, sperm, and blood. The fish were euthanized with MS-222, and their weights and lengths recorded. Blood was collected using a 10 µL microcapillary tube (Drummond Scientific) after cutting off the caudal fin. Plasma was separated from serum by centrifuging the blood at 2400 x g for 12 minutes at 4°C. Plasma samples were stored at -80°C for steroid extractions. For days 14-28 of the exposure, the sexes were combined with 6 fish per tank and 6 tanks per treatment group. Spawning and reproductive success were measured by collecting eggs every other day from days 16-
28. The total number of eggs and fertilization success were counted and calculated. Hatching success was measured on days 15-18 post fertilization. Unhatched *Fundulus* embryos were aerated for the same amount of time per group each day. The number of hatched larvae was recorded immediately following aeration. Hatching percentage was calculated for each parental tank by (cumulative # hatched larvae/total # eggs collected * 100%). Additionally, eggs were raised until 1 and 3 weeks post hatch when larvae were flash frozen, and stored at -80°C for further steroid extraction and analysis. The remaining adults were dissected on day 28 as described above. To achieve six tanks per treatment two separate 28 day exposures were needed. Exposure conditions were 21-24°C, 14:10 light-dark period, and 23-24 ppt salinity. Fish were kept in the tanks for at least 1 week for acclimatization prior to the start of the exposure, and tanks were routinely checked for elevated ammonia. During the BaP exposure, water was changed every 24 hours (90% static water renewal) and tanks were subsequently dosed at approximately the same time each day. Water samples (100 to 200 mL) were collected to confirm nominal concentrations of BaP using gas chromatography/mass spectrometry in selected ion monitoring mode (Wang et al., 2006b).

**Sperm Counts**

In order to quantify the number of sperm, 0.01g of male gonad was homogenized in 50 µL of 18 ppt salinity H2O for 1 min. Then 1 µL of the homogenate was diluted in 99 µL of 18 ppt H2O/trypan blue mix and the solution was vortexed and loaded onto both chambers of a Neubauer hemocytometer and incubated at 26º C for 10 minutes. Sperm were counted according to WHO laboratory manual for the examination and processing of human semen 5th edition at 400x magnification (Olympus BX40). In brief, sperm from
the central grid (number 5) were counted until a total of at least 200 spermatozoa were observed in a complete row. Counts were repeated in the second chamber. The sum and difference of the two numbers were calculated and an acceptability value of the difference was determined. If the difference was acceptable, concentration was calculated. However, if the difference was too high, two new dilutions were prepared and counted. The following equation was used to determine sperm concentrations: 

\[ C = \frac{(N/n) \times (1/100) \times 50\mu L}{g \text{ gonad}} \]

where \( C \) = concentration, \( N \) = number of sperm and \( n \) = volume in hemacytometer; 1/100 was the dilution used, and 50 µL was the volume in which the gonad was homogenized.

Sex Steroid Extraction and Quantification

Plasma samples were thawed on ice and pooled from 2 or 3 fish of the same sex. The ether extraction protocol used for adult Fundulus was adapted from a previous study (MacLatchy et al., 2005). Briefly, pooled samples were added to 100 µL of DI H\(_2\)O in a glass test tube. Then 5 mL of ether was added and each test tube was vortexed for three times of 15 second intervals. Samples were then incubated at room temperature for 10 minutes. The glass tubes were placed in an acetone/dry ice bath for 1 minute and subsequently swirled to ensure that the white precipitate would stick to the bottom. The rest of the clear solution was decanted into a 20 mL scintillation vial. The addition of 5 mL ether and subsequent steps were repeated two more times. The scintillation vials were kept uncapped and allowed to evaporate overnight in the fume hood. Once completely dry, 1 mL of phosgel buffer (40.5 mM Na\(_2\)HPO\(_4\), 9.3 mM NaH\(_2\)PO\(_4\)-H\(_2\)O, 0.1% gelatin, 0.25 mM thimersol) was added and the steroids were allowed to reconstitute into the buffer for 20 minutes at room temperature. The samples were
stored at -20ºC until analyzed by radioimmunoassay as previously described (Dube and MacLatchy, 2001; MacLatchy et al., 2005).

