Mitochondria as a Target of Benzo[a]pyrene Toxicity in a PAH-Adapted and Naïve Population of the Atlantic Killifish (*Fundulus heteroclitus*)

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are important contaminants found in increasing amounts in aquatic ecosystems. One of the sites contaminated by extremely high levels of PAHs is the Atlantic Wood Industries Superfund Site on the Elizabeth River, VA. The Atlantic killifish (*Fundulus heteroclitus*) from this site exhibit increased levels of antioxidants, increased sensitivity to hypoxia, and increased expression of enzymes involved in glycolytic metabolism, suggesting that exposure to PAHs in the environment may induce changes in mitochondrial function and energy metabolism. Normal mitochondrial activity is crucial to an organism's survival. Therefore, gaining a better understanding of how mitochondria are affected by environmental contaminants such as PAHs is an important research objective. This research focused on the effect of benzo[*a*]pyrene (BaP), a representative PAH, on mitochondria in the killifish model and on comparison of the mitochondria of the PAH-adapted killifish from the Elizabeth River Superfund Site to reference site fish. In order to assess the extent of mitochondrial DNA damage in the killifish, a PCR-based assay, Long Amplicon Quantitative PCR (LA-QPCR) for nuclear and mitochondrial DNA (nDNA, mtDNA) damage was adapted to this model and used to test the effect of BaP on DNA damage and in an *ex situ* study examining DNA damage in killifish inhabiting the Elizabeth River Superfund site. With the LA-QPCR, mtDNA and nDNA damage in the killifish from the Elizabeth River Superfund site and from a reference site (King's Creek, VA) that were treated with BaP

were examined. Similar increases in mitochondrial and nuclear DNA damage were observed in King's Creek fish treated with BaP. Killifish from the Elizabeth River showed high levels of basal nDNA and mtDNA damage compared to fish from the reference site, but the level of damage induced due to BaP treatment was much lower in Elizabeth River killifish. Laboratory-reared offspring from both populations showed increased BaP-induced damage in mtDNA, relative to nDNA. Similar to the experiment with adult tissues, the Elizabeth River larvae had higher levels of basal DNA damage than those from the reference site, but were less impacted by BaP exposure. Results suggest that BaP exposure can have important energetic consequences and that multi-generational exposure in the wild may lead to adaptation that dampens DNA damage arising from BaP exposure. Additional experiments showed bothe higher levels of BPDE-dG adducts and chromosomal breakage in the Elizabeth River population. Since the toxic effects of many PAHs are the result of bioactivation by cytochrome P4501A (CYP1A), the existence of enzymes that can potentially metabolize PAHs in mitochondria was verified. Using Western blot, a protein similar in size to microsomal CYP1A was identified with a monoclonal antibody against scup CYP1A in the mitochondrial fraction from livers of adult male killifish. Fish dosed with BaP had increased EROD activity in the liver mitochondrial fraction compared to controls. In killifish larvae dosed with BaP and benzo[*k*]fluoranthene (BkF), CYP1A protein levels as well as enzyme activity were elevated. However, fish from the Elizabeth River

Superfund site showed recalcitrant mitochondrial CYP1A protein levels and enzyme activity in a similar manner to microsomal CYP1A. Finally, the hypothesis was tested that energy metabolism of BaP-treated fish may be different from the control group and that killifish from the Elizabeth River Superfund site may also have altered energy metabolism compared to reference site fish. Respiration of killifish embryos treated with BaP from both populations was measured. Compared to the King's Creek control fish, all other treatment groups showed decrease in oxygen consumption, indicating lower respiration rate. However, no differences in activities of key enzymes involved in glycolysis (PK) and anaerobic metabolism (LDH) were observed in liver of adult killifish in BaP-treated group compared to the control group. Moreover, when we conducted ^{1}H-NMR analysis on BaP treated King's Creek and Elizabeth River killifish to see profile of energy metabolism products, we saw no difference among the four treatment groups. The findings in this thesis contribute to the understanding of how BaP, a common environmental pollutant in the aquatic ecosystem, targets the mitochondria in fish model. Nevertheless, deeper examination of how BaP may impact mitochondrial function in killifish and potentially influence adaptation of killifish at a highly contaminated site is necessary. Further studies will elucidate whether such impacts can potentially affect the energy budget and organism-level fitness in populations in the wild.

Dedication

For my parents...

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Earning a Ph.D. degree was certainly a group effort that could not be achived without my labmates. Dr. Cole Matson had billions of answers and advises for the millions of questions I had. I depended on you so much throughout my time in the lab. Dr. Lauren Wills, what more can I say to someone willing to come into lab at 5am Saturday morning for fish dissection assistance? Dr. Alicia Timme-Laragy, thank you for taking on the role of one-on-one mentor my first year in lab. Bryan Clark, your home made coffee made all the difference in our field trips. Lindsey Van Tiem, thank you for teaching me to laugh even during research disasters. On top of all the help you have

given me as partners in research, thank you for enduring hours and hours of manuscript reading. I would also like to thank Youngeun Cho, and Cole Thompson, two amazing undergrads that I was lucky to work with, and Autumn Bernal for reviving the protein work in the lab.

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1. Introduction

In this dissertation, I used the ecological model *Fundulus heteroclitus* to examine how mitochondria can be a target of toxicity caused by benzo[*a*]pyrene, a common environmental toxicant. In addition, I investigated whether a population of killifish chronically exposed to polycyclic aromatic hydrocarbons (PAHs) shows characteristics of mitochondrial impairment.

1.1 Polycyclic Aromatic Hydrocarbons

1.1.1 Polycyclic aromatic hydrocarbons

PAHs are planar hydrocarbon compounds with at least two aromatic rings fused together. These compounds are primarily produced by incomplete combustion of organic materials. Sources of PAHs include natural processes such as forest fires, and anthropogenic processes such as fuel oil or gasoline spills, combustion of fossil fuels and wood, urban run-off, and creosote release (Walker et al. 2005). As a group, PAHs are listed as number eight on the 2007 Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) priority list by the Agency for Toxic Substances and Disease Registry (ASTDR 2007). Unlike many other environmental contaminants, such as chlorinated hydrocarbons, the level of PAHs in the environment has been increasing since 1970s, correlating with urban sprawl and increased vehicle use

in USA (Van Metre et al. 2000; Van Metre et al. 2005) and urbanized Asian countries (Chang et al. 2006).

Most PAHs are highly lipophilic and can thus enter the cell easily. Within the cell, PAHs can be bioactivated by phase 1 metabolism. The intermediates formed during this process engage in processes such as macromolecule adduct formation from epoxide intermediates and reactive oxygen species (ROS) generation from quinone metabolites. Several studies indicate that mitochondria might be a major target of PAH toxicity. PAHs are localized to the mitochondria, and exposure to them is correlated with decreases in ATP production, loss of mitochondrial membrane potential, and changes in mitochondrial morphology, as well as induction of mitochondria-dependent apoptosis (Zhu et al. 1995; Li et al. 2003; Ko et al. 2004; Xia et al. 2004). However, whether mitochondria are the primary target of PAH toxicity and initiate subsequent cellular effects, or whether mitochondrial damage is a secondary effect, remains to be elucidated.

1.1.2 Benzo[*a*]pyrene

This research examined the effects of an environmentally relevant PAH, benzo[*a*]pyrene (BaP). BaP is listed as ninth on the CERCLA priority list (ASTDR 2007). It is a well-studied chemical that is a potent carcinogen as well as a potent cytochrome P4501A (CYP1A) inducer via the aryl hydrocarbon pathway discussed below. Metabolic activation of BaP is described in Figure 1.1 and Figure 1.2. BaP can be metabolized to BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) and then covalently bind to DNA at the N^2

position of guanine to form a BPDE-DNA adduct (Figure 1.1). BaP can also form a semiquinone and produce ROS, resulting in oxidative DNA damage of macromolecules, including DNA (Figure 1.2). It is assumed that BaP toxicity is somewhat reflective of toxicity of other PAHs as well.

1.2 Aryl Hydrocarbon Receptor Pathway

1.2.1 Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that is activated by many xenobiotics, including planar halogenated aromatic hydrocarbons (pHAHs) and PAHs (Hahn 1998). AhR exists in the cytoplasm as a complex with two heat shock proteins 90 (hsp90) and an X-associated protein2 (XAP2) (Denison et al. 2003). Upon activation, the protein dissociates from the complex, translocates into the nucleus, dimerizes with AhR nuclear translocator (ARNT) and binds to the xenobiotic responsive element (XRE) in the promoter region of AhR target genes, which results in their transcriptional upregulation. AhR target genes include several genes involved in phase I and phase II metabolism (Nebert et al. 2000). Genes that are transcribed by the AhR include *cyp1a*, *cytochrome P4501b1 (cyp1b1)*, *uridine diphosphate glucoronosyltransferase*01* (*ugt*01*), *ugt*06*, *NAD(P)H menadione oxidoreductase*, *aldehyde dehydrogenase*, and *glutathione transferase alpha* (Hahn 1998; Schrenk 1998; Nebert et al. 2000; Denison et al. 2003). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a pHAH, increases mitochondrial reactive oxygen species and induces a loss of mitochondrial

membrane potential in mice in an AhR dependent manner (Shimizu et al. 2000; Senft et al. 2002; Fisher et al. 2005). It is yet unknown whether PAHs can induce mitochondrial dysfunction in an AhR dependent manner.

1.2.2 Cytochrome P4501A

CYP proteins mediate oxidative and reductive biotransformation of endogenous and exogenous compounds (Williams et al. 1998b). Of the over 50 families of CYP proteins, the enzymes in the CYP1 family are under the control of the AhR pathway. They are involved in the metabolism of various planar organic compounds including PAHs (Hahn 1998; Denison et al. 2003; Nebert et al. 2004).

Several different cytochrome P450 proteins exist in animal mitochondria. They have a variety of endogenous roles, including cholesterol side chain cleavage, aldosterol synthesis, vitamin D1, D2, and D3 activation, and bile acid synthesis (Omura 2006). There is evidence that some of these mitochondrial P450 proteins are involved in xenobiotic metabolism (Anandatheerthavarada et al. 1999; Bhagwat et al. 1999; Boopathi et al. 2000; Genter et al. 2006). In mammals, the CYP1A1 gene is targeted to the mitochondria by proteolytic cleavage of the N-terminus in the cytosol (Addya et al. 1997). Studies in mammalian systems have shown that mitochondrial CYP1A1 is localized into the inner membrane of mitochondria after β-naphtoflavone (BNF) or TCDD treatment, and the protein level reaches its highest at a later time point compared to the endoplasmic reticulum (Bhagwat et al. 1999; Genter et al. 2006). Although several

forms of mitochondrial P450s, as well as adrenodoxin, exist in the teleost mitochondria (Sampath-Kumar 1994; Gilman et al. 2003; Leusch et al. 2003; Hagen et al. 2006), the existence of xenobiotic metabolizing P450s in teleost mitochondria has not been confirmed.

1.3 Mitochondria

There are several hundred to several thousand mitochondria per eukaryotic cell. Mitochondria are responsible for the majority of cellular energy production in eukaryotes. In addition, mitochondria are involved in initiating apoptosis, maintaining Ca^{2+} homeostasis, assembly of heme and iron-sulfur clusters, steroid synthesis, and participating in cell signaling (Duchen 2004). In the context of the dynamic energy budget concept (Nisbet et al. 2000), reduction in energy availability (i.e. reduced ATP production) can divert energy utilization from growth and reproduction to just basic metabolic maintenance, thus reducing the organism's chances of survival (Figure 1.3).

1.3.1 Respiration and Energy Metabolism

Mitochondria generate most of cellular ATP (Wallace 1997), with the remainder being generated in the cytosol by glycolysis. ATP generation in the mitochondria is done through oxidative phosphorylation (OXPHOS) in the electron transport chain (ETC) of the inner mitochondrial membrane. Four complexes constitute the ETC. Complex I includes NADH-dehydrogenase, which receives electrons from the reduced form of

nicotinamide adenosine dinucleotide (NADH). Succinate dehydrogenase at complex II receives electrons from succinate via flavin adenine dinucleotide ($FADH_2$). Electrons received at complexes I and II are shuttled by ubiquinone to complex III, where the cytochrome bc_1 complex moves electrons to cytochrome c. Cytochrome c carries electrons to complex IV. Here, cytochrome c oxidase converts oxygen to water via a four electron reduction. During this electron transfer, protons are pumped into the intramembrane space at complexes I, III, and IV, creating a proton gradient across the mitochondrial inner membrane. Protons return to the mitochondrial matrix via the ATP synthase (F_0F_1 ATP Synthase) allowing the conversion of adenosine diphosphate (ADP) to ATP to occur. The efficiency of the ETC and the ATP synthase in coordinating proton pumping and ATP production is termed "coupling efficiency." The tighter the coupling, the more efficiently ATP is generated by the mitochondria (Brandon et al. 2006).

As the site of oxygen consumption, mitochondria are also a major source of endogenous reactive oxygen species (ROS) (Cadenas et al. 2000; Fariss et al. 2005). ROS are generated mainly by complex I and complex III of the ETC. As a consequence, mitochondria are especially susceptible to injuries by ROS (Kowaltowski et al. 1999). Consequently, several mechanisms exist in the mitochondria to counter such oxidative injuries (Andreyev et al. 2005). O_2 is converted to $O_2^{\bullet -}$ (superoxide) by the transfer of electrons in the mitochondria. The $O_2^{\bullet -}$ produced is converted to H_2O_2 by manganese superoxide dismutase (MnSOD), which is then reduced to H_2O by glutathione

peroxidase (GPx) or catalase (Di Giulio et al. 1995). Several studies have demonstrated that BaP induces oxidative stress (Winzer 2001; Burdick et al. 2003; Li et al. 2003). Others show that BaP quinones (e.g. 1,6-quinone) may directly disrupt OXPHOS without generating oxidative stress (Imlay et al. 1992; Zhu et al. 1995). Killifish from the Elizabeth River (a Superfund site highly contaminated with PAHs) have elevated GPx activity (Bacanskas et al. 2004), and MnSOD protein levels (Meyer et al. 2003b). Both enzymes are involved in countering mitochondrial and cellular oxidative stress. In addition, larval and adult killifish liver glutathione levels increased significantly after exposure to the Elizabeth River sediment extract. This suggests that sediment extracts containing PAHs may be inducing mitochondrial oxidative stress and that the killifish at this site are responding to the increased oxidative stress.

1.3.2 Mitochondrial DNA

In the animal kingdom, mitochondria are the only organelles that have their own DNA, RNA, and ribosomes other than the nucleus. Although some proteins are associated with mitochondrial DNA (mtDNA), mtDNA exist in discrete clusters known as nucleoids, unlike nuclear DNA (nDNA), which forms complexes with histone and other proteins (Brown 2008). The proteins encoded by mitochondrial DNA (mtDNA) are essential components of the ETC. The mitochondrial genome is a circular chromosome of double stranded DNA ranging in size from 16 kilobase pairs in humans to 2.4 megabase pairs in plants (Penta et al. 2001). Nevertheless, mitochondrial genome size in

most vertebrates are similar (Scheffler 1999). In mammals, the mitochondrial genome transcribes thirteen proteins in the respiratory chain (Figure 1.4), two ribosomal RNAs (rRNAs), and twenty-two transfer RNAs (tRNAs) (Penta et al. 2001; Van Houten et al. 2006). However, the majority of mitochondrial proteins are transcribed in the nucleus and transported to the mitochondria.

For several reasons, mtDNA is believed to be especially prone to damage. The proximity of mtDNA to the ETC, where ROS are constantly produced, allows for extensive oxidative damage. In addition, DNA repair activity in the mitochondria is relatively less efficient compared to nDNA repair (Shadel et al. 1997). Base excision repair has been observed in the mitochondria, but nucleotide excision repair appears to be lacking. mtDNA is also more accessible to genotoxic species that have not yet been cleared by phase II metabolism (Izzotti 2009). Finally, mtDNA is considered structurally less protected from damage than nDNA (Suliman et al. 2004). Unlike nDNA, mtDNA does not form a hierarchical supercoil and exists as a complex with histone proteins. However, there are studies that suggest that the packaging of mtDNA with mitochondrial transcription factor A (TFA) gives it stability and also some protection from damage (Alam et al. 2003; Kanki et al. 2004). In mammals, mtDNA is especially susceptible to damage from the stable adduct formation by dihydrodiol-epoxide derivative of BaP (Backer et al. 1980). Furthermore, Graziewicz et al. (2004) showed that

mtDNA replication is hindered by adducts formed from BaP and BcP (benzo[*c*]phenanthrene) and consequent misincorporations caused by the adducts.

1.3.3 Mitochondria and PAHs

In mammals, ultrafine particles, the majority of which are PAHs, localize in the mitochondria where they induce functional and structural damage such as mitochondrial membrane potential ($\Delta\Psi_m$) disruption, reduced ATP levels, and membrane shrinkage (Zhu et al. 1995; Li et al. 2003; Xia et al. 2004). In addition, PAHs have been linked to the induction of mitochondria-mediated apoptosis (Ko et al. 2004; Xia et al. 2004; Detmar et al. 2006; Huc et al. 2006). However, the mechanisms underlying PAHs' toxic effects on mitochondria are not fully understood. The few studies that have examined the effects of PAHs on mitochondria in mammalian systems disagree as to how mitochondria are impacted, as well as on exactly how mitochondria may mediate cellular toxicity in response to PAHs (Solhaug et al. 2004; Xia et al. 2004; Huc et al. 2006). Some researchers argue that such mitochondrial effects are secondary to the activation of p53 by nuclear DNA damage in response to PAH exposure (Huc et al. 2006; Matoba et al. 2006). Given the evidence, it is likely that mitochondria are both directly and indirectly affected by PAH toxicity.

In fish, even less is known about the effects of PAHs on mitochondria. As mentioned above, PAHs are an important class of contaminants in the aquatic environment. Therefore, further investigation into how this group of pollutants may

affect the mitochondria in aquatic animals is necessary. In addition, studies that attempt to determine the response and adaptation of fish chronically exposed to such external stress in areas heavily polluted with PAHs can point to the mechanism behind such effects.

1.4 The Atlantic Wood Industries Superfund Site

1.4.1 Atlantic Wood Superfund Site

The Elizabeth River, located in Portsmouth, Virginia (36°48'27.4'' N, 76°17'36.1'' W), is a tributary of the James River, which is in the Chesapeake Bay watershed (Figure 1.5). Historically, creosote and pentachlorophenol were heavily used by a wood-treatment facility that operated on the southern branch of the Elizabeth River from 1926 to 1992. In addition, the Navy used part of the area to dispose of the abrasive blast media, from the sand blasting of ships, and also industrial sludge from the production of acetylene (EPA 1995b). The sediment, groundwater, and soil at the site are contaminated with pentachlorophenol (PCP) and metals, and especially with extremely high concentrations of PAHs (Conrad et al. 2004; Hartwell et al. 2007; Vogelbein et al. 2008). A recent survey showed that total PAH concentration at the most polluted site was as high as 500 μg/g (ppm) dry sediment (Vogelbein et al. 2008). PAHs with the highest concentrations included fluoranthene, pyrene, chrysene, benzo[*a*]pyrene,

benzo[*e*]pyrene, benzo[*a*]anthracene, and phenanthrene (Table 1.1). The site has been designated a National Priorities Superfund site since 1990 (EPA 1995b).

1.4.2 King's Creek reference site

King's Creek is a tributary of Severn River in Gloucester County, Virginia (37°17′52.4″N, 76°25′31.4″W). This site is approximately 54.91 km north of the Elizabeth River Superfund site (Figure 1.5). Killifish from this site has been used in our laboratory for several years as reference fish in comparison studies involving the Elizabeth River Superfund site population (Meyer et al. 2003a; Meyer et al. 2003b; Bacanskas et al. 2004; Meyer et al. 2005; Wills et al. 2009).

1.5 Atlantic Killifish

1.5.1 Killifish

The Atlantic killifish (*Fundulus heteroclitus*, Fundulidae, Cyprinodontiformes) is an estuarine fish broadly distributed along the Atlantic coastline of the North America from Newfoundland to northeast Florida. They have life expectancies of about 3 – 4 years (Wirgin et al. 2004) and can grow to approximately 8-10 cm (Kneib, 1986). Local populations of killifish have limited home range as low as 30 – 40 m during summer (Lotrich 1975). Therefore, these fish are ideal for studying responses to environmental influences such as different salinity, oxygen availability, pH, and temperature as well as local contamination (Burnett et al. 2007). Thus, killifish are widely utilized as models in

laboratory toxicology (Eisler 1986; Wassenberg et al. 2002), genetic adaptation (Nacci et al. 1999), and ecological studies (Nacci et al. 2002a).

1.5.2 Resistant Populations

Different populations of killifish show adapatation to metals (Weis et al. 1999), dioxin-like compounds and PCBs (Munns et al. 1997), and PAHs (Hahn 1998; Meyer et al. 2002b). Killifish inhabiting the Elizabeth River also show adaptation to their PAH-contaminated environment. These fish are resistant to developmental toxicity from the sediment at the site (Ownby et al. 2002; Wassenberg et al. 2004). In response to PAH treatment, they show higher levels of antioxidants, including mitochondrial superoxide dismutase (MnSOD), but are recalcitrant to CYP1A induction (Meyer et al. 2003b; Bacanskas et al. 2004).

Interestingly, adults from this population show increased hepatic lesions (Vogelbein et al. 1990). Field surveys also indicate that there is a positive association between lesion formation and PAH contamination (Vogelbein et al. 2003). In addition, Elizabeth River killifish shows higher sensitivity to photo-enhanced toxicity of PAHs (fluoranthene) and to low oxygen conditions than the King's Creek fish (Meyer et al. 2003a; Meyer et al. 2003b; Meyer et al. 2003c). Furthermore, there are indications that the fish from Elizabeth River may be more dependent on the glycolytic pathway for energy metabolism (Meyer et al. 2005). Such data indicate that there may be trade-offs associated with the adaptation to PAH-contaminated environment.

1.6 Dissertation Objective and Outline

Mitochondria are the major ATP producers of the cell. Maintenance of normal mitochondrial activity is crucial to an organism's survival. With such important potential physiological and ecological implications, gaining a better understanding of how mitochondria are affected by environmental contaminants is an important research objective. To understand changes in cellular bioenergetics of aquatic organisms in response to PAHs, this dissertation focused on whether mitochondria can be a target of PAH toxicity in the killifish model. I examined the cellular mechanism by which the killifish mitochondria may be affected in response to PAH treatment by examining various aspects of the mitochondria in response to a common environmental pollutant, BaP. In addition, I examined whether chronic exposure to PAHs in the field influenced the mitochondrial function of a population of killifish adapted to an extremely contaminated Superfund site, the Atlantic Wood Superfund Site.

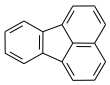
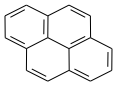
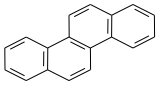
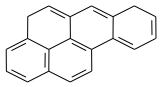
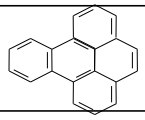
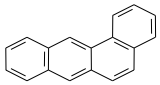
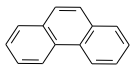
This dissertation is organized into five research chapters that examine various aspects of the killifish mitochondrial response to PAH treatment:

- Chapter 2: To effectively measure mitochondrial DNA damage in Atlantic killifish, the potential of the long amplicon quantitative PCR (LA-QPCR) assay was explored. In addition, the applicability of this method for use as a biomarker of effect was tested.

