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Anthropogenic Pollution Effects on Mitochondrial Energy Metabolism, Gene Expression, and Genotypes of Natural Fundulus Heteroclitus Populations

Xiao Du
University of Miami, xdu2011@gmail.com

UNIVERSITY OF MIAMI

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Xiao Du

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Xiao Du

Approved:

_____ Marjorie F. Oleksiak, Ph.D. Associate Professor of Marine Biology and Ecology	_____ Lynne A. Fieber, Ph.D. Associate Professor of Marine Biology and Ecology
_____ Douglas L. Crawford, Ph.D. Professor of Marine Biology and Ecology	_____ Kevin G. McCracken, Ph.D. Associate Professor of Biology
_____ Sawsan Khuri, Ph.D. Research Assistant Professor of Computer Science Director of Engagement, Center for Computational Science	_____ Guillermo J. Prado, Ph.D. Dean of the Graduate School

DU, XIAO (Ph.D., Marine Biology and Ecology)

Anthropogenic Pollution Effects on Mitochondrial Energy Metabolism, Gene Expression, and Genotypes of Natural *Fundulus heteroclitus* Populations (August 2016)

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Energy balance is a major concern for organisms developing stress tolerance, as combating pollutant toxicity is usually metabolically costly. Mitochondria, which are responsible for cellular energy production, are a potential target of pollutant toxicity. Thus, understanding mitochondrial energy metabolism will shed light on pollution adaptation. This research examines the oxidative phosphorylation (OxPhos) modulations due to chronic pollution exposure, how gene expression changes covary with OxPhos changes, and genotypic changes potentially underlying these phenotypic changes in response to pollution. Mitochondrial energy metabolism was investigated by quantifying hepatocyte OxPhos function in two independent, polluted *F. heteroclitus* populations from Elizabeth River, VA and New Bedford Harbor, MA, which are highly contaminated with polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) respectively. Compared to the respective reference populations, altered OxPhos functions were detected in both Elizabeth River and New Bedford Harbor populations, suggesting OxPhos was affected by pollution. Importantly, both polluted populations show elevated respiratory control ratio and routine respiration, which represent higher ATP production, indicating enhanced, adaptive mitochondrial metabolism in response to chronic pollution.

The divergent changes in proton leakiness (LEAK), complex II, and complex IV activity in New Bedford Harbor *versus* Elizabeth River populations suggest these natural populations' capacity to develop energy balance for stress tolerance in distinct ways. Acute dosing of a representative PAH and PCB elevated OxPhos uncoupling and inhibited ATP production in reference fish but failed to induce any effects in polluted fish, implying resistance to acute toxicity in polluted populations. Heritability of those OxPhos modulations was examined using laboratory-reared F3 generation fish. Result shows the toxicity resistance and enhanced routine respiration in Elizabeth River fish are consistent across generations, suggesting genetic adaptation. This is also supported by the lack of OxPhos differences between field-collected and laboratory-reared F3 generation New Bedford fish. To promote the understanding of OxPhos modulations, gene expression was measured on the same fish to identify potential pathways or processes contributing to OxPhos changes. Approximately 3.4% of genes have potentially adaptive gene expression changes in polluted fish, and these genes are enriched for functional clusters for stress responses and regulation of a variety of metabolic processes. Genes that are significantly linked to OxPhos variations are involved in a variety of energy-related metabolic processes and defense responses. These results suggest that pollution has a significant effect on mitochondrial energy metabolism by both directly modulating energy balance and indirectly elevating energy needs due to detoxification. Finally, to identify genetic changes that may underlie the observed phenotypic changes due to chronic pollution exposure, signatures of adaptation were investigated by examining the genetic variation of thousands of markers derived from genotyping-by-sequencing in *F. heteroclitus* inhabiting the strong pollution cline in New Bedford Harbor, MA. Identified

outliers underlying high genetic variation successfully discerned population genetic structure paralleling geographic PCBs. Gene annotation reveals that the pollutants-correlated outliers are functionally involved in diverse diseases, immune system response, and a variety of metabolic functions (*e.g.*, lipid metabolism and fatty acid biosynthesis), suggesting energy balance is targeted by pollution. Overall, these results suggest that identified outliers are most parsimoniously described as adaptive, and tested functionality of selected outliers supports adaptation. The findings in this thesis contribute to the understanding of how natural populations adapt to pollution from the bioenergetic point of view.

For my family

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Chapter 1 Introduction

1.1 Background

Aquatic organisms inhabiting estuarine and coastal areas are often exposed to pollutants due to urban development and human activities. It is well known that pollution can impair the fitness of natural populations in various ways, *e.g.,* inducing endocrine disruption (Sumpter and Johnson 2005), causing genotoxicity (Rose, French et al. 2000; Jung, Matson et al. 2011), and even triggering cancer (Shimada and Fujii-Kuriyama 2004; Srogi 2007). Yet, it is also well established that populations exposed to environmental stress (*e.g.,* pollution) have the potential to adapt when stress remains constant over generations and traits that enhance successful individuals are inherited (Bijlsma and Loeschcke 2005). The successful individuals that function better under stress are more likely to survive and reproduce, and after generations, populations may be predominated by the selected types, constituting the process of adaptation (Futuyma 1986; Nacci, Coiro et al. 1999). This appears to be the case with the saltmarsh minnow, *Fundulus heteroclitus* inhabiting highly polluted environments, which can adapt to pollution by developing tolerance to their sediment contaminants (Endler 1986; Nacci, Coiro et al. 1999; Meyer and Di Giulio 2002; Ownby, Newman et al. 2002; Meyer and Di Giulio 2003).