The methanol extraction procedure used for whole larvae homogenates was also adapted from a previous study (Morthorst et al., 2013). Five larvae were pooled together in a 1.5 mL epitube. The sample was homogenized for 1 minute in 50 µL of homogenizing buffer consisting of PBS and 1mM EDTA using a pellet pestle. Following homogenization, 200 µL methanol was added and samples were vortexed for 1 minute. They were stored at 4ºC for 1 hour with intermittent vortexing every 15 minutes. Following the incubation period, the tubes were centrifuged at 3,000 x g for 5 minutes. They were frozen on dry ice and the supernatant was transferred into 7 mL glass tubes. The addition of methanol and subsequent steps were repeated two more times. Samples were stored at -80º C until evaporated with a gentle stream of nitrogen gas. Once completely dry, samples were reconstituted with 1 mL of 50 mM acetate buffer, incubated at room temperature for 30 minutes and stored at -20ºC until ready for solid phase extractions (SPE DSC-18, 100mg; Sigma-Aldrich). The larvae samples were passed through the mini columns according to manufacturer's instructions for reversed phase sorbents. Ethyl acetate (1% methanol) was used to collect steroids and the solution was dried under a gentle stream of nitrogen gas. Larvae samples were reconstituted in 210 µL of EIA buffer. Estradiol and testosterone were analyzed with enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions.
Vitellogenin mRNA Expression

*Fundulus* livers were stored in 1 mL RNA later (Ambion, Austin, TX) until RNA was extracted, reverse transcribed, and vitellogenin (vtg) expression was analyzed through real-time PCR. Detailed methods have previously been described (Wang et al., 2006). Primers specific to vtg were synthesized by Invitrogen (Carlsbad, CA) = Forward: 5’-GAGGATCTGTGCTGATGCAGTTGTG-3’; Reverse: 5’-GGGTAGAAGGCAGTCTTTCCC-3’. Use of these primers has been described previously with *Fundulus* (Garcia-Reyero et al., 2004).

Gonad Histology

Gonads were fixed in 20 mL of 4% (w/v) paraformaldehyde overnight, and then processed by dehydration through increasing concentrations of ethanol. Tissues were subsequently rinsed with Clearify (American Master Tech Scientific, Lodi, CA) and were embedded in molten paraffin (paraplast embedding media paraplast X-tra, Sigma, St. Louis, MO). Gonads were sectioned at 5 µm thickness using a microtome (Olympus Cut4055, Olympus American, San Jose, CA) and subsequently stained with hematoxylin and eosin (HE) for ovary/testis staging.

The developing oocytes were divided into three different categories that include previtellogenic (stage 1a-1c), vitellogenic (stage 2), and mature (stage 3). The size based divisions have previously been described (Wallace and Selman, 1985; Dong et al., 2008; Fang et al., 2010) and number of oocytes at certain stages were normalized to normal area of the ovary being examined (n=5-6 fish for 2 wk (1-2 fish/tank; 3 tanks/treatment) and 10-12 fish for 4 wk (1-3 fish/tank; 6 tanks/treatment)).
Within the secondary spermatocyte region of testis, the number of empty follicles and total follicles were counted within six grids of 50,000 pixels² per grid using Image J. Presence of interstitial fibrosis (thickened walls) was recorded for each testis (n = 6 tanks with 1-3 fish/tank) (Bugel et al., 2010). All gonad scoring was done blind to treatment.

Statistics

Results were analyzed by Graphpad Prism 5.0 and presented as mean ± S.E. Statistical differences between treatment groups were determined using unpaired t-test or one-way ANOVA followed by tukey’s post test. Non-parametric data were analyzed by Kruskal-Wallis followed by Dunn’s post test (p<.05).

Results

Water concentrations and survival

Actual water concentrations for 1 µg/L and 10 µg/L BaP averaged 1.34 ± 0.35 µg/L and 18.7 ± 6.99 µg/L, respectively. Female adults had 100% survival for all treatment groups. Males exhibited 90% survival in the controls and 100% for 1 and 10 µg/L BaP.