- Chapter 3: Basal levels of mtDNA and nDNA lesions, as well as the effect of BaP on mtDNA and nDNA damage were examined in adult and larval killifish from Elizabeth River and King's Creek.
- Chapter 4: The existence of mitochondrial CYP1A was investigated in killifish. In addition, the inducibility of mitochondrial CYP1A in Elizabeth River killifish was examined.
- Chapter 5: The effect of BaP treatment on cellular respiration and oxidative metabolism was examined. Population differences between Elizabeth River and King's Creek fish were also examined.
- Chapter 6: Several methods were used as biomarkers to assess the extent of DNA damage in Elizabeth River killifish population

Finally, the findings of this dissertation as well as their implications and future direction are summarized in Chapter 7. In addition, LA-QPCR conditions for the zebrafish (*Danio rerio*) model is described in the appendix.

Table 1.1 Prevalent PAHs at the Elizabeth River Superfund site

Chemical	Structure	Characteristics
Fluoranthene		~ 148 ppm in ER sediment # 110 on CERCLA priority list
Pyrene		~ 78 ppm in ER sediment # 253 on CERCLA priority list
Chrysene		~ 61 ppm in ER sediment # 137 on CERCLA priority list AhR agonist
Benzo[*a*]pyrene		~ 58 ppm in ER sediment # 9 on CERCLA priority list AhR agonist
Benzo[*e*]pyrene		~ 54 ppm in ER sediment
Benzo[*a*]anthracene		~ 44 ppm in ER sediment # 39 on CERCLA priority list AhR agonist
Phenanthrene		~ 34 ppm in ER sediment # 246 on CERCLA priority list

Adapted from Vogelbein and Unger (2003).

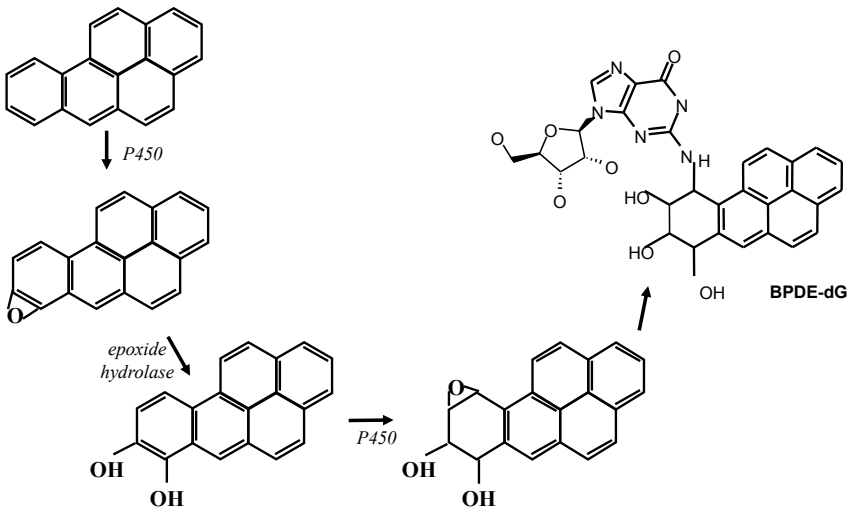


Figure 1.1 Covalent DNA adduct formation by BPDE

BaP can be metabolized to epoxide, which in turn can bind to guanine and form stable adduct with DNA. (Adapted from Miller and Ramos (2001)).

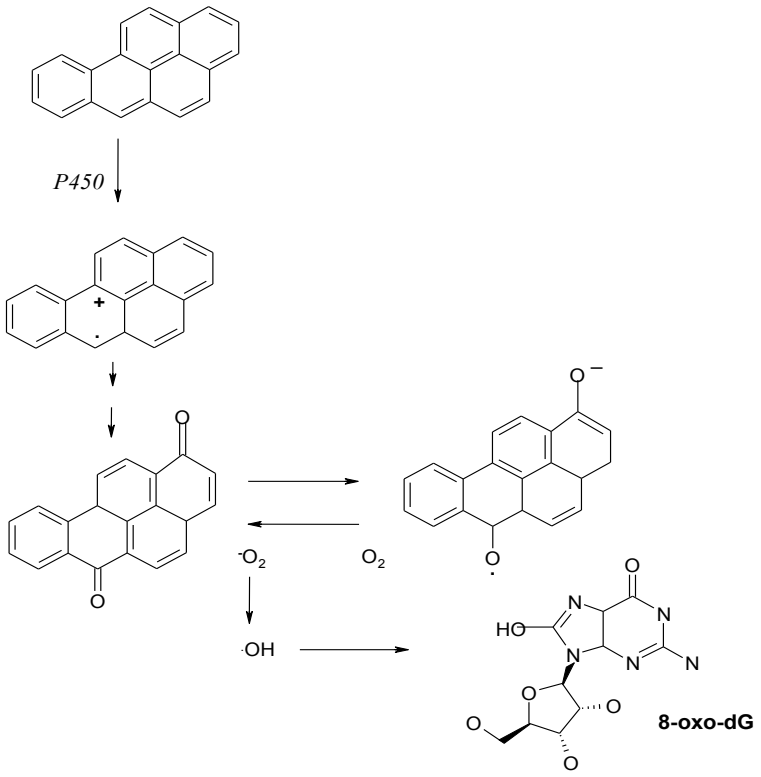


Figure 1.2 Oxidative metabolites of BaP

BaP goes through a series of reactions involving P450s to form quinones that can induce oxidative DNA damage (Adapted from Miller and Ramos (2001)).

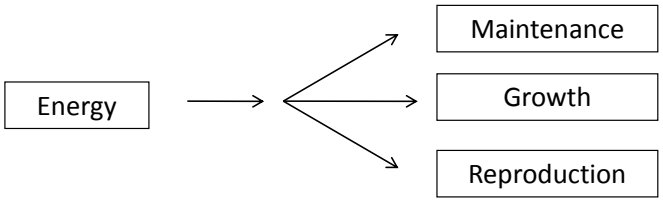


Figure 1.3 Energy Budget

Within an organism, the energy taken up through food is converted to ATP for use in basic maintenance, growth, and reproduction. Decrease in food sources or ATP production can decrease the distribution of energy to growth and reproduction.

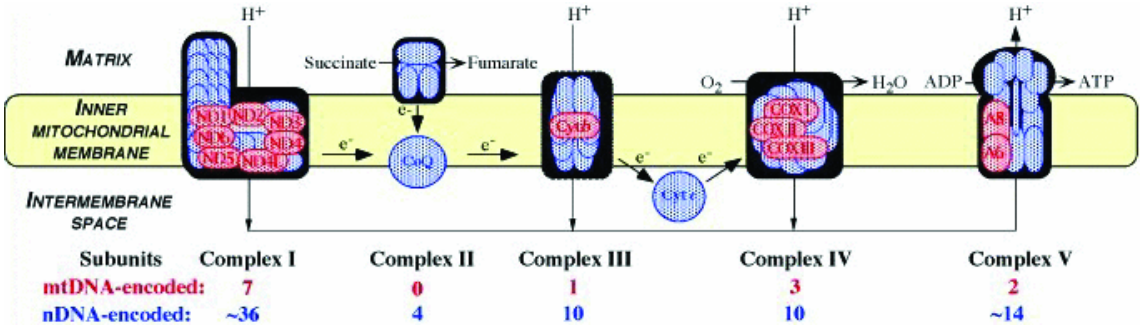


Figure 1.4 OXPHOS genes in the mitochondrial genome

The mitochondrial genome encodes 13 proteins that all make up part of the OXPHOS complex. (DiMauro et al. 2005).

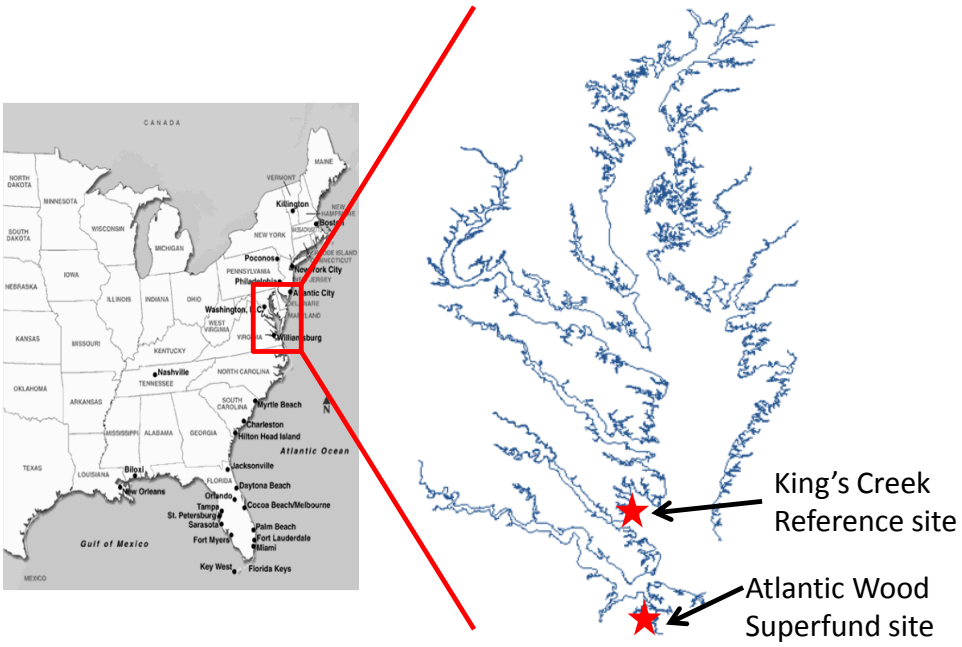


Figure 1.5 Atlantic Wood Superfund site in the Chesapeake Bay wastershed

Atlantic Wood Industries Superfund site is located in the southernmost part of the Chesapeake Bay watershed in Portsmouth, VA. Approximately 55 km north is the King's Creek reference site in Gloucester County, VA. (Maryland Geological Survey, 2000).

2. The Long Amplicon Quantitative PCR for DNA Damage Assay as a Sensitive Method of Assessing DNA Damage in the Environmental Model, Atlantic Killifish (*Fundulus heteroclitus*)

This chapter has been published under the same title in the journal Comparative Biochemistry and Physiology- Part C: Toxicology and Pharmacology, volume 149, issue number 2, pages 182-186, in the year 2009. The authors are Dawoon Jung, Youngeun Cho, Joel N. Meyer, and Richard T. Di Giulio.

2.1 Introduction

DNA damage results from exposure to many contaminants, and is widely used as an indicator or biomarker of biological effects (van der Oost et al. 2003). In addition, DNA damage and repair is an important field of study within ecotoxicology (Theodorakis 2001). The long amplicon quantitative PCR (LA-QPCR) assay, previously referred to as the QPCR assay, provides a sensitive way of assessing DNA damage and alterations to DNA that often lead to damage (Ayala-Torres et al. 2000). The assay measures the fraction of undamaged template DNA by comparing the amplification of very long PCR target (amplicon) in control and experimental samples, under the assumption that lesions and/or structural alterations in the target genomic DNA template will block or slow the progression of the DNA polymerase used in the reaction (Kalinowski et al. 1992). We can then mathematically transform the difference in

amplification to represent lesion frequencies. With parallel amplification of shorter PCR target within the amplified region of the longer target, we can normalize for DNA copy number and compare DNA damage in different DNA sources. The LA-QPCR assay can detect DNA strand breaks, adducts, and many other types of structural modifications such as those caused by oxidative damage. Therefore, it is potentially of particular utility in biomonitoring contexts where the specific types of DNA damage may not be well-defined or predictable ahead of time. It has been used successfully in human cell lines (Yakes et al. 1997; Van Houten et al. 2000) and laboratory model organisms (Chan et al. 2006; Meyer et al. 2007). In these models, the assay has been shown to have a high sensitivity, with a limit of detection of approximately 1 lesion per 10^5 nucleotides (Santos et al. 2006). This assay is a relatively simple method of detecting damage in nuclear DNA (nDNA) and mitochondrial DNA (mtDNA), and if adapted, would be valuable for detecting DNA damage in wildlife populations. However, the use of this assay in an environmental model has not been published.

The Atlantic killifish (*Fundulus heteroclitus*) is an estuarine fish species distributed throughout the coastal marshes along the North American Atlantic Coast. This species has a limited home range (Lotrich 1975), and is considered to be very adaptable to diverse local environments and various stressors that are present in their habitats (Burnett et al. 2007). Different populations show resistance to pollutants such as metals (Weis et al. 1989), dioxin-like compounds and PCBs (Prince et al. 1995a; Prince et al.

1995b; Nacci et al. 1999), and PAHs (Meyer et al. 2002b; Ownby et al. 2002; Meyer et al. 2003a). Furthermore, they are easy to collect and maintain in the laboratory (Burnett et al. 2007). Thus, killifish have been widely utilized in laboratory toxicology (Eisler 1986; Wassenberg et al. 2002), genetic adaptation research (Nacci et al. 2002a), ecological studies (Weis et al. 1989; Nacci et al. 1999), and as a model organism for estuarine system monitoring (Eisler 1986).

Several assays have been developed for assessing contaminant exposures in killifish (Binder et al. 1985; Van Veld et al. 1992; Greytak et al. 2005). Most of these assays are specific to individual or groups of compounds, and do not address the downstream and potentially higher level biological effects of contaminants on this species. Considering the role of killifish as an important environmental model, an assay that can easily detect general DNA damage would be a valuable tool for assessing contaminant effects in this organism.

In this study, we explored the potential of the LA-QPCR assay to measure PAH-induced genetic damage in Atlantic killifish. First, we tested the utility of this method for use in laboratory exposures by quantifying DNA damage in killifish that had been injected with the well-established genotoxin benzo[*a*]pyrene (BaP). Second, in an environmental study, we used LA-QPCR to look for evidence of genotoxicity in Atlantic killifish inhabiting a highly contaminated Superfund site.

2.2 Materials and Methods

2.2.1 Killifish care

Adult killifish (*F. heteroclitus*, Fundulidae, Cyprinodontiformes) were captured using baited minnow traps from King's Creek, a tributary of York River in Gloucester County, VA. This is a relatively unpolluted site that we have used for several years as a reference site (Meyer et al. 2002b; Meyer et al. 2005). After capture, the fish were transported to the Duke University Ecotoxicology Laboratory. Fish were maintained in a recirculating system containing 23-25°C, 25 ppt artificial sea water (Instant Ocean®, Aquarium Systems, Forster & Smith, Rhinelander, WI, USA) with a 14:10 Light:Dark photoperiod. The fish were fed a mixed diet of Tetramin® Tropical Fish Food (Tetra Systems, Blacksburg, VA, USA) and brine shrimp (*Artemia*, Brine Shrimp Direct, Ogden, UT, USA).

2.2.2 BaP treatment and DNA isolation

Male killifish were moved to individual aerated tanks with 3 L of artificial seawater 24h before treatment. Ten fish were injected intraperitoneally with BaP in corn oil. Fish were injected with 5 μL/g wet weight of 10 mg/kg BaP. Additional fish were injected with 5 μL/g wet weight of corn oil as a carrier control. The fish were fed daily and sacrificed 72h post-treatment. Brain, liver, and muscle tissues were dissected out and flash frozen in 20% glycerol, and stored at -80ºC. Tissues were later ground in liquid nitrogen, and total DNA was extracted with the Genomic-tip 20/G kit (Qiagen Inc.,

Valencia, CA, USA) according to the manufacture's protocol. In addition, fish from King's Creek and from the Atlantic Wood Superfund site at the Elizabeth River in Portsmouth, VA were collected and sacrificed within 24h of capture. Previous studies have shown that populations from these two sites are genetically suited to be used in comparison studies (Mulvey et al. 2002; Mulvey et al. 2003). Liver and muscle from the fish were dissected out. The tissues were stored at -80ºC until total DNA was isolated as described above. Since this assay relies upon the amplification of long stretches of DNA, it is critical that the DNA template be extracted as carefully as possible. The extracted DNA should not be exposed to phenol, and should be of high molecular weight. Additional protocol details are available in Santos et al. (2006).

2.2.3 Ultraviolet radiation C (UVC) exposure

Total DNA was isolated from the liver and brain of adult male *Fundulus heteroclitus* using the isolation methods described above. Equal amounts of DNA (50 μL of 3 ng/μL) were exposed to 0, 5, 10, and 20 J/m^2 of ultraviolet radiation (254 nm; hereafter referred to as UVC) using either an ultraviolet lamp (UVLMS-38 EL Series 3UV Lamp, UVP, Upland, CA, USA) in conjunction with a UVX radiometer and UVX-25 sensor (UVP), or a CL-1000 Ultraviolet Crosslinker (UVP) with an emission peak at 254 nm. DNA was immediately frozen until further analysis.

2.2.4 Primer Selection

Primers for large and small nuclear targets were designed for the cystic fibrosis transmembrane conductance regulator gene (CFTR, GenBank assession no. **AY028263**), and large and small mitochondrial targets were designed from a cDNA sequence for cytochrome c oxidase polypeptide VIa, mitochondria (GenBank assession no. **CN984995**). The CFTR gene was selected as it was the only published gene over 10kB long. Primers were designed using PRIMER3 (Rozen et al. 2000). The primer sequences for the large mitochondrial target were obtained from Kim et al (2004). Primers and amplification sizes are described in Table 2.1. All primers were tested to confirm the amplification of a single band of the expected length.

2.2.5 LA-QPCR

LA-QPCR was performed according to a protocol modified from Santos et al. (2006). This assay has previously been referred to as the QPCR for DNA damage assay; we have chosen to refer to it as the LA-QPCR assay to avoid confusion with quantitative PCR (qPCR), the abbreviation frequently used to refer to real-time PCR-based measurement of mRNA levels.

Briefly, 10 ng DNA (5 μL of 2 ng/μL DNA) from each sample was amplified with *rTth* polymerase (Applied Biosystems) using the primers described above. Small nDNA and mtDNA targets were amplified for normalization/verification of DNA concentration and to account for mitochondrial copy number, respectively (described below). We

optimized the elongation temperature, $Mg(OAc)_2$ concentration, and cycle number for each PCR target. The PCR conditions for each set of primers are as follows. For all targets, final concentrations of 1x buffer (provided in the *rTth* polymerase kit), 100μg/mL of BSA, 200 μM of each dNTP, and 0.4 mM of each primer were added in the PCR mix. Water volume was adjusted to make the volume 50 μL for each reaction. For both short targets, 1.2 mM of $Mg(OAc)_2$ was used in the PCR mix. The cycling conditions were 75°C for 2 min; 94°C for 1 min; 94°C for 15 s, 62°C for 45 s, and 72°C for 30s (repeated 24 cycles); and 72°C for 5 min. For the long nuclear target, 1.1 mM of $Mg(OAc)_2$ was used in the PCR mix. The cycling conditions were 75°C for 2 min; 94°C for 1 min; 94°C for 15 s and 68°C for 12 min (repeated 24 cycles); and 72°C for 10 min. For the long mitochondrial target, 1.2 mM of $Mg(OAc)_2$ was used in the PCR mix. The cycling conditions were 75°C for 2 min; 94°C for 1 min; 94°C for 15 s and 65°C for 12 min (repeated 16 cycles); and 72°C for 10 min. We added 5 μL of the *rTth* enzyme (diluted to 1 unit/μL) after 90 s of the 75°C incubation at the beginning of the reaction to initiate the amplification with "hot start." PicoGreen dye (Invitrogen Corporation, Carlsbad, CA, USA) was used to quantify the template and PCR product. DNA concentrations were then converted to lesion frequencies per 10kB DNA by application of the Poisson distribution, as described by Ayala-Torres et al. (2000). This approach defines the control samples as undamaged, and generates a lesion frequency in experimental samples relative to the control samples, based on alterations in amplification efficiency and an

assumption of random distribution of damage. Differences in mitochondrial copy number and variations in template amount were normalized by quantifying short target sequences within the long target. With the assumption that no damage would be detected in short targets, we rationalized that any deference in the product quantity reflects differences in the template, and adjusted the quantity of each long target accordingly. With each PCR reaction, we included 5 ng of one of the control DNAs to monitor amplification quality. Only PCR products in which the amplification of 5 ng DNA was 40-60% of the control DNA (10 ng), indicating that the PCR reaction was quantitative, were used in the analysis.

2.2.6 Statistics

Statistical analyses were performed using SPSS, version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). The assumption of normality was tested for all data sets using the Shapiro-Wilk's test. Analysis of Variance (ANOVA) and Fisher's Protected Least-Significant Differences (LSD) were used to test for differences among groups ($\alpha = 0.05$).

2.3 Results

2.3.1 Adaptation of the LA-QPCR assay

To confirm the success of primer selection and condition optimization for this assay, we exposed purified total DNA in buffer from adult male killifish liver and brain to various doses of UVC and assessed damage (Figure 2.1). A dose-dependent increase

in damage to nDNA and mtDNA exposed to various doses of UVC radiation was detected ($p < 0.001$), but no differences in damage were observed between mtDNA and nDNA at a given dose ($p = 0.775$). The increase in DNA damage fit the linear regression with r^2 values of 0.84 for mtDNA and 0.81 for nDNA respectively, and the lesion frequencies detected were comparable to those obtained previously using DNA purified from human cells in culture or *Caenorhabditis elegans* at the same UV doses (Eischeid et al. 2008).

2.3.2 DNA damage in response to BaP

As expected, adult male killifish dosed with intraperitoneal (i.p.) injection of 10 mg/kg BaP showed increased levels of DNA damage relative to killifish dosed with corn oil for both mtDNA and nDNA (Figure 2.2) in all three tissues examined. Three-way ANOVA showed that there was a significant effect of treatment ($p < 0.001$). However, neither DNA source (mitochondria versus nucleus) nor tissue type significantly affected the result ($p = 0.177$ and $p = 0.493$ respectively). In addition, there was no interaction among any of the independent variables.

2.3.3 Comparison of Elizabeth River and reference site killifish populations

Killifish from the Atlantic Wood Superfund site and King's Creek (reference site) were sacrificed within 24h of capture, and lesion frequencies in mtDNA and nDNA from muscle and liver were examined (Figure 2.3). In this case, brain was not examined due to difficulties in acquiring sufficient amount of tissue. Three-way ANOVA showed that

there was a significant effect of site ($p < 0.001$) and tissue type ($p = 0.047$). However, effect of the DNA source (nuclear or mitochondrial genome) was not significant ($p = 0.839$). There was also a significant interaction between tissue type and population ($p = 0.033$), reflecting the fact that the Elizabeth River killifish seemed to show more sensitivity to nDNA damage in muscle, but to mtDNA damage in liver. However, while statistically significant, this difference does not seem large enough to be of clear biological relevance.

2.4 Discussion

We have shown for the first time that the LA-QPCR assay can be adapted to a widely studied environmental model. We detected significant increases in the frequency of DNA lesions after exposure to an environmentally relevant dose (10 mg/kg) of BaP as well as contaminants present at a Superfund site.

In our experiments with BaP-treated fish as well as with the field-caught fish, we detected no significant differences in damage in response to BaP treatment between mtDNA and nDNA. This was a surprise since previous data in mammalian cell culture studies indicated a much greater susceptibility of the mitochondrial genome to polycyclic aromatic hydrocarbon exposure (Allen et al. 1980; Backer et al. 1980). We do not know the reason for this difference. It seems unlikely to be related to the requirement for metabolic activation: Backer and Weinsten used the reactive metabolite benzo[*a*]pyrene diol epoxide, rather than the parent benzo[*a*]pyrene, but Allen and

Coombs used parent compounds that require activation, as we did. More likely candidates include *in vitro* vs *in vivo* differences (the studies cited above used cell culture systems), DNA damage detection methodology, or species differences. With aflatoxin B_1, another lipophilic chemical that is activated to a DNA-reactive form by CYP proteins, Niranjan et al (1982) saw ~3-fold higher binding to mtDNA than nDNA *in vivo*. While this difference was still significant, it is much less than the *in vitro* differences reported by Allen and Coombs and Backer and Weinstein (40- to 500-fold). Furthermore, the vulnerability of mtDNA to this chemical showed species variation (Niranjan et al. 1986), which may be in part related to mitochondrial enzyme differences leading to differential activation (Niranjan et al. 1985). It will be interesting to further explore the relative vulnerabilities of the mitochondrial and nuclear genomes in fish; mtDNA has been shown to be more sensitive to various genotoxins than nDNA in mammalian studies (Backer et al. 1980; Balansky et al. 1996; Yakes et al. 1997; Sawyer et al. 1999a).

However, another important implication of the at least equal or greater sensitivity of the mitochondrial genome to many pollutants is that unless a specific nuclear-coded gene needs to be targeted to assess DNA damage to specific genes, using the LA-QPCR assay with just mtDNA would be sufficient in many field studies. This is advantageous for many environmental models, such as the Atlantic killifish, as they generally do not have significant nuclear genome sequence data available, particularly the 10 kb or more of contiguous sequence needed to design primers for the LA-QPCR

assay. In such models, it is much easier to design primers for mtDNA, since there is a tremendous amount of mtDNA sequence data available and "universal" primers have already been designed in conserved regions that will amplify the mtDNA of most vertebrates (Kocher et al., 1989). In fact, the primers that we used for *Fundulus* were initially designed for the javeline goby (*Acanthogobius hasta*) (Kim et al. 2004).

Interestingly, liver DNA showed more damage in Superfund site killifish than did muscle DNA, although this difference was not observed in the acute exposure. This may be explained by the fact that the killifish population at the Superfund site has been chronically exposed to a complex mixture of chemicals including primarily several different PAHs, as well as PCP and metals (EPA, 2007). Therefore, this population is exposed continuously to a variety of genotoxic agents through their diet. In this case, liver would be one of the primary targets of the toxic effect. The differences in contaminant mixture, and/or time course and route of exposure, may be why we see differences in the DNA damage profile of Elizabeth River *Fundulus* relative to fish acutely exposed to BaP via i.p. injection. Thus, our data suggest that data from muscle tissue alone might not be as informative as other tissues such as liver. Therefore, an examination of several tissues may be necessary to correctly assess the genotoxic effects of pollutants.

Currently, there are several assays that measure DNA damage. DNA-adduct analysis by ^{32}P-postlabelling method can be used to measure chemical-specific adducts.

The method is considered the most sensitive in detecting PAH-adducts, but is expensive and time consuming (van der Oost et al. 2003). Flow cytometry (Theodorakis 2001; Goanvec et al. 2004; Barbee et al. 2008), single-strand break assays (McFarland et al. 1999a; Bolognesi et al. 2006), and the micronucleus test (Al-Sabti et al. 1995; Hayashi et al. 1998; Arkhipchuk et al. 2005; Cavas et al. 2005; Bolognesi et al. 2006) are also used in laboratory and field studies as genotoxic indicators, but these assays detect gross chromosomal damage or abnormalities from clastogenic and aneugenic effects. The comet assay is one of the most widely used biomarkers of DNA damage in laboratory (Pandrangi et al. 1995; Belpaeme et al. 1996; Nacci et al. 1996; Devaux et al. 1997; Devaux et al. 1998) and field studies (Pandrangi et al. 1995; Devaux et al. 1998; Steinert et al. 2002; Lemos et al. 2005; Yang et al. 2006). However, there is still high study-to-study variation, and standardization of measurement is necessary to overcome this issue (Cotelle et al. 1999; Siu et al. 2004; Lemos et al. 2005).

Considering the issues concerning the assays described above, the adaptation of LA-QPCR assay will be an important and effective means to measure general DNA damage in the environment. With this assay, one can detect general lesions caused by a variety of pollution sources or complex mixtures. At the same time, one can target a specific gene or the entire mitochondrial genome for damage assessment. This ability to easily distinguish mtDNA damage from nDNA damage or total DNA damage is an important advantage of this assay, since there is increasing concern for the vulnerability

of mitochondria to various pollutants (Backer et al. 1980; Sawyer et al. 1999a), and for the lower DNA repair capability of the mitochondria for certain kinds of damage (Yakes et al. 1997; Larsen et al. 2005).

In conclusion, we have successfully adapted the LA-QPCR assay in an environmental model. This assay can be utilized as a sensitive method of detecting general nuclear and mitochondrial DNA damage, and has significant potential as a tool for biomonitoring. Therefore, we propose the use of the LA-QPCR assay for DNA damage for use in environmental assessments.

Table 2.1 Primers used for *Fundulus heteroclitus* LA-QPCR assay

Target	Primer sequences
Large nuclear target	F: 5′- CAGCCGCCCGCAAATTCTCA -3′
11459 bp	R: 5′- CAGAATGCGGGCCTTGCTGA -3′
Small nuclear target	F: 5′- GCCGCTGCCTTCATTGCTGT -3′
234 bp	R: 5′- ATGAGCTGGGTGTGCGCTGA -3′
Long mitochondrial target [a]	F: 5′- TTGCACCAAGAGTTTTTGGTTCCTAAGACC -3′
9416 bp	R: 5′- GATGTTGGATCAGGACATCCCAATGGTGCA -3′
Small mitochondrial target	F: 5′- ATCTGCATGGCCAACGCCTA -3′
264 bp	R: 5′- GGCGGTGCCAGTTTCCTTTT –3′

[a] Adapted from Kim et al. (2004)

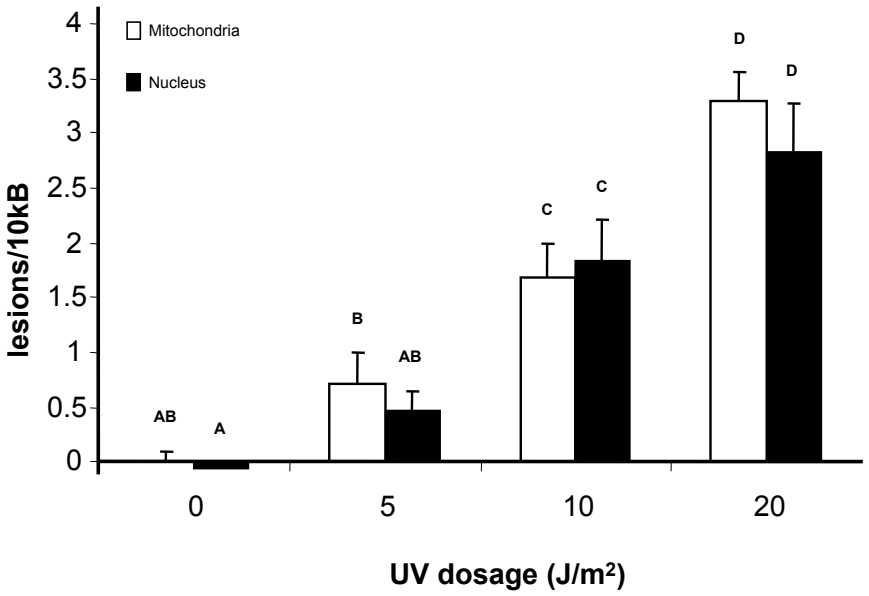


Figure 2.1 DNA damage in UVC-treated killifish DNA.

DNA isolated from liver and brain of adult killifish was exposed to different doses of UVC. There are significant dose-dependent increases in both mitochondrial and nuclear DNA ($p < 0.001$), but no differences between mtDNA and nDNA at a given dose ($p = 0.775$). Different letters indicate significant differences ($p < 0.05$) according to Fisher's LSD. n = 6 per treatment group. Error bars indicate standard error of means.

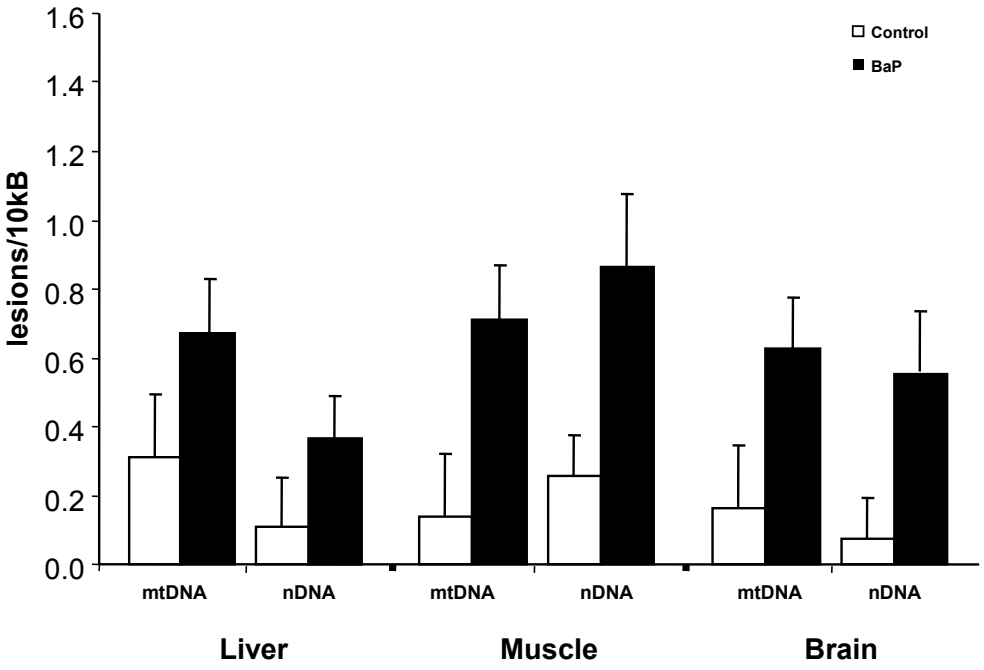


Figure 2.2 Levels of DNA damage in BaP treated adults.

DNA lesion frequencies were measured in adult male fish dosed with 10 mg/kg BaP. BaP treatment was the only significant factor according to three-way ANOVA ($p < 0.001$). Neither DNA source nor tissue significantly affected levels of DNA damage ($p = 0.177$ and $p = 0.493$ respectively). n = 10 per treatment group. Error bars indicate standard error of means.

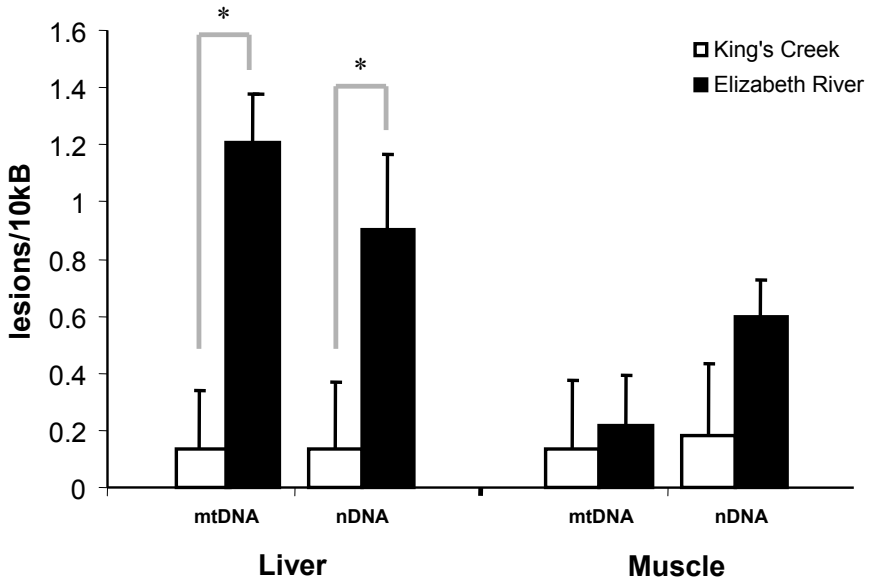


Figure 2.3 Levels of DNA damage in Superfund site and reference *Fundulus heteroclitus* populations.

DNA lesion frequency was measured from liver and muscle of adult fish captured from a Superfund site and a reference site. Population and tissue type significantly affected levels of DNA damage ($p < 0.001$ and $p = 0.047$ respectively) according to three-way ANOVA. However, there was no difference between mtDNA and nDNA ($p = 0.839$). * denotes significant difference ($p < 0.05$) according to Fisher's LSD. n = 5 per treatment. Error bars indicate standard error of means.

3. Effects of benzo[*a*]pyrene on mitochondrial and nuclear DNA damage in the Atlantic killifish (*Fundulus heteroclitus*) from creosote-contaminated and reference sites

Part of this chapter was submitted for publication under the same title in the journal Aquatic Toxicology. The authors are Dawoon Jung, Youngeun Cho, Leonard B. Collins, James A. Swenberg, and Richard T. Di Giulio.

3.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are released into the environment as byproducts of incomplete combustion. These compounds can enter into aquatic ecosystems from anthropogenic sources such as urban runoff, fuel exhaust, and oil shipping and refineries (Latimer et al. 2003). PAHs have been increasing in the aquatic ecosystem in parallel with the rapid urbanization since the 1970s (Van Metre et al. 2000; Van Metre et al. 2005). Thus, the need to understand how these chemicals may affect aquatic systems is gaining in importance.

Several studies in mammalian models indicate that mitochondria are major targets of PAH toxicity within the cell. These studies have shown that PAHs localize to the mitochondria and are correlated with a decrease in ATP production (Zhu et al. 1995), loss of mitochondrial membrane potential and changes in mitochondrial morphology (Li

et al. 2003; Xia et al. 2004), and induction of the mitochondria-dependent apoptotic pathway (Ko et al. 2004; Huc et al. 2006).

Mitochondria are responsible for the majority of cellular energy production in eukaryotes. In addition, mitochondria are involved in cellular calcium homeostasis, cell signaling, and apoptosis (Wallace 1999; Duchen 2004). As a result, disruption of normal mitochondrial function has been linked with a variety of diseases in mammalian systems, including humans (Chan 2006). Thousands of mitochondria can exist in each eukaryotic cell, and within each mitochondrion, several copies of the mitochondrial genome are present. These circular chromosomes are believed to be especially prone to damage (Wallace 1999). Their proximity to the electron transport chain, where reactive oxygen species are constantly produced, makes mitochondrial DNA (mtDNA) susceptible to oxidative damage. In addition, DNA repair activity in the mitochondria is less efficient than nuclear DNA (nDNA) repair (Shadel et al. 1997). Base excision repair (BER) has been observed in the mitochondria, but nucleotide excision repair (NER) has not been detected in the mitochondria yet (Berneburg et al. 2006; Van Houten et al. 2006; Maynard et al. 2009). Therefore, mtDNA may be more affected by bulky adducts, such as those formed by PAH-metabolites, than nDNA. Finally, mtDNA is considered structurally less protected from damage than the more compact DNA-histone complex formed in the nucleus (Suliman et al. 2004). In mammals, mtDNA is especially susceptible to damage from stable adduct formation by the dihydrodiol-epoxide

metabolite of benzo[*a*]pyrene (BaP), a well-studied PAH (Backer et al. 1980; Niranjan et al. 1985). Furthermore, Graziewicz *et al.* (2004) showed that mtDNA replication is hindered by adducts formed from BaP and benzo[*c*] phenanthrene epoxides *in vitro*. Almost nothing is known in terms of the role of xenobiotics, including PAHs, in the integrity and function of fish mtDNA. Since fish are generally efficient in metabolizing PAHs (van der Oost et al. 2003), it is plausible that reactive metabolite formation is elevated in these organisms. Therefore, fish may be quite vulnerable to both mtDNA and nDNA damage formed by bulky adducts as well as oxidative adducts, such as 8-hydoroxyguanosine (8-Oxo-dG), generated by PAHs.

The Elizabeth River, located in Portsmouth, Virginia, is a tributary of the James River in the Chesapeake Bay watershed. The sediment, groundwater, and soil at the Atlantic Wood Industries Superfund site on the southern branch of the Elizabeth River are heavily contaminated with PAHs (EPA 2007; Hartwell et al. 2007). This site has been listed as a National Priorities Superfund site since 1990 (EPA 1995a). Total PAH concentrations at the site can be as high as 500 μg/g dry sediment, with BaP being one of the most prevalent PAHs, accounting for about 11 % of total PAHs (Vogelbein et al. 2008). These concentrations suggest that the Atlantic Wood Industries Superfund site is comparable to some of the most PAH-contaminated rivers/estuaries in the world (Walker et al. 2004).

Atlantic killifish (*Fundulus heteroclitus*) at this site show increased liver tumor formation (Vogelbein et al. 1990; Vogelbein et al. 1999). They also show refractory cytochrome P450 1A (CYP1A) expression and activity (Van Veld et al. 1995; Meyer et al. 2003c). Compared to fish from reference sites, these fish show resistance to developmental toxicity from the sediment collected at the site (Ownby et al. 2002; Meyer et al. 2003c; Wassenberg et al. 2004). Interestingly, this population shows greater sensitivity to hypoxia (Meyer et al. 2003a), upregulated antioxidant defense (Meyer et al. 2003b; Bacanskas et al. 2004), and higher expression levels of enzymes involved in glycolytic energy metabolism (Meyer et al. 2005). Such reports indicate that there may be alterations in mitochondrial structure and/or function in this population. This could have important ecological implications. Fish mitochondria experience high conversion of oxygen to H_2O_2 and episodes of environmental and physiological hypoxia (Abele et al. 2004). At the same time, fish generally have lower mitochondrial antioxidant activity than terrestrial vertebrates (Wilhelm Filho 2007). Added oxidative stress may result in reduced bioenergetic fitness and subsequently, may result in reduced survival of these fish.

In this study, we hypothesized that treatment with BaP would induce damage in killifish mtDNA to a greater extent than in nDNA. We also hypothesized that killifish from the Superfund site would show different patterns of mtDNA damage than the reference site population. To test our hypotheses we compared the basal DNA damage

levels, as well as changes in DNA damage levels in response to BaP for both mtDNA and nDNA of adult killifish collected from Elizabeth River and a reference site, and laboratory-reared progeny from these populations. In addition, we evaluated the amount of oxidative DNA damage in the two populations of fish with or without BaP treatment. Our results showed that BaP does induce mtDNA damage, and that the PAH-adapted Elizabeth River killifish show higher basal level of DNA damage, but some protection from addition damage by BaP treatment.

3.2 Materials and Methods

3.2.1 Fish care

Adult killifish were captured using baited minnow traps from the Atlantic Wood Superfund site at the Elizabeth River in Portsmouth, VA (36°48'27.4'' N, 76°17'36.1'' W) on July 18, 2006. Fish were caught on the same day from King's Creek, a tributary of the Severn River in Gloucester County, VA (37º17'52.4"N, 76º25'31.4"W). After capture, fish were transported to the Duke University Ecotoxicology Laboratory and maintained as described previously (Matson et al. 2008). Fish were depurated in the laboratory for at least four weeks before adult and larval experiments were conducted.

Fish were manually spawned and eggs were incubated on plates with wet filter paper at 27°C. After 14 days, fish were hatched by the addition of 25 ppt artificial sea water (ASW, Instant Ocean®, Aquarium Systems, Forster & Smith, Rhinelander, WI,

USA) and gentle shaking for about 30 minutes. Larvae were kept in 2-liter beakers in 25 ppt ASW and fed brine shrimp daily until initiation of the experiment.

3.2.2 BaP treatment and DNA isolation

For the adult exposure experiment, male fish from both populations were moved to individual aerated tanks with 3 L of 25 ppt artificial seawater (Instant Ocean®, Aquarium Systems, Forster & Smith, Rhinelander, WI, USA) 24 hours before treatment. Ten fish from each population were injected intraperitoneally with BaP dissolved in corn oil. 10 mg/kg of the chemical was injected with an injection volume of 5 μL/g wet weight. Additionally, ten male fish from each population were injected with 5 μL/g wet weight of corn oil as an experimental control. The fish were fed Tetramin® Tropical Fish Food (Tetra Systems, Blacksburg, VA, USA) everyday and sacrificed 72 hours post-treatment.

In the larval experiment, 7 day post hatch (dph) larvae (10-12 from each population per treatment) were exposed to BaP individually in 20-mL scintillation vials containing 10 mL artificial sea water. The water was dosed with equal volumes of DMSO (dimethyl sulfoxide, Sigma-Aldrich, St. Louis, MO, USA) containing BaP (Sigma-Aldrich) to final concentrations of 0, 50, 100, and 200 μg/L BaP. The solvent concentrations did not exceed 0.03 % for all treatments. Larvae were sacrificed 5 days post-treatment.

After sacrifice, individual tissues (brain, liver, muscle) from adults and whole individual larvae were flash frozen in 20% glycerol and stored in -80°C until further analysis. Tissues were later ground in liquid nitrogen, and total DNA was extracted with Genomic-tip 20/G (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol.

3.2.3 Long amplicon quantitative PCR

Long amplicon quantitative PCR (LA-QPCR) is a method that indirectly measures general structural DNA damage. When extremely long PCR targets (~ 10 kB) are amplified in control and experimental groups, DNA templates with lesions and/or structural alterations hinder DNA polymerase activity. Quantification of amplification product allows one to calculate lesion frequency in the target genome. With different primers, one can simultaneously measure both mtDNA and nDNA in the same sample.

LA-QPCR was performed as described previously (Santos et al. 2006; Jung et al. 2009). Briefly, 10 ng DNA was amplified with *rTth* polymerase (Applied Biosystems, Foster City, CA, USA) using primers for long mitochondrial and nuclear targets as described by Jung et al. (2009). Small mtDNA and nDNA targets were amplified simultaneously for normalization. PicoGreen dye (Invitrogen, Carlsbad, CA, USA) was used to quantify the template and PCR product, and relative amplifications were converted to relative lesion frequency per 10 kB DNA.

3.2.4 DNA damage analysis with LC-MS/MS

For DNA adduct analysis, additional adult male killifish were treated with either BaP or corn oil (vehicle control) as described above. At 72 hours post-treatment, fish livers were harvested, flash frozen in liquid nitrogen, and kept at -80°C until further analysis. For liver samples weighing less than 100 mg, livers from two individuals were pooled to ensure that enough DNA could be isolated. For isolation of DNA, 2,2,6,6-tetramethyl-piperidinoxyl (TEMPO, 20 mM final concentration, Sigma-Aldrich) was added to all solutions. Frozen tissues were thawed at 4°C and homogenized in PBS with a Polytron® (Kinematica, Lucerne, Switzerland). After centrifugation at 1700 x g for 10 min, the nuclear pellets were incubated in lysis buffer (Qiagen. Valencia, CA, USA) overnight at 4°C with proteinase K (Applied Biosystems, Foster City, CA, USA). Protein was precipitated with protein precipitation solution and centrifugation at 2000 x g for 10 min. The DNA and RNA in the supernatant were precipitated by mixing with isopropanol for subsequent centrifugation at 2000 x g for 5 min at 4°C. The DNA/RNA pellet was washed with 70% ice cold ethanol and centrifuged at 2000 x g for 3 min at 4°C. After air drying, it was resuspended in cell lysis solution and incubated with Ribonuclease A (Qiagen) for 30 min at 37°C. The enzyme was precipitated with protein precipitation solution (Qiagen) followed by DNA precipitation by propanol and washing with 70% ethanol as described above. The washed and dried DNA was

resuspended in sterilized double distilled water. DNA concentration was measured using a NanoDrop (Thermo Scientific, Wilmington, DE, USA) and DNA was stored at -80°C until assayed.