Steroid concentrations

Steroid concentrations determined at both the 2 week and 4 week time were not statistically different so data was pooled. Control male testosterone and female estrogen concentrations were 2510 ± 691 pg/mL and 7540 ± 1450 pg/mL plasma, respectively (Fig. 3 A and D). Testosterone in the males was significantly reduced by 10 µg/L BaP to 355 ± 242 pg/mL plasma. Estradiol concentrations were also significantly
reduced to 3510 ± 1010 pg/mL plasma. *Fundulus* larvae at 1 and 3 weeks post hatch did not exhibit any significant changes to testosterone (Fig. 3 B and C) and estradiol (Fig. 3 E and F) concentrations when whole larvae were homogenized.

Gonado- and liver somatic indexes (GSI and LSI)

Males did not experience any changes to GSI after 2 weeks exposure, but exhibited a significant decrease after 4 weeks in the 10 µg/L treatment group (n = 5-6 tanks; 2-3 fish/tank; Fig. 4). There were no changes in female GSI at either time point between treatment groups, but female controls did have a significantly higher GSI at the 4 week time point compared to the 2 week controls (Fig. 4 and 5). The LSI for both sexes was not significantly altered from the exposure (Fig. 6).

Fertilization success, egg production, and hatching success

From days 14-28 of the exposure, sexes were combined and eggs were collected every other day. Although total egg production was not significantly altered (Fig. 7A), fertilization success was significantly lower in the 10 µg/L treatment group (Fig. 7B). Total egg production averaged 287 ± 24.4, 270 ± 47.3, and 398 ± 78.6 per tank while fertilization success averaged 91 ± 1.0, 72 ± 9.3, and 71 ± 5.9 percent for control, 1 BaP, and 10 µg/L BaP, respectively. Hatching success was measured at days 15-18 post fertilization (dpf) (Fig. 8). By 18 dpf, 93.4 ± 1.1 percent of F1 control embryos had hatched whereas only 79.8 ± 5.7 percent of F1s from the high dose BaP-exposed parents had hatched (p=0.024, one-tailed, two sample T-test).
Gonad morphology and sperm concentrations

Testes were evaluated for the number of empty follicles and presence of increased interstitial fibrosis. The percentage of empty follicles increased from 13.8% ± 2.81 for controls to 24.7% ± 6.6 for 10 μg/L BaP exposed males (p=0.07, one-tailed, two-sample T-test) (Fig. 9). No significant differences in the number of sperm produced in each treatment were found during either sampling period (Fig. 10). Treatment and exposure time did not alter the proportions of differently staged oocytes (Fig. 11).

Vitellogenin expression

Quantitative RT/RT-PCR was used to quantitate vtg mRNA expression in male and female liver. In males, vtg mRNA expression was very low and highly variable. When both males and females from all treatment groups were normalized to the 2 week control males, females expressed at least 3000-fold more vtg expression (Fig. 12). In the females, expression was high but also highly variable. After 4 weeks of 10 μg/L BaP exposure, females had significantly higher vtg mRNA expression compared to 1 μg/L, but not compared to controls (Fig. 13B).
Discussion

Short-term reproductive bioassays are used to quantitate the effects of EDCs on fish reproduction and endocrine-related biomarkers. In *Fundulus* these studies have been developed and optimized using primarily 17α-ethynylestradiol as the stressor (Blewett et al., 2013; Bosker et al., 2009; Bosker et al., 2010; Doyle et al., 2013; MacLatchy et al., 2003). Yet, there is evidence both from wild fish populations (Pait and Nelson, 2009; Bugel et al., 2010) and mammalian studies that PAHs, including BaP, also adversely impact reproductive potential. For example, pregnant women who smoke or are exposed to second-hand smoke have increased concentrations of BaP in their follicular fluid (Neal et al., 2008). Furthermore, this PAH exposure has been correlated with increased difficulty in conceiving, increased rates of miscarriage, and developmental abnormalities in their offspring (Lee et al., 2011; Neal et al., 2008; Wu et al., 2010). As reported here, in *Fundulus* exposed to 10 µg/L waterborne BaP, there was a more than 20% decrease in the fertilization success and a 14% decrease in F1 hatching success/survival.