For enzymatic hydrolysis, 50 μg DNA was mixed with 50 μl of 80 mM Tris-HCl, 20 mM $MgCl_2$ buffer (pH 7) and appropriate standards ([$^{15}N_5$]8-Oxo-dG internal standard (Cambridge Isotope Laboratories, Andover, MA, USA) for all samples and 8-Oxo-dG analyte standard (Sigma-Aldrich) for positive controls). The hydrolysis was started by addition of 32 U DNAse I (Sigma-Aldrich) and incubation at 37°C for 15 min. 3.4 mU phosphodiesterase I (Sigma-Aldrich) and 2.5 U alkaline phosphatase (Sigma-Aldrich) were then added and the samples were incubated again at 37 °C for 60 min. The final volume of each sample containing all reagents and internal standard was 300 μl. The samples went through an enzyme removal process by centrifugal filtration using pre-washed Centricon YM-10 microcentrifuge filters (Millipore, Bedford, MA, USA).

Samples of hydrolyzed DNA were chromatographed on an Agilent 1200 HPLC system with an automated fraction collector to separate 8-oxo-dG from matrix. Separation was performed on an Ultrasphere ODS C18 4.6 × 250 mm 5 μm column (Beckman, Fullerton, CA) using a gradient of 10 mM ammonium formate in water (adjusted to pH 4.3 with formic acid) and methanol. Methanol composition was held at 7% from 0 to 22 min, then increased linearly to 80% in 1 min, was held at 80% for 6 min to elute TEMPO, decreased to 7% in 1 min, and held at 7% for 6 min for column re-

equilibration. A 275-μL aliquot of sample was injected, and the flow rate was 1 mL per min. The column oven, autosampler tray, and fraction collector chamber temperatures were maintained at 30°C, 4°C and 4°C, respectively. The retention time of 8-oxo-dG, which was determined by using 2'-deoxyguanosine (dG) as a retention time marker and multiplying its retention time by 1.5, was ~18 min. Fractions containing 8-oxo-dG were automatically collected from 1.5 min before until 1.5 min after its predicted retention time. The fraction collection tubes were placed in a SpeedVac concentrator (ThermoFinnigan, San Jose, CA) and evaporated to dryness. Sample residue was transferred to autosampler vials via 2 x 130 μL washings with 50:50::water:methanol, evaporated to dryness in a SpeedVac concentrator, and finally redissolved in 20 μL HPLC grade water for subsequent analysis by LC-MS/MS. The dG amount in each sample was determined during fraction collection by comparison with dG calibration standards using UV detection at 264 nm.

The quantitative analysis of 8-oxo-dG was performed with an Acquity UPLC (Waters, Milford, MA) coupled to a TSQ-Quantum Ultra triple-quadrupole mass analyzer (ThermoFinnigan) using heat assisted electrospray ionization (HESI) in positive mode. Separation was performed on a 2.1 × 100 mm HSS T3 C18, 1.8 μm column (Waters, Milford MA) with gradient elution at a flow rate of 200 μL per min using 0.1% acetic acid in water and methanol. Methanol composition started at 1% and increased linearly to 5% B in 2 min, was held at 5% for 8 min, increased linearly to 80% in 2 min,

held at 80% for 2 min, decreased to 1% in 1 min, then held at 1% for 4 min for column re-equilibration. The retention time of 8-oxo-dG was 9 min, and the total run time was 20 min. The analyte and internal standard were detected in selected reaction monitoring mode (SRM), monitoring the transitions of m/z 284.1 to 168.05 and m/z 289.1 to 173.05 for 8-oxo-dG and [$^{15}N_5$]8-Oxo-dG, respectively. The electrospray conditions were as follows: positive mode, spray voltage of 3000 V, vaporizer (HESI) temperature of 250°C, sheath gas flow rate 35 (arbitrary units), auxiliary gas flow rate 30 (arbitrary units), capillary temperature of 285°C, and collision energy of 12 eV.

For BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) -dG analysis, DNA was isolated and prepared as above, excluding the filtration step with Centricon YM-10 microcentrifuge filters. Quantitative analysis was performed using the procedure described in Chapter 6.

3.2.5 Statistics

Statistical analyses were performed with SPSS, version 15 for Windows (SPSS Inc., Chicago, IL, USA). Normality was checked for all data sets using the Shapiro-Wilk test. Student's *t*-test and analysis of variance (ANOVA) with Fisher's Protected Least-Significant Differences (LSD) were used where appropriate ($\alpha = 0.05$).

3.3 Results

3.3.1 Basal DNA damage in King's Creek and Elizabeth River population

There was a significant overall difference in the basal lesion frequency levels in the mtDNA (Figure 3.1A) and nDNA (Figure 3.1B) of liver, brain, and muscle between King's Creek and Elizabeth River fish ($p = 0.003$). Three-way ANOVA was performed to test the effect of population, genome type, and tissue type. Significant differences were seen between the populations ($p < 0.001$). Highest lesion frequencies were seen in the liver and brain mtDNA of Elizabeth River population. Within the Elizabeth River population, higher lesion frequencies were observed in the mitochondria than in the nucleus, but the difference was not significant ($p = 0.071$). Additionally, there was no difference among tissue types ($p = 0.860$). Among the tissues, the liver and brain mitochondria basal lesion frequencies were significantly different between the populations ($p = 0.009$ and $p = 0.005$ respectively) according to Student's *t*-test.

3.3.2 DNA damage in response to BaP in adults

When treated with BaP, there was also an overall significance among treatment groups ($p = 0.014$) (Figure 3.2). Population was a significant factor influencing the differences ($p = 0.002$), and the two populations had significantly different responses to BaP treatment ($p = 0.002$). BaP-treated King's Creek fish showed significant increases in DNA damage in both genome types in muscle and brain compared to the control fish,

but there were no differences in DNA damage in Elizabeth River killifish in any of the tissues. Specifically, King's Creek fish demonstrated two to seven fold increases in DNA damage when treated with BaP. However, Elizabeth River fish showed less than a two-fold increase in damage in the BaP treated group compared to the control group.

3.3.3 DNA damage in response to BaP in larvae

When larvae from the two sites were compared, the lesion frequencies of both mtDNA (Figure 3.3A) and nDNA (Figure 3.3B) were significantly increased by BaP treatment ($p < 0.001$). This increase was dose-dependent ($p = 0.001$). There was significantly higher damage in the mtDNA compared to the nDNA ($p = 0.002$), and population also had a significant effect on the differences ($p < 0.001$). However, there was no significant interaction between any of the independent variables. Post-hoc analysis showed that all treatment groups except the Elizabeth River control group were significantly different from the King's Creek control group ($p \leq 0.05$).

3.3.4 LC-MS/MS analysis

To further investigate the nature of the DNA damage detected with the LA-QPCR method, we performed LC-MS/MS analysis on adult male killifish dosed with BaP to investigate the presence of 8-oxo-dG, a biomarker of oxidative DNA damage. There were no significant differences in 8-oxo-dG frequency among the groups ($p = 0.304$)

(Figure 3.4). Neither treatment nor population had significant effects ($p = 0.834$ and $p = 0.173$ respectively). However, when the BPDE-dG frequency was measured in King's Creek fish, BaP-treated group had significantly higher levels of adduct compared to the control group ($p < 0.01$) (Figure 3.5).

3.4 Discussion

We examined basal levels of DNA damage, as well as the effect of BaP treatment on nDNA and mtDNA damage in killifish from the Elizabeth River Superfund site and a reference site. The Elizabeth River population had higher levels of DNA damage compared to the reference population both in the adults brought to the laboratory and in the F1 generation reared in the laboratory. These differences were seen in both mtDNA and nDNA. Our previous results on field-caught samples using the same DNA damage detection method showed similar results as the current study in that significantly higher levels of mtDNA and nDNA damage were seen in the liver of the Elizabeth River fish compared to the reference fish (Jung et al. 2009). Using ^{32}P-post labeling analysis, Rose et al (2000) found DNA adducts in spleen, liver, and anterior kidney in adult killifish collected from the Elizabeth River.

An interesting distinction between the previous studies and this study is that, previous studies assessed the damage levels right after field capture, whereas in this study, fish from both populations were reared in the laboratory for at least four weeks

for depuration purposes. Therefore, the fish from the Elizabeth River appear to retain substantial levels of DNA damage well after removal from contaminant exposure. Generally, bulky adducts formed by covalent binding of PAH-metabolites are repaired by NER. Studies using mammalian cell cultures show that even though BaP-adducts can be readily repaired, different types of PAH-adducts as well as different co-exposures with other chemicals can affect repair efficiencies (Maier et al. 2002; Lagerqvist et al. 2008). Elizabeth River fish are exposed to a complex mixture of PAHs that can form various bulky adducts. Future studies will explore how such exposure history can affect the efficiency of DNA repair in these populations.

F1 generation Elizabeth River killifish reared in the laboratory still had higher levels of DNA damage in the control group when compared to the King's Creek fish. Wills et al (2009) reported that F1 generation fish from the Elizabeth River population exhibited slower metabolism of PAHs and that these fish seem to retain the parent compound much longer than F1 fish from the reference site. Given these data, it is plausible to infer that what we see in this study in terms of DNA damage is due to the fact that the Elizabeth River killifish are still retaining PAHs their body, and there is a possibility that there was maternal transfer of the chemicals into the eggs in the Elizabeth River fish.

In response to BaP treatment, King's Creek fish showed expected increases in DNA damage in all tissues regardless of the genome type. There was a 2-fold to 7-fold

increase in the level of DNA damage depending on the tissue type and the source of DNA. It is notable that the muscle and brain showed higher relative BaP-induced DNA damage than the liver. In contrast to the reference site population, there were no significant differences in DNA damage estimates for BaP-treated and control Elizabeth River fish with BaP-treated group showing less than 1.5-increase compared to the control group. It should be noted that, although the relative damage due to BaP-treatment is different, the actual levels of DNA damage in the Elizabeth River population, whether BaP-treated or not, are comparable to the BaP-treated King's Creek fish. Interestingly, a similar trend was seen in the experiment with the F1 generation. F1 fish from both King's Creek and Elizabeth River had significantly higher levels of DNA damage in response to BaP ($p < 0.001$). However, the magnitude of increase was different in the two populations. Elizabeth River F1 fish had between 1.1 and 5.7 fold increase in lesion frequencies in response to BaP treatment, whereas the fold increase in the King's Creek population was from 18.2 to 76.9. This indicates that, although the F1 fish from both populations showed higher level of lesion formation with BaP treatment in larvae compared to the adults, the Superfund site larvae fish appear to exhibit some protection from BaP-induced DNA damage. This protection may be caused by slower metabolism of BaP into reactive metabolites due to the refractory CYP1A induction of the F1 generation seen in previous studies (Meyer et al. 2002a; Meyer et al. 2002b).

A previous study with killifish adapted to a site with high concentrations of dioxin-like compounds also observed that adapted fish had reduced DNA adduct formation compared to reference site fish when treated with BaP (Nacci et al. 2002b). The authors suggested that constant exposure to aryl hydrocarbon receptor (AhR) agonists would result in a refractory AhR pathway, and therefore reduced metabolism of such compounds. As a result, there would be reduced DNA adduct formation in these fish when exposed to chemicals that are metabolized via the AhR pathway. This would result in reduced DNA damage from treatment with genotoxins that are activated through the AhR pathway. However, due to constant exposure to these chemicals, there would still be higher levels of DNA damage *in situ*. Analysis of PAH metabolism in the F1 generation of Elizabeth River fish supports this hypothesis (Wills et al. 2009).

McFarland et al. (1999b) found that brown bullhead (*Ameiurus nebulosus*) from sites heavily contaminated with PAHs had refractory CYP1A activity, as well as decreased DNA single strand breakage in comparison to fish at sites where chronic exposure to PAH began more recently. The authors argued that there might be increased DNA repair and cell turnover rates in the population with a longer exposure history. This may explain our data in which the Elizabeth River population exhibits no difference in DNA damage following BaP treatment, although the time frame of 72 hrs may be inadequate for this to occur. However, this does not explain the high damage level in the Elizabeth River population "depurated" in lab. Nevertheless, it would be interesting to

explore whether there are inherent differences in response to DNA damaging agents and whether these differences are dependent on the AhR-pathway.

There was no difference between the two populations in the abundance of the 8-oxo-dG adduct following BaP exposure. This indicates that the majority of the relative increase in DNA damage we see in BaP-dosed fish is due to covalent binding of BaP metabolites with DNA, rather than from oxidative damage due to reactive oxidative metabolite formation. This was supported by our analysis of the BPDE-dG adduct measurement in the King's Creek fish treated with BaP. Previous studies have shown that Elizabeth River fish have a higher antioxidant capacity than reference fish (Meyer et al. 2003b; Bacanskas et al. 2004). However, our results show that oxidative DNA damage may not be a major form of DNA damage in these fish. Nevertheless, whether oxidative DNA damage is caused by exposure to complex mixture has not been explored. Further analysis using more sensitive techniques will allow us to determine the extent of oxidative DNA damage that occur in the Elizabeth River killifish.

One question that we wanted to explore was whether there were differences in the damage level of mtDNA and nDNA in response to BaP treatment. On the organism level, loss of mitochondrial function would translate to decreased energy production, and therefore decreased energy allocation on growth and reproduction. (Kooijman et al. 1996; Woodford et al. 1998). Unlike nDNA, which less than 5% is actively transcribed, the entire mtDNA is continuously involved in transcription. Therefore, damage in the

mtDNA can result in deleterious functional consequences (Izzotti 2009). In this study, we observed at least equal levels of DNA damage in the mitochondria as in the nucleus in response to BaP treatment both in adults and in larvae. Therefore, we can speculate that the integrity of mtDNA is affected by BaP treatment and that this may have implications for normal mitochondrial function and other cellular functions in which mitochondria are involved. In addition, Elizabeth River control groups in both adults and larvae showed higher levels of mtDNA damage. This implies that the Superfund site fish population may be chronically dealing with a lower energy budget compared to reference site fish, which may affect their general well being in addition to more severe toxic effects. In addition, lower energy budget arising from defective oxidative metabolism would affect reproduction of these fish (Figure 1.3), and ultimately change population dynamics of these fish (Woodford et al. 1998). However, it is yet to be determined whether the Elizabeth River killifish show reduction in fecundity or changes in reproduction. In addition, if there are such differences, correlation of such phenomena with decreased energy budget should also be examined.

It should be noted that our results are in agreement with our previous study in killifish (Jung et al. 2009), but contradict previous results from early mammalian studies that show as much as 50 to 500 times higher BaP adduct formation in mtDNA than in nDNA (Allen et al. 1980; Backer et al. 1980; Sawyer et al. 1999b). Although we observed higher basal levels of damage in the mtDNA compared to nDNA in both adult and in F1

larvae, there were no statistically significant differences between the genome types in BaP-induced damage in adults. Mitochondria have no NER capacity that can remove bulky adducts caused by PAHs (Shadel et al. 1997; Sawyer et al. 1999a). Although, it is debatable whether fish have lower NER capability compared to their mammalian counterparts (Regan et al. 1982; Weimer et al. 2000; Willett et al. 2001; Notch and Meyer 2009), it may be that there is universally reduced NER in the fish nucleus as well as in the mitochondria, and that the damages that we detected in both genomes were due largely to bulky adducts. However, it remains to be seen whether mitochondria are more efficient in DNA repair against oxidative damage and have a different adduct profile from nDNA.

Of the three tissues examined, the difference in response to BaP in the two populations was most pronounced in the brain. Although little is known concerning BaP metabolism in brain tissue of fish, Ericson et al. (1999) showed that BaP adducts in the brain of juvenile northern pike (*Esox lucius*) are not much higher, but are more persistent relative to other tissues. In addition, CYP1A proteins have been identified in localized areas such as the globus pallidus in the rat brain (Kapitulnik et al. 1987; Strobel et al. 2001). This indicates that there may be higher rate of BaP metabolism in specific areas of the brain. Neurological effects of PAHs have been gaining in research attention recently (Saunders et al. 2006; McCallister et al. 2008; Gesto et al. 2009; Perera et al. 2009). High levels of DNA damage in the brain could have long-term behavioral effects, such as

foraging behavior as well as flight response from predators, likely decreasing survival. In addition, it has been shown that BaP induces apoptosis (Huc et al. 2003; Ko et al. 2004). Apoptosis in terminally-differentiated cells, such as neurons, may also enhance damage of the brain tissue. Further investigation may increase our understanding of whether exposure to BaP and other chemicals may affect important behavioral aspects in aquatic animals.

In conclusion, killifish from the Elizabeth River Superfund site show high levels of basal nuclear and mitochondrial DNA damage compared to fish from the reference site, even after several weeks of depuration. However, the level of damage induced due to BaP treatment is lower in this population versus killifish from a relatively unpolluted site. This same trend is seen in the F1 larvae of the Superfund site population that were hatched in the laboratory. Our results indicate that mitochondria are important targets of BaP toxicity in teleosts. Additional studies are needed to determine the consequences of this mtDNA damage in terms of mitochondrial protein production, aerobic metabolism, and organismal fitness.

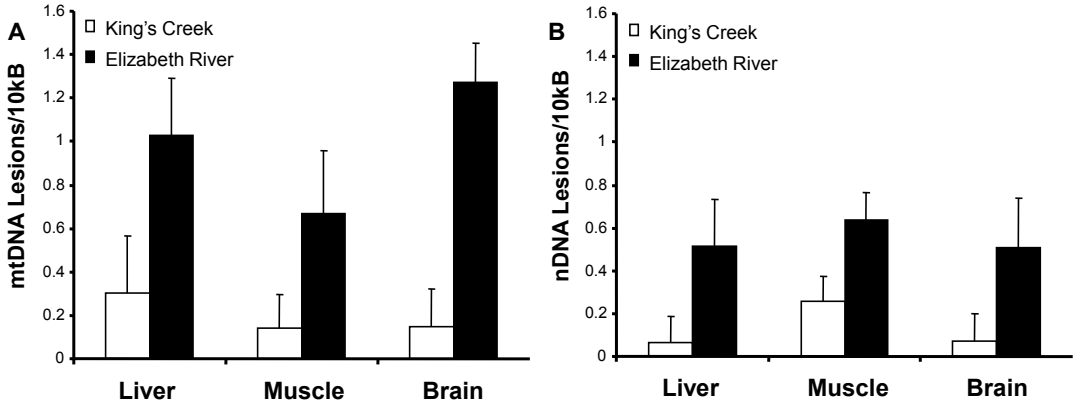


Figure 3.1 Relative basal DNA damage levels in adult killifish from King's Creek and Atlantic Wood Superfund Site.

Comparison of the basal level of DNA damage in both the mitochondria (A) and the nucleus (B) between the two populations using LA-QPCR method show that Atlantic Wood fish have higher levels of DNA damage than fish from King's Creek ($p < 0.001$).

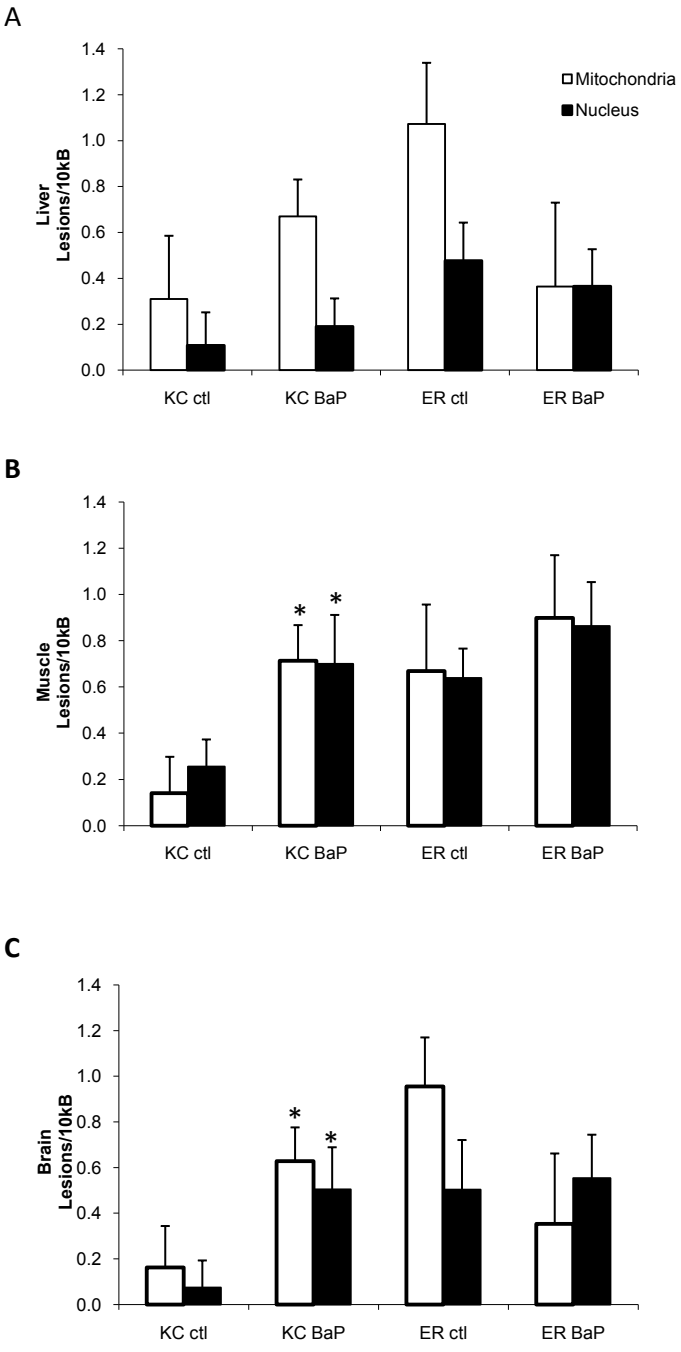


Figure 3.2 General DNA damage in response to BaP treatment in adults from King's Creek and Elizabeth River Superfund Site.

(Figure 3.2) DNA damage in response to i.p. injection of corn oil or 10 mg/kg BaP treatment were measured in liver (A), muscle (B), and brain (C) of adult killifish. BaP-treated group in the King's Creek population exhibited higher levels of DNA damage compared to the control group ($p < 0.001$). No differences were seen in the Elizabeth River population. Open bars indicate mtDNA, and closed bars indicate nDNA. * indicates significant difference ($p < 0.05$) compared to the KC control group. Error bars represent standard error of means.

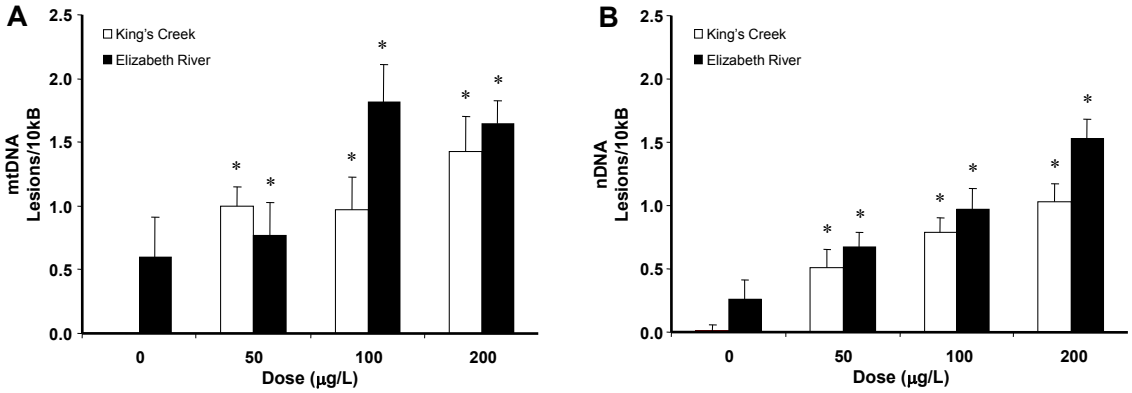


Figure 3.3 DNA damage levels in larval killifish from King's Creek and Atlantic Wood Superfund Site.

There is overall significant difference among the group according to one-way ANOVA ($p < 0.001$). BaP treatment induces higher rates of DNA damage in the mitochondria (A) compared to the nucleus (B) ($p = 0.002$). Fish from the Superfund site show higher basal levels of DNA damage and are differently affected by BaP treatment than the reference site ($p < 0.001$). * indicates significant difference ($p < 0.05$) compared to the King's Creek control group according to Fisher's LSD.

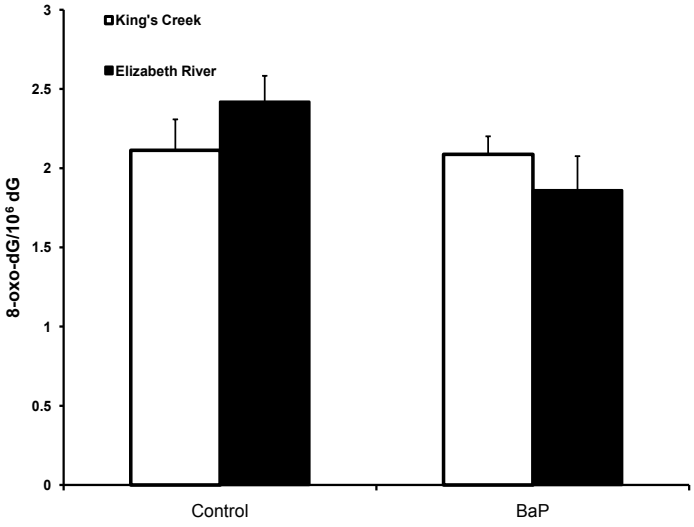


Figure 3.4 8-oxo-dG adduct levels in adult killifish liver treated with BaP.

8-oxo-dG adduct levels did not differ between control and treated groups ($p = 0.304$). Error bars represent standard error of means.

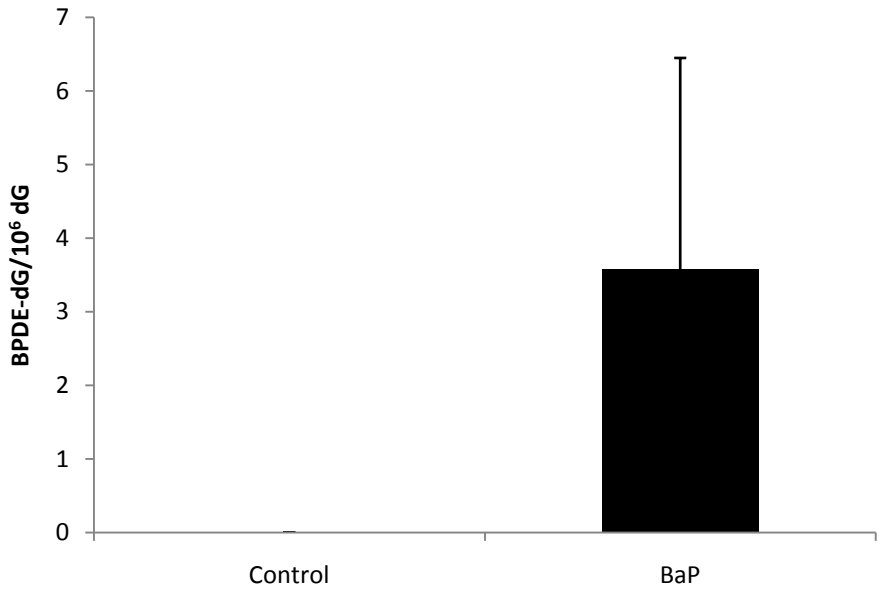


Figure 3.5 BPDE-dG adduct levels in adult killifish liver treated with BaP.

BPDE-dG adduct levels in BaP-treated fish were significantly higher than control fish ($p < 0.01$). Error bars represent standard error of means.

4. Identification of mitochondrial cytochrome P450 induced in response to polycyclic aromatic hydrocarbons in the Atlantic killifish (*Fundulus heteroclitus*)

This chapter was submitted for publication under the same title to the journal Comparative Biochemistry and Physiology- Part C: Toxicology and Pharmacology. The authors are Dawoon Jung and Richard T. Di Giulio.

4.1 Introduction

Cytochrome P450 proteins (CYPs) are hemoproteins that exist in most organisms from bacteria to humans. There are 57 known families of cytochrome P450 proteins (CYPs) in humans (Furge et al. 2006; Myasoedova 2008). These proteins are involved in a variety of cellular functions such as drug metabolism, fatty acid metabolism, and bile acid biosynthesis. Within these protein families are the CYP1 family of proteins regulated by the aryl hydrocarbon receptor (AhR) pathway. These proteins are involved in the metabolism of planar organic carbons such as polycyclic aromatic hydrocarbons (PAHs) (Hahn 1998; Denison et al. 2003; Nebert et al. 2004).

While most CYPs are located and function in the endoplasmic reticulum (microsomes), some CYP proteins are localized to the mitochondria. The existence of mitochondrial CYPs in vertebrates have been known for over forty years (Harding et al. 1964; Omura 2006). These proteins are involved in the synthesis of steroid hormone and

bile acid, and in the conversion of Vitamin D to its active form (Omura 2006). Nevertheless, less is known regarding the presence and function of mitochondrial CYPs that are involved in the metabolism of xenobiotic chemicals similar to microsomes. Since the 1980s, several studies in mammalian systems have shown that several CYP proteins with 'microsomal characteristics' (i.e. CYPs involved in the metabolism of xenobiotics chemicals) are present in the mitochondria (Niranjan et al. 1984; Niranjan et al. 1985; Anandatheerthavarada et al. 1997). Mitochondrial CYPs that can be detected with antibodies against microsomal CYP1, CYP2, and CYP3 families have been identified in various tissues in mammals (Niranjan et al. 1985; Bhagwat et al. 1995; Anandatheerthavarada et al. 1997; Genter et al. 2006). Further analyses have shown that these proteins are induced by the same xenobiotic compounds that induce CYPs in the endoplasmic reticulum (Niranjan et al. 1985; Niranjan et al. 1988; Boopathi et al. 2000). It is now accepted that these proteins are transcribed by the same genes that code for the microsomal CYP proteins, but are post-translationally modified in the cytosol, either by phosphorylation or truncation of the N-terminus, and targeted to the mitochondria (Addya et al. 1997; Genter et al. 2006).

In several teleost fish species, microsomal CYP proteins involved in xenobiotic metabolism, especially as part of the AhR pathway, have been investigated in detail (Stegeman et al. 1991; Williams et al. 1998a; Wirgin et al. 2004). Mitochondrial CYPs that are involved in steroid hormone and bile acid synthesis have also been identified

(Leusch et al. 2003; Hagen et al. 2006). However, unlike the mammalian system, the existence of mitochondrial CYPs involved in xenobiotic metabolism has not been reported in fish.

It is known in mammals that PAHs are localized to the mitochondria (Zhu et al. 1995), and PAHs have been shown to adversely impact mitochondrial function (Li et al. 2003; Ko et al. 2004; Xia et al. 2004; Huc et al. 2006). Hence, identification of functionally active CYP1A proteins that have the potential to metabolize PAHs in fish mitochondria may be important in understanding the effect of such xenobiotics in the mitochondria of these aquatic organisms. This would further enhance our understanding of how pollutants metabolized by the AhR pathway may affect the organism's energy metabolism.

As poikilotherms, fish have to respond to changes in external temperature (Wilhelm Filho 2007; Fangue et al. 2009). In addition, frequent episodes of hypoxia can increase oxidative stress in aquatic organisms (Abele et al. 2004). Therefore, mitochondrial integrity is important in fish. The Atlantic killifish (*Fundulus heteroclitus*) is a well-established environmental model in the field of aquatic toxicology (Burnett et al. 2007). Activation of the AhR pathway in response to contaminants in the environment have been examined in detail in this species (Binder et al. 1985; Prince et al. 1995a; Prince et al. 1995b; Hahn 1998; Nacci et al. 1999; Bello et al. 2001; Meyer et al. 2002b; Nacci et al. 2002b; Powell et al. 2004; Wassenberg et al. 2004; Matson et al. 2008). Several populations

of killifish inhabiting areas with high levels of PAHs and dioxin-like compounds show refractory CYP1A induction in responses to these chemicals (Prince et al. 1995a; Elskus et al. 1999; Nacci et al. 1999). Previous studies from one such population with this refractory CYP1A expression, the killifish inhabiting the Atlantic Wood Superfund site at the Elizabeth River in Portsmourth, Virginia (U.S.A), indicate that these fish may have impaired mitochondrial function compared to fish from uncontaminated sites. These fish have higher tolerance to oxidative stress and upregulated antioxidant defenses, such as manganese superoxide dismutase, (Meyer et al. 2003b; Bacanskas et al. 2004), greater sensitivity to hypoxia (Meyer et al. 2003a), indications of more reliance on anaerobic metabolism (Meyer et al. 2005), and higher levels of mitochondrial DNA damage (Jung et al. 2009).

In this study, we confirmed the presence of mitochondrial CYP1A proteins in the killifish and examined the induction of mitochondrial CYP1A proteins in adult and larval killifish. In addition, we compared the protein level and enzyme activity of killifish from the Elizabeth River Superfund site to fish from a reference site to test whether the Elizabeth River fish exhibit refractory induction of mitochondrial CYP1A protein level and activity, as is the case for microsomal CYP1A in this population.

4.2 Materials and Methods

4.2.1 Fish care

Adult killifish were captured from King's Creek (KC), a tributary of the Severn River in Gloucester County, VA (37°17′52.4″N, 76°25′31.4″W), and from the Atlantic Wood Superfund Site at the Elizabeth River (ER) in Portsmouth, VA (36°48′27.4′′ N, 76°17′36.1′′ W) using baited minnow traps. The fish were moved to the Duke University Ecotoxicology Laboratory and reared in lab as described previously (Wills et al. 2009). The fish were kept in controlled conditions for at least four weeks before experiments were conducted.

Fish eggs were collected from egg boxes stationed in each tank. Eggs were plated out and incubated at 27°C for 14 days. Fish were manually hatched by adding 20 ppt artificial sea water (ASW, Instant Ocean®, Aquarium Systems, Rhinelander, WI, USA) to the plates and shaking for 30 min. Hatched larvae were kept in 2-L beakers until initiation of experiments. Larvae were fed brine shrimp (Brine Shrimp Direct, Ogden, UT, USA) daily, and water was changed every other day during maintenance.

4.2.2 Chemical treatment

Adult male fish were moved to individual 3-L tanks 24 hrs prior to the start of the experiment. In the treatment group, fish were dosed with 10 mg/kg benzo[*a*]pyrene (BaP, Sigma-Aldrich, St. Louis, MO, USA) dissolved in corn oil via intraperitoneal injection. An equal volume per weight of corn oil (5 ml/kg) was injected in the control

group. The water in each tank was changed by statical renewal every other day and the fish were fed Tetramin® Tropical Fish Food (Tetra Systems, Blacksburg, VA, USA) every day. At the end of 3d, fish were sacrificed via cervical dislocation. The liver was harvested from each individual, flash frozen and stored at -80°C until further analysis.

Larvae were dosed 10d after hatching with either 100 μg/L BaP (Absolute Standards, Inc., Hamden, CT, USA) or 100 μg/L benzo[*k*]fluoranthene (BkF, Absolute Standards, Inc.), a potent CYP1A inducer, via water-borne exposure. Fish were dosed in groups of ten in 100 mL of 20 ppt ASW. Larvae were fed brine shrimp daily during exposure. The dosing solution was changed by statical renewal every other day throughout the exposure period. At the end of 5d, fish from each group were flash frozen together, and kept at -80°C until further analysis.

4.2.3 Mitochondria and microsome isolation

Mitochondrial and cytosolic fractions were isolated according to a protocol modified from Harada and Omura (1980), Ivanina and Sokolova (2008), and Dong et al. (2009). Briefly, isolated livers or whole embryos were blotted and washed with homogenization buffer (300 mM sucrose, 50 mM KCl, 50 mM NaCl, 8 mM EGTA, 30 mM HEPES, pH 7.5). Tissues were then homogenized in homogenization buffer with 1 mM PMSF (phenylmethanesulfonyl fluoride, Sigma-Aldrich), 1 μg/mL leupeptin (Sigma-Aldrich), and 1 μg/mL aproponin (Sigma-Aldrich) with a hand-held tissue homogenizer. The homogenate was centrifuged at 500 g for 15 min at 4°C. Supernatant was moved to a

new tube, and the pellet was homogenized again and centrifuged at 500 g for 15 min at 4°C. Supernatant from the second run was combined with the supernatant from the first run and spun at 1,000 g for 15 min at 4°C. The supernatant was moved to a new tube and centrifuged at 10,000 g for 15 min at 4°C. The pellet was resuspended with wash buffer (30 mM HEPES, 500 mM sucrose, pH 7.5) and centrifuged again at 10,000 g for 15 min at 4°C. This procedure was repeated three more times. After the fifth run, the pellet (mitochondria) was resuspended with suspension buffer (0.25 M sucrose, 1 mM EDTA, 0.1 M Tris-HCl in 20% glycerol, pH 7.4), aliquoted, flash frozen in liquid nitrogen, and stored at -80°C.

For adult samples, the supernatant after the first 10,000 x g centrifugation was moved to a new tube and centrifuged at 18,000 g for 30 minutes at 4°C, and the pellet was discarded. The supernatant was spun at 105,000 g for 60 min at 4°C, and the pellet (microsomes) was washed with wash buffer, resuspended in suspension buffer, aliquoted, and stored at – 80°C.

4.2.4 Western blot

Proteins were separated by SDS-PAGE loaded with equal volumes of protein. The amount of protein loaded varied between 10-30 μg, depending on the antibody used for probing. Separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes. Blots were first probed with monoclonal antibody 1-12-3 (3 μg/mL) against scup (*Stenotomus chrysops*) CYP1A (Park et al. 1986; Kloepper-Sams et al. 1987), then

probed with goat anti-mouse IgG horseradish peroxidase secondary antibody (1:10,000 dilution, Jackson laboratory, Bar Harbor, ME, USA). Additional blots were also probed with monoclonal antibody against the endoplasmic reticulum marker protein, bovine liver protein disulfide isomerase (anti-PDI) (1:1000 dilution, Stressgen, Ann Arbor, MI, USA), and monoclonal antibodies against two mitochondrial marker proteins, cytochrome c oxidase subunit I (anti-COX I, 1: 1000 dilution, MitoScience, Eugene, OR, USA) and cytochrome c oxidase subunit IV (anti-COX IV, 1: 1000 dilution, MitoScience) and with secondary antibody as described above. The blots were visualized on x-ray film by enhanced chemiluminescence (SuperSignal® West Pico Chemiluminescence Substrate, Thermo Scientific, Rockford, IL, USA) following the manufacturer's protocol. Band intensity was measured using Image J (Abramoff et al. 2004).

4.2.5 Enzyme activity assays

The glucose 6-phosphatase assay was conducted using the protocol modified from Greenawalt (1974). Briefly, triplicates of 20 - 30 μg of protein from samples were incubated with assay buffer (200 mM imidazole-HCl, 500 mM glucose-6-phosphate, 50 mM NAD^+, 100 mM EDTA, 0.1 IU mutarotase, 1 IU glucose dehydrogenase) in a 96-well microplate and the increase in NADH was measured at 340 nm. Absorbance was converted to activity using 6.3 $mM^{-1} \cdot cm^{-1}$ as the molar extinction coefficient for NADH.

The *in vitro* ethoxyresorufin-*O*-deethylase (EROD) assay was conducted on isolated mitochondrial and microsomal fractions according to the protocols of Meyer et

al. (2002b) and Kennedy et al. (1993). Three replicates of 20 to 50 μg of protein from each sample were incubated with 2.5 μM 7-ethoxyresorufin in cofactor buffer (100 mM HEPES, NADPH (102 μM NADPH, 120 μM NADH, and 5 mM $MgSO_4$) in a 96-well microplate. The florescence of resorufin (reaction product) was measured at 530/590 nm and molar specific activity (pmols resorufin per mg protein per minute) was calculated.

4.2.6 Phosphorylation site prediction

Potential phosphorylation site in the N-terminal region of the killifish CYP1A protein was predicted using the NetPhosK 1.0 server, http://www.cbs.dtu.dk/services/NetPhosK/ (Blom et al. 2004), with the *Fundulus heteroclitus* CYP1A protein sequence obtained from GenBank (Accession number AAD01809).

4.2.7 Statistics

Statistical analyses were performed using SPSS, version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Student's *t* test or analysis of variance (ANOVA) with Fisher's Protected Least-Significant Differences (LSD) were used where appropriate ($\alpha = 0.05$).

4.3 Results

4.3.1 Verification of isolation method

To confirm that the mitochondrial fractions isolated were not significantly contaminated by microsomes, we performed Western blots with an antibody against

protein disulfide isomerase (PDI), commonly used as a microsomal biomarker, and an antibody against cytochrome c oxidase subunit I (COXI), a part of the mitochondrial respiratory chain. We could detect the COXI polypeptide in the mitochondrial fraction, but not in the microsomal fraction. Similarly, PDI was detected in the microsomal fraction only (Figure 1). In addition, we tested the activity of glucose 6-phosphatase, another common method utilized to identify the presence of microsomes. The enzyme activity in the mitochondrial fraction was about 8% of the enzyme activity in the microsomal fraction. These results confirmed that there was minimal cross contamination between mitochondria and microsomes.

4.3.2 Adult liver CYP1A expression and in vitro EROD activity

In adult male fish from our reference site, CYP1A protein was detected in the mitochondria (Figure 2). CYP1A levels were increased in fish treated with 10 mg/kg BaP by roughly 2.11 fold. In contrast, another nuclear-transcribed mitochondrial protein, COX IV, was not different between treatment groups, which demonstrates that the increase in CYP1A protein was specifically due to BaP treatment. BaP-treated fish liver mitochondria showed significantly higher EROD activity level compared to the control group (Figure 3, $p = 0.001$). The level of increase was similar to that of microsomal CYP1A activity ($p = 0.12$).

4.3.3 Comparison of CYP1A protein expression and activity in adapted and reference populations

Compared to larval killifish from the reference site (KC), larval Elizabeth River killifish (ER) showed refractory mitochondrial EROD activity as well as refractory protein induction when dosed with 100 μg/L BaP (Figure 4A). Only KC fish treated with BaP showed increased protein level and activity ($p < 0.001$). Similar results were seen in fish treated with 100 μg/L BkF, a more potent CYP1A inducer (Figure 4B). Only the BkF-treated KC larvae showed increased protein levels and activity ($p < 0.001$). In both cases, the mitochondrial CYP1A protein levels and microsomal CYP1A protein levels (indirectly measured from the cytosol) were similar.

4.3.4 Prediction of potential phosphorylation site

Analysis of the killifish CYP1A protein sequence for potential PKC-mediated phosphorylation site using NetPhosK 1.0 identified Thr-31 to be a probable PKC phosphorylation site (score = 0.72).

4.4 Discussion

Previous studies have shown that BaP can be metabolized in the mitochondria *in vitro* and subsequently induce mitochondrial DNA damage (Niranjan et al. 1984; Niranjan et al. 1985), indicating that mitochondrial CYPs can be involved in the activation of BaP in the mitochondria. However, Raza and Avadhani (1988) showed that BaP metabolism by mitochondrial CYP1A1 *in vitro* was only about 10% of microsomal

CYP1A1 by measuring the metabolite concentration of [^{3}H] BaP incubated with subcellular fractions. Similarly, a recent study by Dong et al (2009) using knock-in mice showed that mice lacking signals for endoplasmic reticulum-targeting in the *Cyp1a1* gene showed similar BaP-induced toxicity symptoms as *Cyp1a1*(-/-) knockout mice, whereas mice lacking the mitochondria-targeting signal were not much different from wild type mice. Therefore, the authors concluded that most of the detoxification of orally administrated BaP was achieved by microsomal CYP1A1, and that mitochondrial CYP1A1 had only minor role in the detoxification process. Although it seems that mitochondrial CYP1A may not play a great role in the acute toxicity of BaP in the organismal level, the role these proteins play in terms of affecting mitochondrial function remains unclear. This is especially true in light of our results. *In vitro* EROD activity in the mitochondria of BaP dosed fish was similar to that of the microsome, suggesting that the mitochondrial CYP1A protein is as active as the microsomal protein. Further studies on actual metabolism of BaP in the two cellular components of the killifish will be necessary to understand the role of mitochondrial CYP1As in the killifish model.

The Elizabeth River Superfund site killifish population seems to have lowered energy metabolism level as well as increased antioxidant defense mechanism (Meyer et al. 2003b; Bacanskas et al. 2004; Meyer et al. 2005). At the same time, this fish population is more vulnerable to low oxygen levels compared to reference site killifish (Meyer et al.

2003a). In addition, previous studies in our laboratory show that the basal mitochondrial DNA (mtDNA) damage level is higher in both wild-caught fish and larval F1 fish that were fertilized in the laboratory (Jung et al. 2009, Chapter 3). Interestingly, although we saw that BaP treatment to KC fish results in increased level of mitochondrial DNA damage, the same treatment did not induce higher level of mitochondrial DNA damage compared to the corn oil-treted group in the ER population. These studies suggest that adaptation of the ER killifish to the PAH-contaminated habitat may have fitness cost to the mitochondria.

The ER killifish show refractive microsomal CYP1A induction in response to PAH treatment in both the adult fish and the F1 generation fertilized in laboratory (Elskus et al. 1999; Nacci et al. 1999; Meyer et al. 2002b; Meyer et al. 2003c). In the present study, we verified that these fish also have refractive mitochondrial CYP1A protein levels as well as reduced activity. The recalcitrant CYP1A proteins in the ER killifish occur at the transcriptional level (Meyer et al. 2003c). Since it is accepted that CYP1A translocation into the mitochondria occurs after post-translational modification (Genter et al. 2006), it is not surprising to see this refractory characteristic in the mitochondria as well. It is quite likely that the quantity of CYP proteins targeted to the mitochondria would also be reduced in this population due to the lower total amount of the gene product. Since genes in the AhR pathway are not induced by PAHs in these fish, the proportion that are translocated into the mitochondria are also not increased compared

to the KC fish. Therefore, this recalcitrant phenotype is probably not a protective mechanism directly related to the mitochondria, but is another result of the repression of the AhR pathway as a whole. However, how this refractive character affects BaP-mediated toxicity in the mitochondria is an intriguing question that has yet to be explored. If PAHs, such as BaP, are metabolized and activated in the mitochondria by mitochondrial CYP1As (Niranjan et al. 1984; Niranjan et al. 1985), the smaller amount of these proteins in the ER killifish could protect from activation of BaP in the mitochondria. This hypothesis is somewhat supported by our previous study that examined mtDNA damage (Jung et al. submitted; chapter 3).