The most significant concern associated with decreased reproductive success from EDC environmental exposure is that there could be population level impacts. Precedent for potential population crashes was provided by whole lake experiments wherein fathead minnow populations crashed following chronic low dose ethynylestradiol exposure (Kidd et al., 2007). While it is more difficult to scale laboratory-based studies to population effects, a study with wild salmon used a projection matrix model in order to show 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). Ankley and coworkers (Ankley et al., 2008) projected that a 50% decrease in fathead minnow plasma estradiol concentrations would correlate with a 92% decrease in population size after five years. BaP 10 μg/L exposure did cause a 53% reduction in Fundulus female estradiol concentrations so the potential for population impact from PAHs is conceivable. The environmentally relevant doses used in this study bracket the pure water solubility of BaP (4 μg/L) (Mackay and Shiu, 1977), but fish from contaminated locations would be expected to be exposed to potentially higher concentrations from sediment (Kimbrough and Dickhut, 2006).

In this study, fertilization success and not egg production was statistically decreased, thereby suggesting potential male sensitivity. In fact, male GSI and plasma testosterone concentrations were decreased by 38% and 86%, respectively. Furthermore, when histopathological analysis of testes was done, BaP exposure caused an increasing trend in the number of empty follicles (p=0.07) and increased interstitial fibrosis. Similar lesions were found in Fundulus collected in Newark Bay though the lesion incidence was not statistically higher than in fish from Tuckerton (a less contaminated site) (Bugel et al., 2010). Increased interstitial fibrosis of gonad connective tissue particularly in males has been noted in a number of fish studies following EDC exposure (reviewed in (Dietrich and Krieger, 2009). Importantly, despite the impacts on male reproductive parameters mentioned, sperm counts were not statistically decreased. However, in this study sperm fitness or motility were not assessed. In mammals, there is precedent for the sensitivity of male fertility to BaP. Sperm counts and sperm motility were decreased and testicular malformations increased in multiple generations of mice following a F0 6-week, daily oral BaP exposure (Mohamed et al., 2010).
A classic biomarker of exposure to environmental estrogens is altered vtg expression, an egg yolk protein, and especially its induction in male fish (Jones et al., 2013; Hutchinson et al., 2006). While many studies measure circulating vtg protein expression, a quantitative PCR approach for liver mRNA expression was used here. Using PCR, *Fundulus* males exposed to estradiol had ten-times higher expression compared to control females whereas nonylphenol was a weak inducer (Garcia-Reyero et al., 2004). In this study, BaP did not cause a dose-dependent effect on vtg mRNA expression in either males or females, though as would be expected, vtg expression was increased in reproductively active females (4 week vs 2 week, Fig. 6).

In females, despite a significant reduction in estradiol concentrations, egg production was not significantly affected. Conservation of egg production capability despite exposure to high concentrations of EDCs may be a *Fundulus*-specific phenomenon. Doyle and coworkers (Doyle et al., 2013) note that in many freshwater fish laboratory models relatively low doses of ethynylestradiol decrease egg production, and *Fundulus* are resistant possibly because of their ability to maintain lipid transport into the ovary. This insensitivity may explain how *Fundulus* populations can survive in contaminated environments (e.g. adjacent to Superfund sites) (Prince and Cooper, 1995; Bello et al., 2001a; Meyer and DiGiulio, 2003; Greytak et al., 2005). In contrast to *Fundulus* ovo-insensitivity, in mice, BaP causes ovotoxicities including increased primordial follicle activation, developing follicle atresia, and effects on membrane fluidity that impair fertility; all toxicities that are consistent with PAH-exposure related premature ovarian failure and infertility in humans (Sobinoff et al., 2012).
The 53% decrease in circulating estradiol concentrations is consistent with the reduction in brain aromatase mRNA expression found in both *Fundulus heteroclitus* larvae and adults (Dong and Willett, 2008) and ovarian aromatase enzyme activity after waterborne exposure to BaP (Patel et al., 2006). The reduction in testosterone concentrations in the adult males was perplexing because the reduction in aromatase expression would hypothetically lead to an increase in circulating androgens. A study in rats also showed a significant decrease in testosterone after BaP exposure and provided a plausible mechanism for the change. Through acetylation-mediated epigenetic changes, the expression of the steroid acute regulatory protein (StAR) was significantly reduced (Liang et al., 2012). StAR is an important protein upstream in the steroid biosynthesis pathway (Manna et al., 2003) responsible for transporting cholesterol from the outer to the inner mitochondrial membrane where another P450 side chain cleavage enzyme converts cholesterol to the obligate steroid precursor pregnenolone. To our knowledge, BaP’s effects on StAR expression have not been studied in *Fundulus*, and additional insight into these upstream enzymes and steroids will contribute to better understanding of mechanisms related to the reproductive and developmental toxicities associated with PAH exposure.

The recognition of BaP as an endocrine disruptor is generally considered secondary to its established carcinogenicity. This study clearly indicates that BaP has multiple effects on classic markers of endocrine function in *Fundulus*. Yet, when conducting bioassay testing, it is important to distinguish biomarkers of exposure that provide potential insight into mechanism and true adverse effect measures that may be population relevant (Hutchinson et al., 2006). Importantly, in this study BaP exposure
caused both a decrease in fertilization success as well as the multigenerational impact on F1 hatching success. A previous study using similar concentrations of BaP and fathead minnows has reported that even unexposed F2 embryos had decreased survival, but potential mechanisms for the multigenerational toxicity were not explored (White et al., 1999). Our study indicates that while BaP caused a >50% decrease in female circulating estrogen concentrations, this depression had no significant effect on fecundity or gonad morphology. In contrast, BaP effects on male reproductive endpoints (GSI, decreased circulating testosterone, testis histopathology) were more susceptible to change and could be related to the decreased fertilization success in the subsequent generation.
Figure 3. **Testosterone (A-C) and estradiol (D-F) concentrations.** In the adult *Fundulus*, 10 µg/L BaP treatment significantly reduced circulating testosterone concentrations in the males (A) (Kruskal-Wallis, p<0.05, n=7, 2-3 males/pool; bars with the same letter are not statistically different) and estradiol concentrations in the female (D) (ANOVA, p<0.05, n=7, 2-3 females/pool). Whole body steroid concentrations of larvae (N=5-6, 5 larvae pooled per sample) at 1 week post hatch (WPH) (B, E) and 3 weeks post hatch (C, F) were not altered by either 1 or 10 µg/L BaP treatment.
Figure 4. **Male (A) and female (B) gonadosomatic index (GSI).** In males, no significant changes were observed in GSI after 2 weeks exposure to BaP, however, 10 µg/L BaP treatment for 4 weeks significantly reduced male GSI. In the females, GSI was not altered at either of the time points or BaP doses (ANOVA, p<0.05, n=5 tanks at 2 wk with 2-3 fish/tank; and n=6 at 4 wk with 3 fish/tank; bars with the same letter are not statistically different).
Figure 5. **Female control gonadosomatic index (GSI).** Control female gonadosomatic indexes were significantly increased during the second half of the exposure period (ANOVA, p<0.05, n=5 tanks at 2 wk with 2-3 fish/tank; and n=6 tanks at 4 wk with 3 fish/tank).
Figure 6. **Male (A) and female (B) liver somatic index (LSI).** No significant differences were found in either sex at both sampling time points. (ANOVA, p<0.05, n=5 tanks at 2 wk with 2-3 fish/tank; and n=6 tanks at 4 wk with 3 fish/tank).
Figure 7. **Total eggs produced (A) and percent fertilization success (B) per tank during days 14-28 of adult exposure.** Waterborne BaP exposure significantly reduced percentage of eggs fertilized at 10 µg/L BaP (n=6 tanks [total egg production], n = 5 tanks [percent fertilization success], 2-sample t-test, p<0.05, 3 males + 3 females/tank; bars with the same letter are not statistically different; ANOVA did not show significant changes between treatment groups). Total egg counts were not significantly altered by BaP exposure.
Figure 8. **Hatch success of F1 embryos**. Parental exposure to 10 µg/L BaP caused a significant decrease in percent hatch at 16, 17 and 18 dpf compared to control (2-sample t-test, p<0.05, n=5 parental tanks; points with the same letter are not significantly different; ANOVA did not show significant changes between the three treatment groups).
Figure 9. **Testis morphology of control and 10 μg/L BaP-treated males (A), number of empty follicles (B) and incidence of increased interstitial fibrosis (C).** The percentages were calculated as (# of empty follicles/total follicles) x 100% and increased fibrosis scored as present or not (n = 6 tanks/treatment group with 1-3 testes/tank at 4 weeks). * Indicates empty follicle; arrow indicates fibrosis, bar = 200 μm.
Figure 10. **Sperm concentration in males.** No significant differences were found at either time point (ANOVA, n=3 tanks at 2 wk with 2-3 fish/ tank; and n=4 tanks at 4 wk with 3 fish/tank).
Figure 11. **Percentage of different developmental stages of follicles represented in female ovaries.** The percentages were calculated as (# follicles at certain stage/ total #) x 100%. No significant differences were found (n = 6 ovaries analyzed at 2 weeks and 12 ovaries at 4 weeks/ treatment group). Follicle stages 1a-1c represent the previtellogenic phase. Stage 2 and 3 are considered vitellogenic and mature follicle stages, respectively.
Figure 12. **Liver vtg mRNA expression.** When vtg mRNA expression was normalized to control males (after 18sRNA normalization), females had significantly more vtg mRNA expression compared to the males in all treatment groups. Both 2 week and 4 week data are combined (ANOVA, p<0.0001; n= 11 tanks; 2-3 fish/tank).
Figure 13. Male (A) and female (B) liver vtg mRNA expression measured by qRT/RT-PCR. Fold induction is expressed relative to controls after normalization to 18S rRNA by the $2^{-\Delta\Delta C_t}$ method. After 4 weeks of exposure, female fish from 10 $\mu$g/L BaP had a significant induction of vtg compared to 1 $\mu$g/L BaP but not compared to controls (ANOVA, $p<0.05$; n= 6 tanks with 3 fish/tank at 4 wk; n = 5 tanks with 2-3 fish/tank at 2 wk; bars with the same letter are not statistically different).
CHAPTER 3: CONCLUSIONS