There is evidence that translocation of CYP1A to mitochondria can be signaling-mediated through endoprotease activity or protein kinase C (PKC) activity (Dasari et al. 2006). More specifically, the N-terminal of the CYP1A protein consists of endoplasmic reticulum-targeting sequences. When this sequence is cleaved by endoprotease, a cryptic mitochondria-targeting sequence is revealed. Alternatively, phosphorylation of Thr-35 by PKC in mammals diminishes the protein's affinity for the endoplasmic reticulum. Genter et al. (2006) speculated that events such as reactive oxygen species (ROS) production may trigger this signaling event. In populations of fish adapted to sites such as the Elizabeth River where fish are continuously exposed to a complex mixture of chemicals (Hartwell et al. 2007; Vogelbein et al. 2008), alterations in cellular pathways in addition to the AhR pathway are not entirely unexpected. Our results suggest that there

is a potential PKC-mediated phosphorylation site at Thr-31 in the killifish CYP1A protein. Nevertheless, alterations in the PKC signaling pathway in the ER killifish have not been examined. More in-depth investigation into the mechanism behind translocation of mitochondrial CYP1As in this killifish population may be worthwhile.

In conclusion, we have identified for the first time in teleost fish, mitochondrial CYP proteins that are induced by AhR agonists and involved in xenobiotics metabolism. Our finding implies that PAH metabolism and its consequences should also be examined in the mitochondria, when considering the aquatic ecosystem. In addition, we have confirmed that the ER killifish show recalcitrant mitochondrial CYP1A protein level and activity in response to BaP treatment. Further investigation into the implications this has for the function of killifish mitochondria and how this affects the survival of the animals in the wild is warranted.

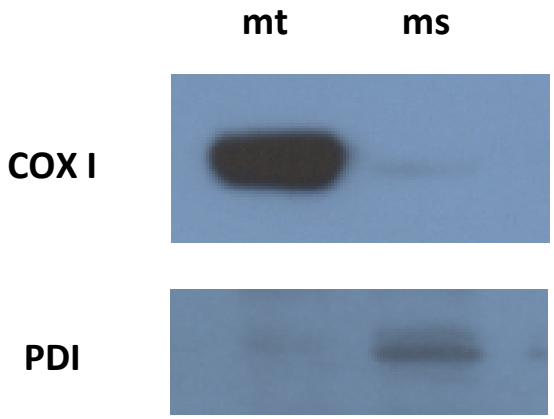


Figure 4.1 Expression of mitochondrial and microsomal marker proteins.

Equal amounts of proteins loaded for SDS-PAGE followed by probing for mitochondrial and microsomal fractions with antibodies specific to mitochondria (COX I) and endoplasmic reticulum (PDI). There is minimal cross contamination between mitochondria and microsome. mt: mitochondria, ms: microsomes, cytochrome oxidase subunit I: COX I, protein disulfide isomerase: PDI.

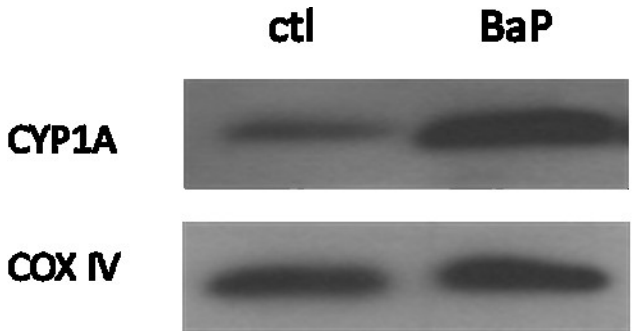


Figure 4.2 CYP1A and COX IV protein expression in mitochondria of adult killifish.

Adult male killifish were dosed with corn oil or BaP (10 mg/kg) and mitochondria from the liver of each individual was isolated 72 hrs after treatment. There is about 2.11 fold increase in CYP1A expression in response to BaP injection. However, COX IV protein, a nuclear-encoded mitochondrial protein does not show difference between treatment groups. ctl: control, BaP: BaP treated, COX IV: cytochrome oxidase subunit IV.

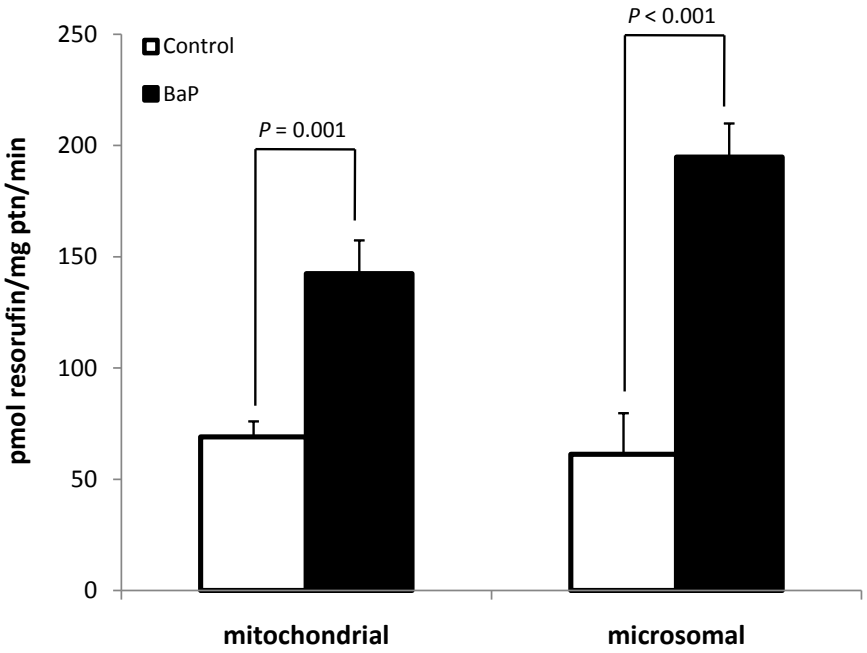


Figure 4.3 *In vitro* EROD activity level in mitochondrial and microsomal fractions of adult killifish.

Proteins were isolated from livers of adult male killifish dosed with corn oil (control) or BaP (10 mg/kg). *In vitro* EROD activity was increased in the BaP-treated fish. Mitochondrial EROD activity of control fish was set as reference. The level of increase was similar to that of microsomal CYP1A activity ($p = 0.12$). n = 5 per treatment. Error bars represent standard error of means.

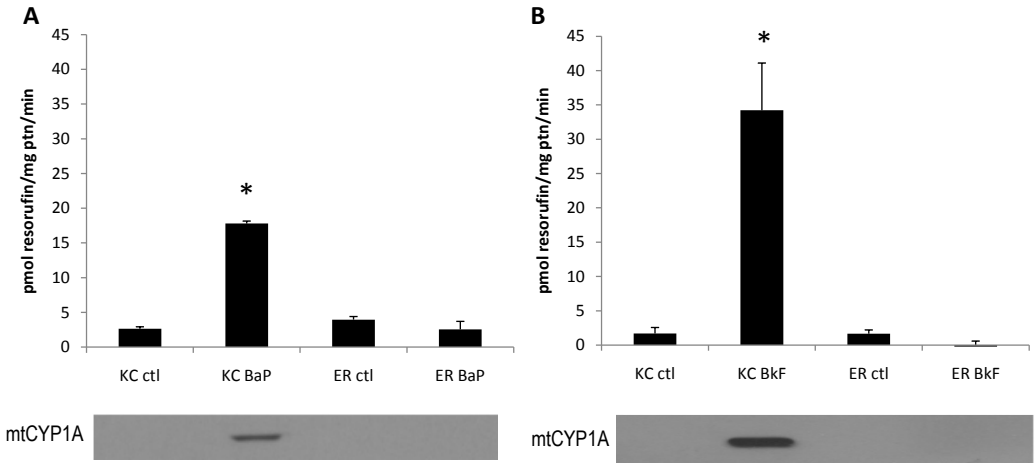


Figure 4.4 *In vitro* EROD activity level and CYP1A protein expression in larval killifish mitochondria.

Protein isolated from the mitochondria of pooled individuals of 10 day-old larvae dosed with DMSO or 100 μg/L BaP (A) and DMSO or 100 μg/L BkF (B) were examined for EROD activity and CYP1A protein expression. *In vitro* EROD activity and CYP1A protein level were increased only in the BaP and BkF-treated King's Creek fish. KC: King's Creek fish, ER: Elizabeth River fish, ctl: control, BaP: BaP treated mtCYP1A: mitochondrial CYP1A. n = 3 to 4 of pooled samples of 10. Error bars represent standard error of means. * indicates $p < 0.001$.

5. Energy metabolism in the two populations of fish

This chapter is product of a collaborative effort of Dawoon Jung and Cole P. Thompson (Duke), John H. Grimes, Thomas M. O'Connell, and Jeffrey M. Macdonald (UNC-Chapel Hill).

5.1 Introduction

In eukaryotic organisms, the majority of cellular energy is produced in the form of adenosine triphosphate (ATP) in the mitochondria via aerobic metabolism. The ATP is utilized in cellular functions that contribute to basic maintenance (homeostasis and metabolism), growth, and reproduction in the organism. Therefore, in addition to the direct effects toxicants may have at the cellular level, energetic budget theory predicts that toxicants that affect mitochondrial function can have indirect adverse effects on an organism by decreasing the energy supply (van Noordwijk et al. 1986; De Coen et al. 2001; Ricklefs et al. 2002). This can result in decreased reproduction and low survival, with potential population-level impacts (Sokolova et al. 2008).

Aquatic organisms, including fishes and invertebrates, are exposed to various environmental stressors including changes in oxygen, pH, temperature, and salinity. Temperature changes in the environment affect ectotherms, since the kinetics of the enzymatic processes in the body change according to changes in the temperature (Portner et al. 2007b). Poikilotherms acquire tolerance to temperature changes in part by

modifying mitochondrial structure and density (Portner et al. 2007a). At the same time, extreme thermal conditions induce energetic stress to the animal by decreased ATP formation capacity in the mitochondria in low temperatures and increased demand in oxygen as well as increased mitochondrial proton leakage in high temperatures (Portner et al. 2006; Wilhelm Filho 2007). Damage to mitochondria by pollutants may decrease thermal tolerance of aquatic ectotherms.

Polycyclic aromatic hydrocarbons (PAHs) are common organic pollutants released into the environment from various anthropogenic sources such as diesel exhaust, industrial burning, urban runoff, and creosote release (Latimer et al. 2003). Previous studies involving laboratory models have shown that PAHs may affect mitochondrial function. Ultrafine particles, including PAHs from diesel exhaust, can enter the mitochondrial membrane (Li et al. 2003) and be metabolized and activated by mitochondrial CYP1As (Niranjan et al. 1984; Niranjan et al. 1985). Genotoxic PAHs, such as benzo[*a*]pyrene (BaP), metabolized in the mitochondria can induce mitochondrial DNA damage (Backer et al. 1980; Backer et al. 1982; Niranjan et al. 1985; Jung et al. 2009). PAHs can also induce oxidative stress that results in mitochondrial dysfunction (Li et al. 2003; Xia et al. 2004). In addition, Zhu et al. (1995) have shown that a quinone metabolite of benzo[*a*]pyrene (BaP) can directly disrupt mitochondrial structure and ATP synthesis without causing oxidative stress.

The Atlantic Wood Superfund site located on the southern branch of the Elizabeth River in Portsmouth, VA (USA) is heavily contaminated with PAHs from creosote released from the former wood treatment facility (Hartwell et al. 2007; Vogelbein et al. 2008). The population of Atlantic killifish (*Fundulus heteroclitus*) found at this site show recalcitrant CYP1A levels both in the mitochondria (Chapter 4) and in the endoplasmic reticulum (Meyer et al. 2002b). They have lower tolerance to hypoxia, but show higher antioxidant parameters (Meyer et al. 2003a; Meyer et al. 2003b; Bacanskas et al. 2004). In addition, these fish have damaged mtDNA both in the adult tissues and in the F1 larvae (Chapter 3). Moreover, differential display analyses indicated that these fish may have impaired mitochondrial energy metabolism (Meyer et al. 2005).

In this study, we examined the effect of BaP on aerobic respiration, energy production, and anaerobic metabolism. In addition, we compared the energy metabolism products of PAH-adapted killifish from the Elizabeth River Superfund site to killifish from a reference site.

5.2 Materials and Methods

5.2.1 Fish care

Adult killifish (*Fundulus heteroclitus*) were captured using baited minnow traps from King's Creek, a relatively unpolluted area tributary of the Severn River in Gloucester County, VA (37°17'52.4"N, 76°25'31.4"W), and from the Atlantic Wood

Superfund Site at the Elizabeth River in Portsmouth, VA (36°48′27.4′′ N, 76°17′36.1′′ W). Additional fish were collected from Main's Creek in Chesapeake, VA (37°45′15.7′′ N, 76°17′15.0′′ W), a site relatively less polluted compared to the Superfund site. The fish were transported to the Duke University Ecotoxicology Laboratory and reared in lab as described previously (Wills et al. 2009). For embryo studies, fish were manually spawned from fish acclimated to laboratory conditions for at least one month and fertilized *in vitro* in 20 ppt artificial sea water (ASW, Instant Ocean®, Aquarium Systems, Forster & Smith, Rhinelander, WI, USA). For larval studies, embryos were manually hatched by gentle shaking in ASW for 30 min. Larvae were fed brine shrimp (Brine Shrimp Direct, Ogden, UT, USA) daily, and water was changed every other day during maintenance.

5.2.2 Chemical treatment

At 24 hours post-fertilizations (hpf), individual embryos were placed in 10 ml of ASW in 20 ml scintillation vials dosed with 100 μg/L BaP dissolved in DMSO or equal volume of DMSO. The volume of DMSO was below 0.3 % of total volume for all experiments conducted. Embryos were scored for deformities at 96 hpf. Little deforminty was observed, and fish with no apparent deformities were kept and used for oxygen consumption assay at 120 hpf.

Three day-post hatch larvae were exposed to water-borne dose of 0, 50, 100, and 200 μg/L BaP for four days. Water was changed once during the experiment. At the end of the experiment, ten larvae were pooled from each treatment, flash frozen and kept in -80°C until enzyme activity measurement.

Adult male fish (5.4 – 14.8 g) from Elizabeth River and King's Creek were moved to 3-L dosing chambers 24 hrs before the start of the experiment for acclimation purposes. Fish were intraperitoneally injected with 10 mg/kg BaP dissolved in corn oil or the same volume of corn oil for control fish. Fish were fed Tetramin® Tropical Fish Food (Tetra Systems, Blacksburg, VA, USA) daily, and water was changed every other day during the exposure period. Three days after dosing, fish were sacrificed via cervical dislocation. Livers were collected and immediately processed for metabolomic analysis as described below. Adult male fish from Main's Creek (5.6 – 18.1 g) were dosed dosed as above and liver and dorsal muscle tissues were collected, flash frozen, and kept at -80 °C for enzyme activity assays described below.

5.2.3 Oxygen consumption

Embryos were washed and placed in ASW until measurement. Five embryos per treatment were moved to ASW saturated with oxygen in respiration chamber (Qubit Systems, Kingston, Canada) at room temperature. The total decrease in dissolved oxygen in the chamber was measured for five minutes.

5.2.4 Enzyme activity assays

Pyruvate kinase (PK) and lactate dehydrogenase (LDH) activity were measured in liver and muscle tissues according to a protocol modified from Couture et al.(2007) and Pelletier et al. (1994). Tissues were homogenized in 10x volume of homogenization buffer (20 mM HEPES, 1 mM EDTA, 0.10 % Triton X-100, pH 7.5) and protein concentration was determined using BioRad DC Protein Assay dye reagent (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard. For all assays, triplicates of 30 to 50 μg of protein were analyzed for each sample. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

For PK measurement, 195 μL of PK reaction buffer (50 mM imidazole, 20 mM $MgCl_2 \cdot 6H_2O$, 70 mM KCl, 0.5 mM β–NADH, 20 mM ADP, 500 mM phosphoenolpyruvate, excess lactate dehydrogenase, pH 7.5) was added to each well containing 5 μL of sample. Absorbance was monitored at 340 nm for 15 minutes. As above, 195 μL of LDH reaction buffer (0.1 mM KH_2PO_4, 0.1 mM K_2HPO_4, 0.5 mM β–NADH, 1.0 mM pyruvate, pH 7.0) was added to each well containing 5 μL of sample and absorbance was monitored at 340 nm for 15 minutes. A millimolar extinction coefficient of 6.22 $mM^{-1} \times cm^{-1}$ for NADH was used to calculate the enzyme activities of both assays.

5.2.5 ^{1}H-NMR spectroscopic analysis

The liver of BaP or DMSO treated Elizabeth River and King's Creek fish (described above) was dissected out from each individual, wet weight was measured, and immediately moved to ice-cold 65% methanol. Samples were then sonicated for 10 seconds, repeatedly flash frozen and thawed, and centrifuged to extract metabolites from tissue. Resulting methanol extracts were dried down and kept at -80°C until further analysis.

For NMR analysis, 200 µl of D_2O (0.10% NaN_3; 1 mM 3-(trimethylsilyl)-1-propanesulfonic acid (DSS)) was added to each sample. Each sample was then sonicated, vortexed, and spun down for 10 minutes at 16,200 g. Fifty µl of the material were spun down at 1500 rpm for 20 minutes. Supernatants were run on a 600MHz NMR with a 2 second pre-saturation sequence and a 20 millisecond CPMG sequence (Carr, Purcell, Meiboom and Gill sequence) to minimize the effects of chemical exchange and narrow the residual water signal.

5.2.6 Statistics

Statistical analyses for treatment and population were generally performed using SPSS, version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). The assumption of normality was tested for all data sets using the Shapiro-Wilk's test. Student's *t* test or analysis of variance (ANOVA) and Fisher's Protected Least-Significant Differences (LSD) were used to test for differences among groups ($\alpha = 0.05$). For the data obtained

from NMR spectroscopy, principal component analysis (PCA) and partial least square (PLS) analysis were performed using Simca P+ version 11.5 (Umetrics, Umeå, Sweden).

5.3 Results

5.3.1 Oxygen consumption

Oxygen consumption rates of killifish embryos dosed with either 100 μg/L BaP or DMSO are shown in figure 5.1. There was an overall significant effect among the treatment groups ($p < 0.01$). Within the King's Creek killifish, oxygen consumption in the BaP-treated group was significantly less than consumption by DMSO-treated fish ($p = 0.015$). In contrast, BaP-treated Elizabeth River killifish consumed significantly more oxygen compared to the control group from the same population ($p = 0.019$). Two-way ANOVA showed a significant interaction between population and treatment ($p = 0.005$). The oxygen consumption of both treatment groups of Elizabeth River killifish embryos was lower than the King's Creek control group.

5.3.2 Enzyme activity assay

Given the above result, we wanted to test whether treatment with BaP would affect glycolysis and anaerobic metabolism of killifish. Since there was a possibility that the difference in the LDH activity of the King's Creek killifish and the Elizabeth River killifish is due to a difference in the northern and southern populations (Crawford et al. 1989; Schulte et al. 2000), we used killifish from another reference site, Main's Creek,

located upstream of the Atlantic Wood Superfund site, to avoid this potential difference. Main's Creek fish were used to establish the time-point at which to conduct a comparison study of the PAH-adapted and naïve populations of fish. LDH activity was not significantly different between the treatment groups in either the liver (Figure 5.2A) or the muscle (Figure 5.2C). Similarly, PK activity was also not different between the treatment groups in the liver (Figure 5.2B) or the muscle (Figure 5.2D). LDH to PK ratio, which indicates the ratio of anaerobic metabolism to general energy metabolism was not different among any treatments either (data not shown). In addition, LDH activity of whole larval homogenates from the two populations dosed with 100 μg/L BaP showed similar trend (Figure 5.3).

5.3.3 ^{1}H-NMR Spectroscopic analysis

PCA of all samples gave a prediction values (Q_2(cum)) of 0.403. PCA analysis of the control samples yielded Q_2(cum) = 0.306 , the benzo[*a*]pyrene samples gave Q_2(cum) = 0.396. PCA analysis of the Superfund site gave Q_2(cum) = 0.407, the clean site gave Q_2(cum) = 0.357. All of these Q_2 values are of magnitudes that indicate poor predictivity for models built from this data. PLS analysis with benzo[*a*]pyrene samples classified as 1 and the control as 0 did not generate a statistically significant model. PLS analysis with the Superfund site gave a Q_2(cum) of -0.1 meaning it was not statistically significant. In addition, the adenine phosphate levels of all samples were not different from the

background noise (Figure 5.5). Similar analysis with ^{31}P NMR gave the same resultes (data not shown).

5.4 Discussion

When exposed to toxic chemicals, organisms have to increase their energy production to counter increased basal metabolism caused by detoxification (Woodford et al. 1998; Nisbet et al. 2000; Sousa et al. 2006). Therefore, toxicants that can affect mitochondrial function could show enhanced toxicity by potentially interfering with ATP production. Previous studies suggest that BaP, a well-studied chemical known to induce DNA damage and ROS production, may also affect mitochondrial function (Zhu et al. 1995; Li et al. 2003; Xia et al. 2004). Therefore, a decrease in oxygen consumption by embryos treated with BaP seems to indicate that aerobic metabolism was decreased by BaP treatment.

When King's Creek and Elizabeth River killifish were compared, the two populations showed a difference in their basal level of oxygen consumption. This may indicate that the Superfund site killifish have a lower metabolic rate compared to the reference site fish. Interestingly, the BaP-treated group in the Elizabeth River population had a higher rate of oxygen consumption compared to the control group. This could be because the fish are responding to increased energy demand due to BaP metabolism. However, it is unexpected that the King's Creek and Elizabeth River killifish showed the

opposite response to BaP treatment. Possible explanation would be that the dose (100 ppb) we used was biologically effective enough in the King's Creek fish to experience destruction of cellular functions and result in oxygen consumption. In contrast, the dose may have been relevant enough to increase energy expenditure (e.g. increased energy requirements for detoxification), but not enough to destruct cellular functions.

LDH is an enzyme involved in anaerobic metabolism. Measurement of LDH activity in the muscle or liver can inform us of the anaerobic metabolism capacity of the organism (De Coen et al. 2001; Vieira et al. 2008). Previous studies measuring LDH after PAH exposure have shown mixed results. For example, Viera et al. (2008) saw increased LDH in common goby (*Pomatoschistus microps*) dosed with as low as 1 μg/L BaP, and Oikari and Jimenez (1992) saw similar results in sunfish hybrid (*Lepomis macrochirus* x *L. cyanellus*) treated with 8 mg/kg BaP. In contrast, Tintos et al. (2008) did not see any changes in the LDH levels in rainbow trout (*Oncorhynchus mykiss*) dosed with 10 mg/kg BaP. Given these conflicting studies, we hypothesized that LDH activity would increase in the BaP-treated group. However, no significant differences were observed among any groups. PK is an enzyme involved in glycolysis, and is also used as a marker of energy metabolism (Osman et al. 2007). No significant differences were observed in activities of this enzyme, either. Our results indicate that anaerobic metabolism may not be affected by treatment with BaP, or that Elizabeth River killifish are not likely to depend more on anaerobic metabolism for energy production. This result contradicts the respiration data

as well as a previous study in which differential display showed mRNA signals that suggested higher glycolytic metabolism in Elizabeth River killifish (Meyer et al. 2005). One explanation for this result may be that killifish may respond to mitochondrial damage by reducing total energy expenditure instead of switching to anaerobic metabolism. This could result in the reduced growth and reproduction, as depicted in Figure 1.3.

To examine the metabolic changes induced by BaP, we carried out a ^{1}H-NMR-based metabolomic experiment using adult male killifish from King's Creek and Elizabeth River dosed with BaP. ^{1}H-NMR spectroscopy is a method that can detect a broad-based and unbiased metabolic profile (Ong et al. 2009). Recently more attempts are being made to use this approach on aquatic species to measure changes in energy metabolism (Parsons et al. 2007; Southam et al. 2008; Viant 2008). However, in our experiment, we did not see any differences in the metabolic profile of any of the treatment groups. This was another unexpected finding, considering indications from previous results and evidence in the literature that BaP treatment would switch respiration to anaerobic pathway (Zhu et al. 1995; Ko et al. 2004; Salazar et al. 2004). Moreover, metabolomic analysis revealed no difference between the two populations, with or without BaP treatment. One caveat in the NMR experiment is that we used adult fish that were acclimated to laboratory conditions for over five months. The fish may have acclimated to the uncontaminated environment and regained a more "normal"

energy metabolism. However, the hypothesis assumes that any difference in mitochondrial energy metabolism is strictly a physiological adaptation. Obviously, more detailed work would be necessary to understand BaP's effect on energy metabolism, as well as what potential metabolic effect chronic exposure to PAHs may have in the Elizabeth River killifish population.

In conclusion, we hypothesized that treatment with BaP would result in increased anaerobic metabolism and changes in metabolomic profile in the killifish model. In addition, we hypothesized that killifish from the Elizabeth River Superfund site exhibit increased anaerobic metabolism and would show a different response to BaP treatment. However, neither hypothesis was supported in this study. Future studies involving examination of mitochondrial enzyme activity as well as ATP production may further elucidate the role BaP might have on mitochondrial function.

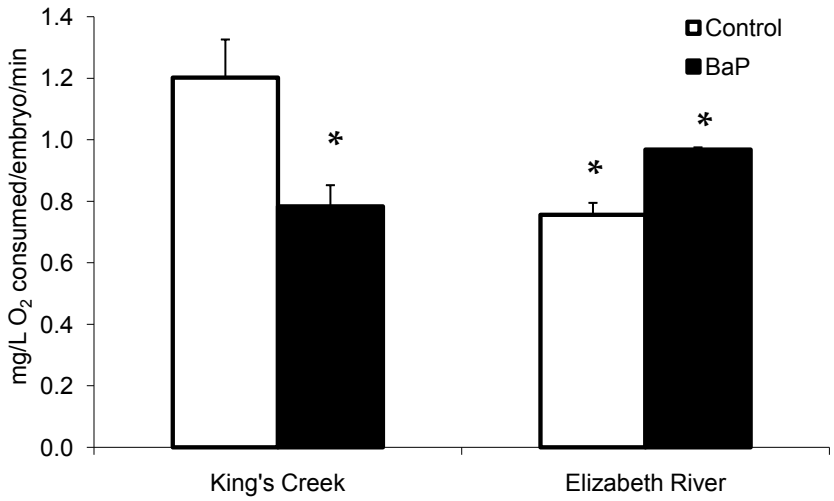


Figure 5.1 Oxygen consumption of embryos dosed with BaP.

Oxygen consumption levels of embryos dosed with 100 μg/mL were measured. Compared to the King's Creek control group, King's Creek BaP group and both treatments of the Elizabeth River showed significant decrease in the oxygen consumption level ($p < 0.05$). Two-factor ANOVA showed that there was significant interaction between treatment and population. n = 5 pools of 5 individuals per treatment. Error bars indicate standard error of means.

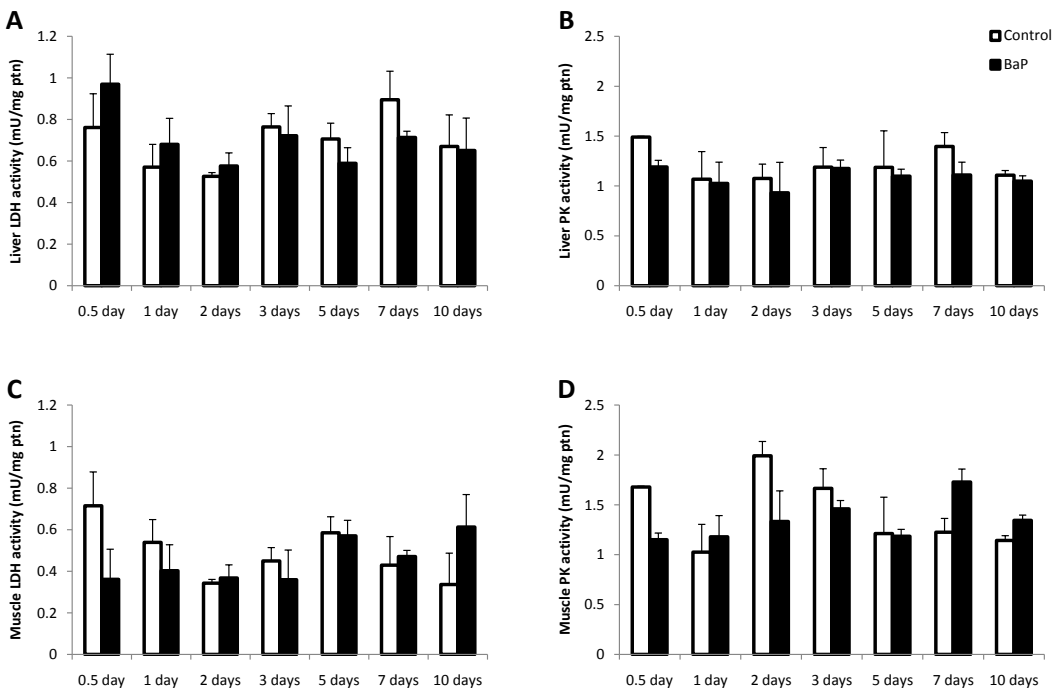


Figure 5.2 Enzyme activity of Main's Creek killifish dosed with BaP.

Adult killifish from Main's Creek were dosed with 10 mg/kg BaP and liver and muscle were isolated for LDH and PK activity. (A) Liver LDH,(B) Liver PK, (C) Muscle LDH (D) Muscle PK. Open bars indicate control group, closed bars indicate BaP group. Error bars indicate standard error of means.

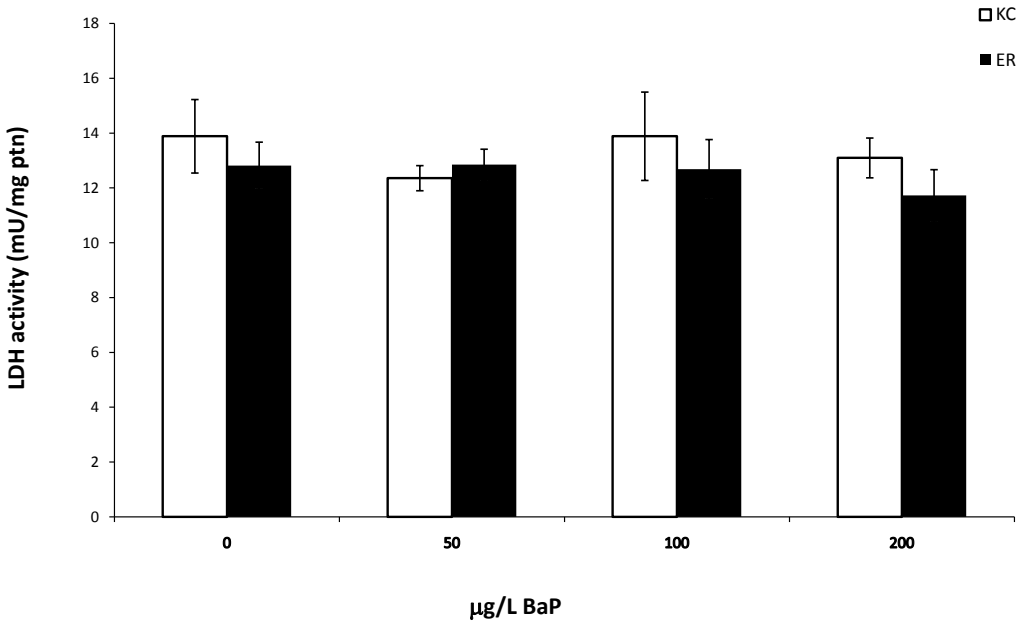


Figure 5.3 LDH activity of larval killifish dosed with BaP.

Larval killifish from Elizabeth River and King's Creek were dosed with 100 µg/L BaP (n = 3 of pooled group of 10 individuals) and whole homogenates were measured for LDH activity. Open bars indicate King's Creek fish, closed bars indicate Elizabeth River fish. Error bars indicate standard error of means.

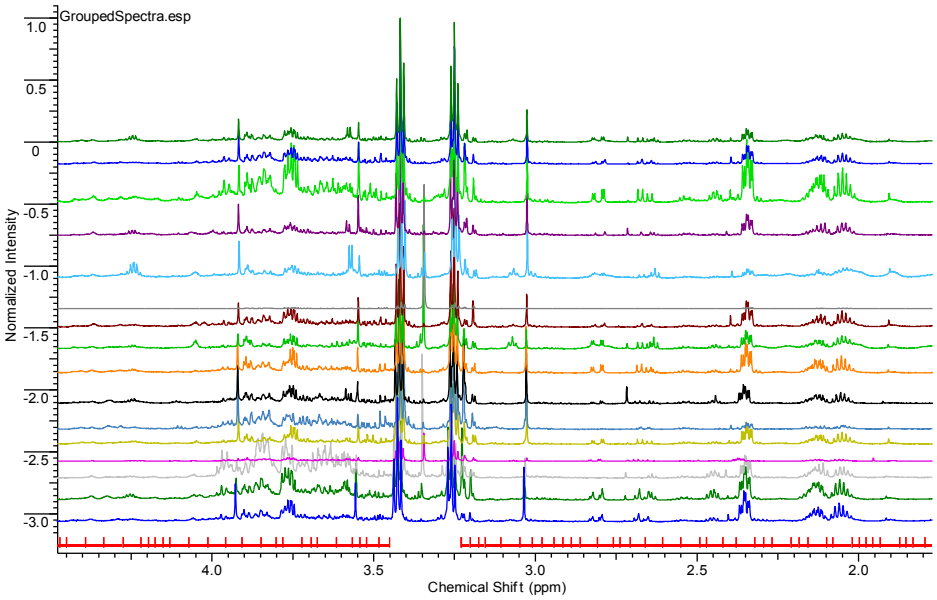


Figure 5.4 Chemical shift spectrum of ^{1}H-NMR.

Adult male fish from Elizabeth River and King's Creek were dosed with either 10 mg/kg BaP or corn oil. Liver was isolated 72 hrs later and ^{1}H-NMR analysis was conducted. Each different colored line represents each individual sample. X-axis represents chemical shift from the standard chemical. Although the spectral data was of good quality, and some differences are seen in the peaks, PCA analysis showed that there was no difference among the populations or treatment groups.

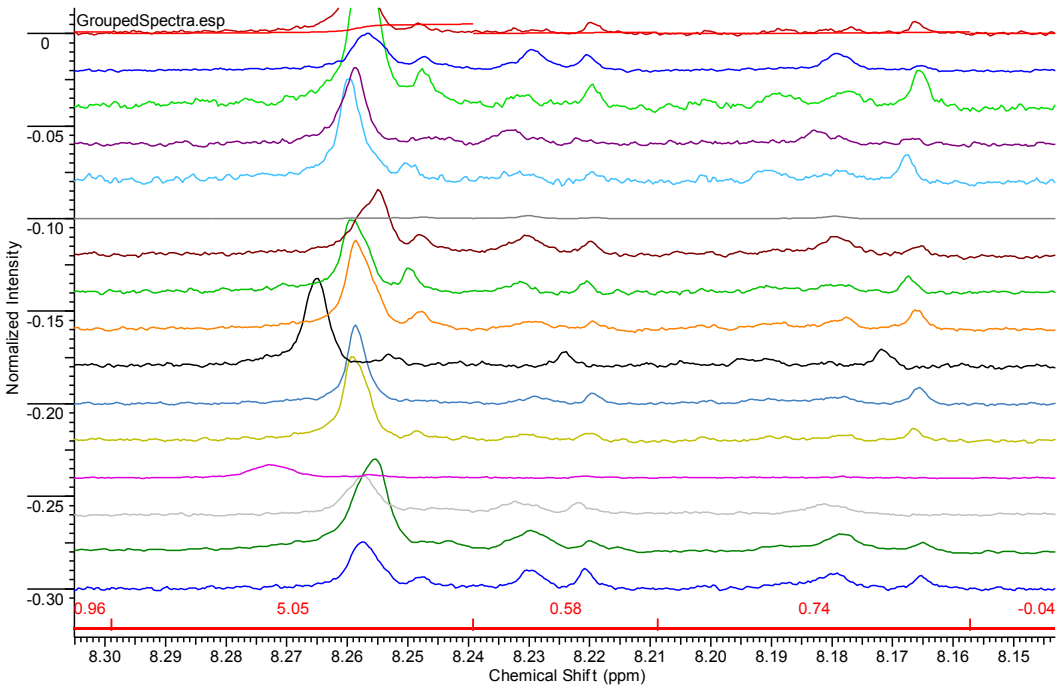


Figure 5.5 Chemical shift spectrum of adenine phosphate region on ^{1}H-NMR

Regions in the chemical shift spectrum that indicate the adenine phosphate levels show that the peaks in the region are not significantly different from noise shifts. Each different colored line represents each individual sample. X-axis represents chemical shift from the standard chemical.

6. Comparison of different biomarkers of genotoxicity

This work was conducted as part of a study which will be submitted for publication with Cole W. Matson, Dawoon Jung, Leonard B. Collins, Geoff Laban, Heather Stapleton, James A. Swenberg, John W. Bickham, and Richard T. Di Giulio as co-authors.

6.1 Introduction

In the field of ecotoxicology, assessing the level of damage pollutants can cause to the organism is an important issue. Several methods of assessing the genotoxic effects of pollutants are utilized in the field of ecotoxicology (discussed in Chapter 2). While developing the long amplicon quantitative PCR (LA-QPCR) method for the ecological model Atlantic killifish (*Fundulus heteroclitus*), we became interested in using different established methods to assess the extent of genotoxicity in the killifish population at the Atlantic Wood Superfund Site on the Elizabeth River.

The Atlantic Wood Superfund Site on the Elizabeth River in Portsmouth, VA is a site heavily contaminated with creosote from a wood treatment facility. Concentrations of polycyclic aromatic hydrocarbons (PAHs) at this site are as high as 500 ng/g sediment, with a high abundance of carcinogenic PAHs, such as benzo[*a*]pyrene, chrysene, and dibenzo[*a*,*h*]anthracene (Vogelbein et al. 2008). Therefore, the killifish at the Elizabeth River Superfund Site are constantly exposed to extremely high concentrations of several

carcinogens. Moreover, previous studies have reported high occurrences of hepatic lesions in these killifish (Vogelbein et al. 1990; Vogelbein et al. 1999).

In this study, we assessed the genotoxicity of the killifish exposed *in situ* using two different types of methods; BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) DNA adduct measurement using liquid chromatography-mass spectrometry (LC-MS), and chromosomal damage estimation using flow cytometry.

6.2 Materials and Methods

6.2.1 Sample collection

Killifish were captured at the Atlantic Wood Superfund Site (Portsmouth, VA) and the King's Creek Reference site (Gloucester County, VA) using baited minnow traps. Within 24 h of transfer back to the laboratory, blood and liver were harvested from five adult males (5 – 13 g) from each population. Blood samples for chromosomal damage analysis were collected into heparinized collection tubes (Fisher Scientific, Pittsburgh, PA, USA), flash frozen, and kept at -80°C. Liver samples for DNA adduct analysis were also flash frozen and kept at -80°C.

6.2.2 DNA adduct measurement

For measuring oxidative (8-OH deoxyguanosine, or 8-oxo-dG) and covalent (BPDE-dG) DNA adducts, DNA from killifish liver harvested as mentioned above was isolated and enzymatically hydrolyzed as described in Chapter 3 with some

modification- only samples for 8-oxo-dG analysis were filtered with Centricon YM-10 microcentrifuge filters. The final volume of each sample containing all reagents and internal standard was 300 μl. The [$^{15}N_5$]8-Oxo-dG internal standard for all samples was purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and 8-Oxo-dG analyte standard was purchased from Sigma-Aldrich. BPDE-dG standards ([^{15}N]BPDE-dG internal standard for all samples and *N*^{2}-BPDE-dG analyte standard for positive controls) were generous gifts from Dr. Natalia Tretyakova (University of Minnesota).

Samples of hydrolyzed DNA were chromatographed on an Agilent 1200 HPLC system with automated fraction collector. Separation was performed on an Ultrasphere ODS C18 4.6 × 250 mm 5 μm column (Beckman, Fullerton, CA) using a gradient of 10 mM ammonium formate in water (adjusted to pH 4.3 with formic acid) and methanol. Methanol composition was held at 7% from 0 to 22 min, then increased linearly to 80% in 1 min, was held at 80% for 6 min to elute TEMPO, decreased to 7% in 1 min, and held at 7% for 6 min for column re-equilibration. A 275 μL aliquot of sample was injected, and the flow rate was 1 mL per min. The column oven, autosampler tray and fraction collector chamber temperatures were maintained at 30°C, 4°C and 4°C, respectively. The retention time was determined by using 2′-deoxyguanosine (dG) as a retention time marker and multiplying its retention time by 1.5 (~18 min for 8-oxo-dG and ~23.5 mi for *N*^{2}-BPDE-dG). Targeted fractions were automatically collected from 1.5 min before until 1.5 min after its predicted retention time. The fraction collection tubes were placed in a

SpeedVac concentrator (ThermoFinnigan, San Jose, CA) and evaporated to dryness. Sample residue was transferred to autosampler vials via 2 x 130 μL washings with 50:50::water:methanol, evaporated to dryness in a Speedvac concentrator, and finally redissolved in 20 μL HPLC grade water for subsequent analysis by LC-MS/MS. The 2'-deoxyguanosine (dG) amount in each sample was determined during fraction collection by comparison with dG calibration standards using UV detection at 264 nm.

The quantitative analysis of 8-oxo-dG was performed with an Acquity UPLC (Waters, Milford, MA, USA) coupled to a TSQ-Quantum Ultra triple-quadrupole mass analyzer (ThermoFinnigan) according to the protocol described in chapter 3. The quantitative analysis of N^2-BPDE-dG was performed with a nanoAcquity UPLC (Waters) coupled to a TSQ-Quantum Ultra triple-quadrupole mass analyzer (ThermoFinnigan) using the nanospray source in positive mode. The trap column was a Waters 180 μm x 20 mm Symmetry C18 5 μm, and separation was performed on a 100 μm × 100 mm BEH300 C18 1.7 μm column (Waters). The mobile phase consisted of 10 mM ammonium acetate, 0.1% acetic acid in water and methanol. The sample was first injected onto the trap column at a flow rate of 10 μL per min for 1.5 min at an initial methanol concentration of 5%, then flow was directed in-line with the analytical column. Gradient elution was performed at a flow rate of 600 nL per min. The methanol composition started at 5% and increased linearly to 80% in 10 min, was held at 80% for 2 min, decreased to 5% in 2 min, then held at 5% for 6 min for column re-equilibration.

The retention time of N^2-BPDE-dG was 12 min, and the total run time was 20 min. The analyte and internal standard were detected in selected reaction monitoring mode (SRM), monitoring the transitions of m/z 570 to 257 (collision energy 15 eV) and m/z 570 to 454 (collision energy 35 eV) for N^2-BPDE-dG and m/z 575 to 257 (collision energy 15 eV) and m/z 575 to 459 (collision energy 35 eV) for [$^{15}N_5$]N^2-BPDE-dG. Other nano-electrospray conditions were as follows: positive mode, spray voltage of 2000 V, capillary temperature of 280°C.

6.2.3 Chromosomal damage estimation

Chromosomal damage in blood samples was estimated using a flow cytometric method as described in Matson et al. (2005). Samples were run on a Beckman Coulter (Fullerton, CA) Cytomix FC 500. Genome size half-peak coefficients of variation are used as a measure of chromosomal damage. Variability in genome size is likely the result of the unequal distribution of chromosomes or chromosome fragments during cell division. This can result from clastogenicity or aneugenicity. Thus the flow cytometric method provides a generalized estimate of large-scale DNA (i.e. chromosomal) damage.

6.3 Results

6.3.1 DNA damage

The result from both 8-oxo-measurement and BPDE-dG measurement are shown in figure X.1. For 8-oxo-dG, one of the more common oxidative DNA adducts, no

difference was observed between the two populations (p = 0.146). We also measured the levels of BPDE-dG, formed by the covalent binding of BPDE- a common metabolite of benzo[*a*]pyrene (BaP)-to DNA. Elizabeth River killifish showed clearly elevated, though not significant, levels of BPDE-dG adduct compared to the King's Creek killifish (p = 0.060). Lack of statistical significance is a product of the extremely small sample sizes (n = 3), and does not suggest a lack of biological significance.

6.3.2 Chromosomal damage

As measured by flow cytometry, there were higher levels of chromosomal damage in the Elizabeth River killifish compared to the King's Creek killifish (p = 0.042, Figure X.2).

6.4 Discussion

The Elizabeth River Superfund Site is polluted with a complex mixture of contaminants including PAHs, metals, and pentachlorophenol (Hartwell et al. 2007; Vogelbein et al. 2008). Therefore, the fish inhabiting this site are exposed to a several chemicals that can act as genotoxicants. Among these, we chose to measure BPDE-dG adduct frequency since BaP is one of the most abundant PAH at the Atlantic Wood Superfund site (~11% of total PAHs by mass) (Vogelbein et al. 2008). We had expected that a large portion of the DNA damage may be induced by specific adduct formations

such as BPDE-DNA adduct. The adduct level in the Elizabeth River killifish was high compared to King's Creek killifish.

No significant differences were observed in the extent of oxidative DNA damage as represented by the 8-oxo-dG frequency. Oxidative DNA damage can be caused by various chemicals that are found in the sediments of the Atlantic Wood Superfund site. Therefore, we were expecting a higher degree of difference between the two populations. Perhaps, it is possible that the 8-oxo-dG measurement may not be as sensitive of a method in measuring oxidative DNA damage in wild populations. More studies that involve measuring different types of oxidative adducts, such as (3-(2′-deoxy-β-D-erythro-pentofuranosyl)-pyrimido[1,2-a]-purin-10(3H)-one, may shed more light on this issue (Jeong et al. 2008).