We successfully utilized a short-term reproductive fish bioassay in order to assess BaP’s role as an endocrine disruptor. The changes in steroid concentrations and gonad development in the parental generation coincided with a significant reduction in fertilization success and hatching rate of the F1 embryos.

Additional studies need to focus upstream within the steroid biosynthesis pathway in order to investigate the mechanisms behind testosterone reduction. As mentioned, the modulation in StAR expression is a potential culprit, but additional enzyme expression could be examined through whole pathway analysis using transcriptomic approaches. Potential molecular changes to the feedback loop associated with the HPG axis also need to be assessed. An important upstream biomarker deals with LH/FSH expression from the pituitary gland. We originally tried to utilize immunohistochemistry to measure both hormones, but our Fundulus pituitaries were compromised during dissections. Instead of trying to isolate the brain, whole Fundulus heads should be fixed and sectioned in order to avoid any chance of losing the pituitary gland.

While the lack of effects on egg production could have resulted from resistant females, there is also the possibility that we did not have enough statistical power from the reproductive bioassay. Due to constraints with exposure settings, we were not able to measure fecundity during a pre-exposure phase and exclude outliers. This could be
one of the main factors causing inter-tank variability and lack of BaP mediated effects on egg production. For our study, a sample size of six tanks only had 9.7% power to detect a 20% difference in total eggs produced. In order to achieve the 80% goal, at least 82 tanks would be needed to account for the variability present. Future exposures could utilize tank pre-selection in order to better assess fecundity parameters.

Overall, this study is significant because we made considerable progress in further establishing BaP-mediated toxicological endpoints including phenotypic and molecular consequences using an environmentally relevant species. We also further established the fish model for investigating reproductive and developmental deficits associated with toxicant exposure.

We showed that parental BaP exposure can cause multigenerational consequences in the F1 generation reflected by reduced hatch. The mechanism for BaP’s F1 toxicity may be due to parental reduced reproductive fitness or acute BaP/BaP-metabolite exposure deposited in the embryos by maternal transfer. Further research is needed to understand the mechanisms of and implications for multigenerational BaP toxicity.
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Type:  Poster
Meeting: South Central Society of Toxicology (SCSOT)

Title: USING FUNDULUS HETEROCLITUS TO STUDY BENZO[A]PYRENE EFFECTS
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Date:  March 2011
Type:  Poster
Meeting: Society of Toxicology (SOT)
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Date: April 2011
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Meeting: Graduate Student Council (GSC)

Title: ASSESSING THE REPRODUCTIVE AND DEVELOPMENTAL ALTERATIONS ASSOCIATED WITH BENZO[A]PYRENE EXPOSURE USING FUNDULUS HETEROCLITUS
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Date: March 2013
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