In a previous study, we measured the extent of general DNA damage in the mitochondria and the nucleus of killifish collected from King's Creek and the Elizabeth River Superfund site using long amplicon-quantitative PCR (LA-QPCR) method (Chapter 2). In that study, significantly higher levels of mtDNA and nDNA damage was observed in the liver of the Elizabeth River killifish relative to King's Creek reference fish (Figure 2.3). This increased discrimination observed between the populations by LA-QPCR method compared to the current study is most likely due to the fact that the LC-MS/MS method measures very specific types of DNA adduct, whereas the LA-QPCR method measures any type of polymerase-inhibiting DNA damage (Ayala-Torres et al.

2000). In an environment where organisms are exposed to a complex mixture of genotoxic chemicals, such as the Atlantic Wood Superfund site, DNA damage is likely caused by more than one type of chemical.

Flow cytometry measures chromosomal damage and loss and this method has been used widely as a biomarker of genotoxicity (Theodorakis 2001; Goanvec et al. 2004; Matson et al. 2005; Matson et al. 2009). Generally, this method measures more severe damage in the organism's DNA than the above-mentioned methods. In this study, Elizabeth River killifish showed significantly higher levels of chromosomal damage than King's Creek killifish. This result suggests that this population suffers from substential chromosomal damage-most likely due to high levels of several genotoxic compounds.

In conclusion, the killifish at the Elizabeth River Superfund Site exhibit higher levels of DNA damage. The damage is severe enough to cause significant damage in the chromosomal structure. Metabolites of BaP seem to be partially responsible for the DNA damage, but oxidative DNA adducts do not seem to play a role in the genotoxicity of the killifish.

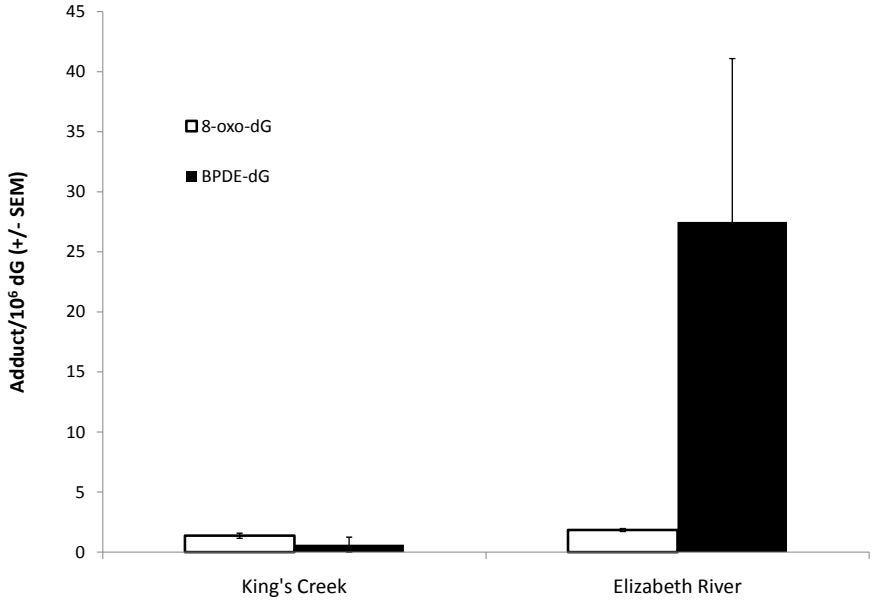


Figure 6.1 Levels of DNA adducts in Superfund site and reference *Fundulus heteroclitus* populations.

Oxidative DNA damage (8-oxo-dG) was not significantly different between the population, but BPDE-dG level was elevated in the Elizabeth River population (p = 0.060).

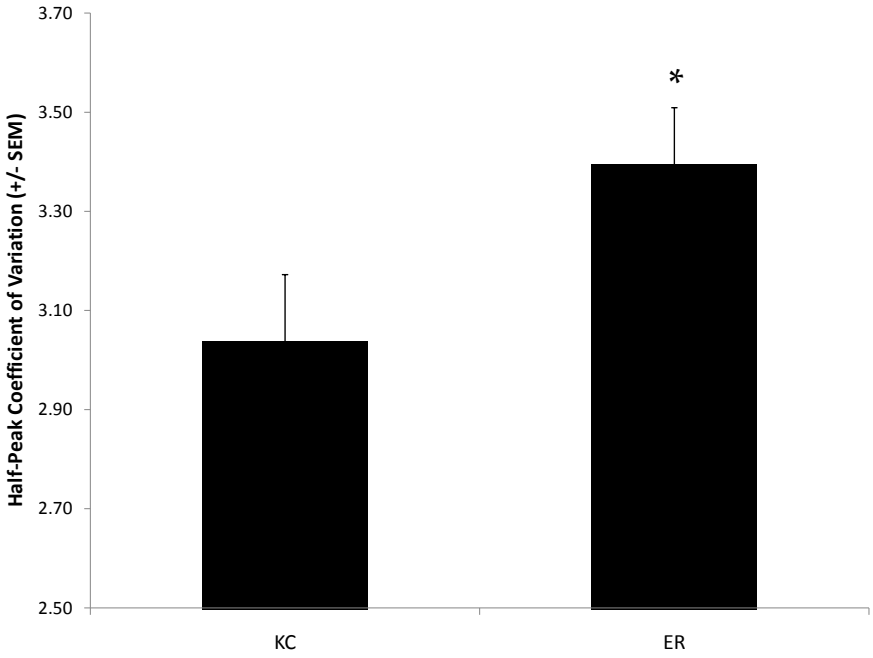


Figure 6.2 Chromosomal damage measured by flow cytometry in Superfund site and reference *Fundulus heteroclitus* populations.

* indicates significant difference ($p = 0.042$).

7. Conclusion

7.1 Summary

In this dissertation, I examined the mechanisms by which benzo[*a*]pyrene (BaP), a well-studied polycyclic aromatic hydrocarbon may affect different aspects of the mitochondria in the environmental model *Fundulus heteroclitus* (Atlantic killifish). In addition, I examined whether a population of killifish inhabiting the Atlantic Wood Industries Superfund site on Elizabeth River (Portsmouth, VA) showed compromised mitochondrial function due to chronic exposure to a complex mixture of chemicals. Three basic questions about BaP's effect on the mitochondria were addressed in this dissertation: (1) does BaP treatment result in mitochondrial DNA damage; (2) are there enzymes in the mitochondria that have the potential to metabolize BaP; (3) are there functional consequences regarding energy metabolism from BaP treatment.

In order to assess the extent of mitochondrial DNA damage in the killifish, I adapted a PCR-based assay (LA-QPCR) for nuclear and mitochondrial DNA damage for use in an important environmental model, the Atlantic killifish (*Fundulus heteroclitus*) (Chapter 2). I validated this method in killifish by measuring DNA damage in response to UVC. Next I measured damage in liver, brain, and muscle of fish dosed with BaP and in liver and muscle tissues from killifish inhabiting a Superfund site (Elizabeth River, VA, USA) to confirm the utility of this method for biomonitoring.

Next, I compared mtDNA and nDNA damage in the killifish from Elizabeth River to fish from a reference site (King's Creek, VA, USA) with or without BaP (Chapter 3). In King's Creek killifish, BaP reatment resulted in a similar level of damage in the mitochondrial and nuclear DNA. Killifish from the Elizabeth River showed high levels of basal nDNA and mtDNA damage compared to fish from the reference site, but the level of damage resulting from BaP treatment was much lower in Elizabeth River killifish. Laboratory-reared offspring from both populations showed increased BaP-induced damage in mtDNA, relative to nDNA. Similar to the experiment with adults, the Elizabeth River larvae had higher levels of basal DNA damage than those from the reference site, but were less impacted by BaP exposure. However, measurements with 8-oxo-dG indicated that oxidative DNA damage was not significant.

Since the toxic effects of many PAHs are the result of metabolism by CYP1A, I investigated whether active forms of CYP1A enzymes are present inside the mitochondria (Chapter 4). Using Western blot, CYP1A was identified in the mitochondrial fraction from adult male killifish livers. BaP-treated adult fish increased protein level and EROD activity in the liver mitochondrial fraction compared to controls. Similarly, in killifish larvae dosed with 100 μg/L BaP and 100 μg/L benzo[*k*]fluoranthene (BkF), CYP1A protein levels as well as enzyme activity were elevated. However, fish from the Elizabeth River Superfund site showed recalcitrant induction of mitochondrial CYP1A protein expression and activity.

Next, I tested the hypothesis that energy metabolism of BaP-treated fish may be different from the control group and that killifish from the Elizabeth River Superfund site may also have altered energy metabolism compared to reference site fish (Chapter 5). First, we compared oxygen consumption of killifish embryos treated with BaP from both populations. Compared to the King's Creek control fish, all other treatment groups showed decrease in oxygen consumption, indicating lower respiration rate. However, when we measured the activity level of PK and LDH, two key enzymes involved in glycolysis and anaerobic metabolism, respectively, in adult killifish liver and muscle, we did not see any difference in the activity of either enzyme. Moreover, metabolomic analysis on BaP-treated King's Creek and Elizabeth River killifish showed no difference in the profile in all four treatment groups. Therefore my hypothesis was not supported.

Lastly, I utilized several methods as biomarkers to assess the extent of DNA damage in Elizabeth River killifish population (Chapter 6). Results showed that the Elizabeht River killifish population exhibited high levels of DNA damage in terms of structural damage, adduct formation, and chromosomal aberration.

7.2 Future directions

Although this dissertation examined various aspects of the mitochondria as a target of BaP toxicity, the actual effect of BaP on the function of mitochondria was not fully explored. The activity assays conducted on LDH and PK (chapter 5) did not

indicate differences in the BaP-treated group compared to the control group. In addition, preliminary metabolomic examination also showed no difference between the two groups. However, these are only indirect studies. More direct study into the function of mitochondrial enzymes involved in Krebs cycle and oxidative phosphorylation such as aconitase, cytochrome *c* oxidase, or ATP synthase may shed some light on this aspect. In addition, some studies have measured mitochondrial density indirectly by measuring CS and COX activity and mRNA level (Portner et al. 2006; Fangue et al. 2009). Using this method, along with gene expression patterns of other nucleus-transcribed mitochondrial proteins, might prove to be promising.

Partly to address the question of energy metabolism, I attempted a metabolomic analysis of killifish dosed with BaP using ^{1}H-NMR. To our surprise, we did not see any difference among the two populations or in fish dosed with BaP versus control, in the water-soluble phase of the metabolites. Perhaps the lipids may have gone through more intensive changes, which we were not able to capture with the aqueous fraction analysis (Jeffrey Macdonald, UNC-Chapel Hill, personal communication). A more thorough examination of metabolomics using ^{1}H-NMR may help further address this issue of potential metabolic change in fish exposed to BaP.

Another potential route to take is to examine mitochondrial structural differences in fish treated with BaP. For example, Gernhöfer et al (2001) showed mitochondrial structural damage in the gill, liver and kidney of brown trout (*Salmo trutta* f. *fario*) when

exposed to mixed PAHs. In addition, Lemaire et al. (1992) saw structural perturbation in the mitochondria of liver cells and intestine cells of sea bass (*Dicentrarchus labrax*) exposed to high levels of BaP. It would be interesting to see whether structural damage occurs in the mitochondria of killifish exposed to environmentally relevant levels of BaP.

In this dissertation, I exclusively used BaP to investigate the basic mechanism of BaP toxicity in the mitochondria. However, PAHs exist in the environment as mixtures of individual chemicals. PAHs are collectively listed as number eight on the 2007 Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) priority list as a group (ASTDR 2007), indicating the importance of understanding how complex mixture of PAHs may affect various cellular mechanisms. Therefore, understanding whether this class of compounds can cause more damage to the mitochondria as a group, compared to the effect of BaP alone, would be a more environmentally relevant question. Working with coal tar or crude oil may be a way to approach this issue. This also holds true in terms of other chemicals, such as metals, that co-exist in polluted sites such as the Elizabeth River Superfund site. For example, it is known that metals such as cadmium can affect the mitochondrial function in aquatic organisms (Sokolova et al. 2005; Banerjee et al. 2008; Belyaeva et al. 2008; Lemire et al. 2008). How these chemicals interact to exert toxicity is an important matter. Therefore, the next step would be to understand the response of killifish mitochondria to treatment with complex mixtures such as the Elizabeth River sediment extract.

In the estuarine environment, fish are periodically exposed to a wide range of environmental conditions. Such conditions include periods of hypoxia and temperature extremes. In the context of such natural stresses, mitochondrial stability of the organism is a very relevant matter. Studies have shown that mitochondrial components or the organelle itself plays a role in the dealing with thermal changes as well as oxidative stress caused by reoxygenation after hypoxic episodes (Kraemer et al. 2004; Portner et al. 2006; Wilhelm Filho 2007; Richards et al. 2008; Fangue et al. 2009). Hypoxia and increasing global temperature are of growing concern in recent years (Schiedek et al. 2007; Diaz et al. 2008; Noyes et al. 2009). Whether such environmental conditions can have an interactive effect with PAHs on energy metabolism of killifish could also be further pursued.

A second overarching question that I wanted to answer in this dissertation was to examine whether PAH-adapted killifish at the Elizabeth River Superfund Site also show adaptation against mitochondrial damage. Previous studies in our laboratory indicated that this population will have impaired mitochondrial function (Meyer et al. 2003a; Meyer et al. 2005; Jung et al. 2009). In the present study, killifish from the Elizabeth River show higher level of DNA damage. However, these fish did show some protective characteristics (chapters 3 and 4). Nevertheless, these differences do not seem to translate to differences in the function of the mitochondria in this population compared to reference population. However, as mentioned above, a more detailed

functional analysis is necessary in the killifish mitochondria, and in the same sense, more detailed functional analysis should be conducted to determine whether Elizabeth River killifish exhibit characteristics of impaired mitochondria and reduced fitness, or whether this population shows mitochondrial adaptation from chronic exposure to PAHs.

One issue that I stumbled upon was that our reference site killifish from King's Creek may not be a suitable reference group in which to study energy metabolism. Northern and southern populations of killifish do show some differences in energy metabolism (Burnett et al. 2007; Fangue et al. 2009). In addition, isolated populations geographically in the range of southern population also exhibit northern characteristics (Crawford et al. 1989). Therefore, to completely resolve this issue, it may necessary to include another population of fish inhabiting geographically south of Elizabeth River Superfund site.

In conclusion, this thesis addressed the mechanism in which mitochondria of the Atlantic killifish are affected by BaP, a well-studied PAH. The adaptation of the Elizabeth River population, inhabiting a PAH-polluted area, to potential mitochondrial damage in response to additional toxicant exposure was also explored. The findings in this thesis contribute to the understanding of how a common environmental pollutant targets the mitochondria. Further studies will elucidate whether such impacts can

potentially affect the energy budget and organism level fitness in populations in the wild.

Appendix. Long amplicon quantitative PCR conditions for *Danio rerio* (zebrafish) model

The objective of this work was to develop the PCR condition for the long amplicon quantitative PCR (LA-QPCR) in the zebrafish model. This study was a collaborative effort of Dawoon Jung and Youngeun Cho.

X.1. Development of PCR primers and conditions

PCR primer sets were designed from zebrafish mitochondrial whole genome (accession number **AC024175**) and aryl hydrocarbon receptor 2 genomic sequence (accession number **BX908726**), using PRIMER3 (Rozen et al. 2000).

Table X.1 Primers used for *Danio rerio* LA-QPCR assay

Target	Primer sequences
Long nuclear target	F: 5′- AGAGCGCGATTGCTGGATTCAC -3′
10759 bp	R: 5′- GTCCTTGCAGGTTGGCAAATGG -3′
Short nuclear target	F: 5′- ATGGGCTGGGCGATAAAATTGG -3′
233 bp	R: 5′- ACATGTGCATGTCGCTCCCAAA -3′
Long mitochondrial target	F: 5′- TTAAAGCCCCGAATCCAGGTGAGC -3′
10304 bp	R: 5′- GAGATGTTCTCGGGTGTGGGATGG -3′
Short mitochondrial target	F: 5′- CAAACACAAGCCTCGCCTGTTTAC -3′
198 bp	R: 5′- CACTGACTTGATGGGGGAGACAGT –3′

Table X.2 Amplification conditions for each target

Short mitochondrial target	Short nuclear target	Long mitochondrial target	Long nuclear target
75º 2′ 94º 1′ 94º 15′′ 62º 45′′ 72º 30′′ 72º 5′ 8º ∞	75º 2′ 94º 1′ 94º 15′′ 60º 45′′ 72º 30′′ 72º 5′ 8º ∞	75º 2′ 94º 1′ 94º 15′′ 68º 12′ 72º 10′ 8º ∞	75º 2′ 94º 1′ 94º 15′′ 69º 12′ 72º 10′ 8º ∞
21 cycles (variable)	27 cycles (variable)	19 cycles (variable)	24 cycles (variable)

X.2 Verification of LA-QPCR method

To verify whether the method can be adapted to the zebrafish model, we exposed zebrafish embryo cells (ZF4 cell line, ATCC CRL-2050) to various doses of UVC. As shown in Figure X.1, dose-dependent damage ($p < 0.05$ for both mtDNA and nDNA) was detected with the newly developed primer sets and PCR conditions.

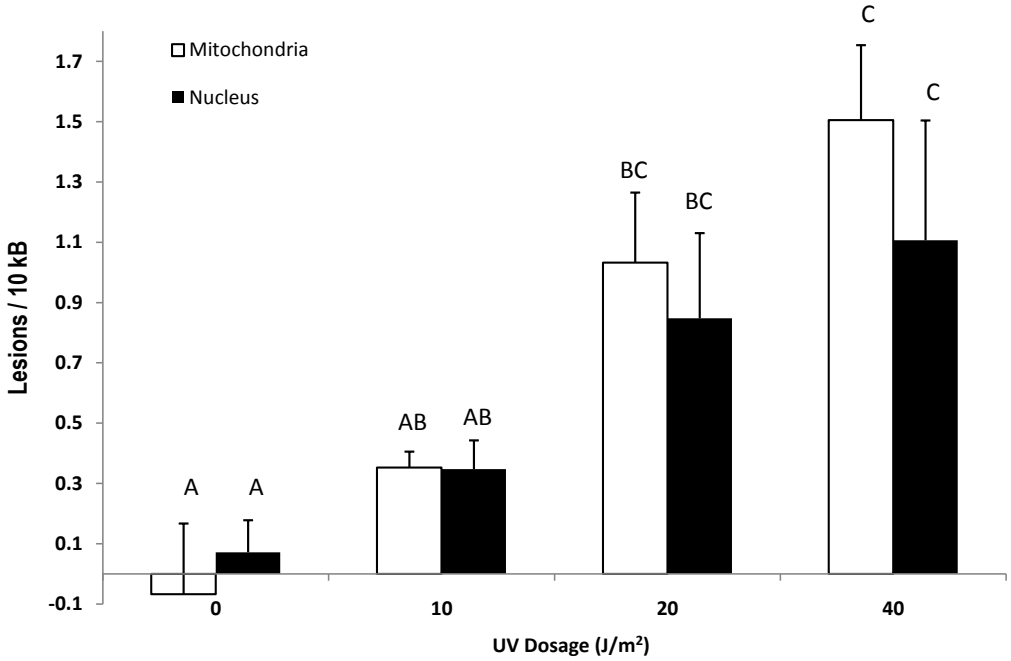


Figure X.1 DNA damage in UVC-treated zebrafish cells.

X.3 Zebrafish DNA damage repair capacity

To test whether there is nucleotide excision repair (NER) capacity in the nucleus and in the mitochondria of the zebrafish, a preliminary study was conducted as described below. Petri dish (10 mm diameter) containing roughly 10^6 cells/ml were drained free of culture medium and treated with 10 J/m^2 of UVC. The dishes were immediately filled with new culture media and wrapped in aluminum foil to prevent any exposure to light. Then the plates were kept in normal incubation condition for 0, 2, 4, 8, 12, and 24 hours. After each designated time, DNA was isolated from three random plates per time point, and damage of mtDNA and nDNA were measured using LA-

QPCR. As seen in Figure X.2, there was significant dose dependent decrease in nDNA damage (p <0.01). However no significant differences in damage were seen in mtDNA.

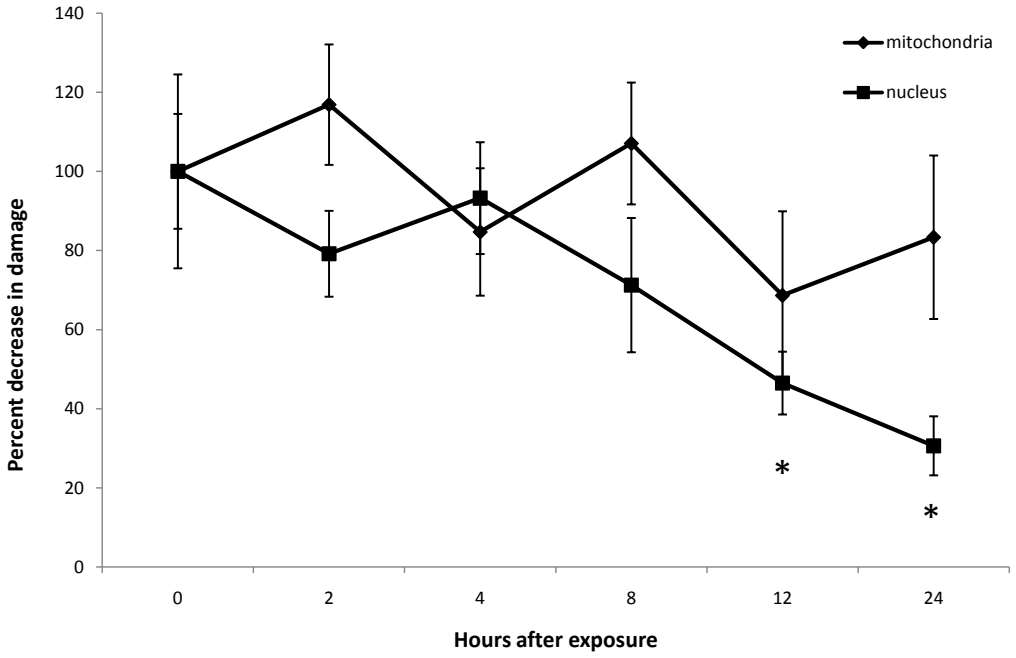


Figure X.2 DNA damage repair in the zebrafish cell line ZF4. * indicate significant difference from 0 hrs (p < 0.01)

The result above indicates that NER capacity does exist in the zebrafish nucleus. In addition, the result also confirms that NER does not exist in teleost mitochondria.

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Biography

Dawoon Jung was born in Seoul, Korea on 22nd June, 1975. She received Bachelor of Science degree in Molecular Biology from Seoul National University in February 1998. She received Master of Science degree in Molecular Biology from the same university in February 2000. Her thesis title was *Community Structure of Small Crustaceans Inhabiting River Han.*

The following publications have been authored by Dawoon Jung:

Jung D., Y. Cho, J.N. Meyer, R.T. Di Giulio. 2009. The long amplicon quantitative PCR for DNA damage assay as a sensitive method of assessing DNA damage in the environmental model, Atlantic killifish (*Fundulus heteroclitus*). *Comparative Biochemistry and Physiology-Part C: Toxicology and Pharmacology*. 149(2): 182-186.

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