Energy balance is a major concern for organisms developing stress tolerance, as combating pollutant toxicity is usually metabolically costly (Calow 1991). Energy acquisition limitation leads to tradeoffs between the metabolic cost of stress tolerance and the energy costs of fitness-related functions such as growth, development, and reproduction (Sokolova, Frederich et al. 2012). That is, focus on energy balance directly

links physiological stress effects to organismal fitness, facilitating predicting population-level consequences (Sokolova, Frederich et al. 2012). Thus, energy balance needs to be considered to better understand how organisms tolerate and adapt to pollution. Moreover, mitochondria, which are responsible for the majority of cellular energy production in eukaryotes, have been proposed as a potential target of pollutants toxicity, especially persistent organic pollutants (POP), in a variety of physiological studies that report a link between POP exposure and mitochondrial function deficiency (Sivalingan, Yoshida et al. 1973; Zhu, Li et al. 1995; Li, Sioutas et al. 2002; Ko, Kim et al. 2004; Xia, Korge et al. 2004). This suggests that understanding mitochondrial energy metabolism will shed light on pollution adaptation, as mitochondria function is both directly targeted by pollutants toxicity and indirectly affecting organisms' energy balance, which is crucial for developing pollution tolerance.

Therefore, in this dissertation I used the ecological model *Fundulus heteroclitus* to examine how the oxidative phosphorylation (OxPhos) pathway, the biochemical pathway for most cellular energy production, is altered in response to pollutant toxicity and whether any alterations are heritable. In addition, I examined the gene expression differences of the same individuals and linked them to OxPhos differences to gain a better understanding of how pollution affects energy metabolism. Lastly, I investigated how the genotypes of a *F. heteroclitus* population inhabiting a strong pollution cline are affected by pollution.

1.2 Mitochondria

The mitochondrion, which is a dual membrane-enclosed structure found in most eukaryotic cells, is responsible for producing the majority of cellular energy in the form

of adenosine triphosphate (ATP) and reducing equivalents in the form of NADH and $FADH_2$. These latter two molecules are used in the oxidative phosphorylation (OxPhos) pathway to create the electro-chemical gradient that is used to produce the majority of ATP in our cells. Moreover, mitochondria play a critical role in the maintenance of cellular energy stores, thermogenesis, and apoptosis. Many human diseases (inherited or acquired) are correlated with alterations in mitochondrial function, *e.g.,* Parkinson disease, Leber's hereditary optic neuropathy (LHON), and Leigh's syndrome (fatal neurological syndrome) (Wallace 1999).

1.2.1 Oxidative Phosphorylation Pathway

ATP generation in mitochondria is realized through the oxidative phosphorylation (OxPhos) pathway in the electron transport chain of the inner mitochondria member. The OxPhos pathway consists of 89 proteins that form five multisubunit OxPhos enzyme complexes (Fig.1.1) imbedded in the inner mitochondrial membrane (Hatefi 1985; Smeitink et al., 2001; Pagliarini and Rutter 2013). Complexes I and II accept reduction equivalents from NADH and succinate *via* $FADH_2$ respectively. Electrons received at complexes I and II are shuttled by ubiquinone to complex III, where they are transferred to cytochrome c. Cytochrome c carries electrons to complex IV, where four electrons provide the energy needed to convert oxygen water. At the same time, energy harvested from electron transfer drives respiratory complexes I, III, and IV to pump protons into the mitochondrial intermembrane space. This pumping activity creates a proton gradient across the inner membrane that powers complex V, the ATP synthase, to generate ATP (Hatefi, 1985; Boyer, 1997; Schultz and Chan, 2001). Since protons can leak across the inner membrane thus relieving the proton gradient, OxPhos is considered incompletely

coupled (Divakaruni and Brand 2011). The efficiency in coordinating proton pumping and ATP production is termed the "coupling efficiency" of OxPhos. Two protein families that influence proton leak are adenine nucleotide translocase (ANT) and uncoupling proteins (UPC) (Divakaruni and Brand 2011). Genetic variation in these proteins or their expression levels could alter proton leak and affect mitochondrial membrane potential.

1.2.2 Mitochondrial Regulation

Mitochondria have their own circular DNA genome (mtDNA, ~16.5 kb in vertebrates). The proteins encoded by mitochondrial DNA (mtDNA) constitute essential components of the electron transport chain. However, the majority of mitochondrial proteins are transcribed in the nucleus and transported to the mitochondria. Thus, mitochondrial function relies on a cross-talk between nuclear and mitochondrial genes. Specifically, respiratory complexes I, III, IV and V consist of subunits derived from both mtDNA and nuclear DNA in contrast to complex II, whose four subunits are only encoded by nuclear DNA. All 13 mitochondrial-encoded proteins and 77 nuclear proteins (Table 1.1) interact to form the five enzyme complexes in the OxPhos pathway.

Due to the fact that the mitochondrial encoded genes are a small fraction of the total number of genes necessary to sustain the biological functions of this organelle, nuclear genes make a major contribution to mitochondrial metabolic systems and molecular architecture (Garesse and Vallejo 2001). The nuclear genes contribute with the catalytic and auxiliary proteins to mitochondria enzymatic activity (Cannino, Di Liegro et al. 2007), regulate the expression of nuclear and mitochondrial OxPhos genes *via* nuclear-encoded factors such as Tfam, TFB1M, NRF-1 and NRF-2 (Scarpulla 2006), and encode factors responsible for the import, assembly, and final localization of

mitochondrial polypeptides (Neupert 1997; Koehler 2004). Moreover, nuclear activity can be modulated by signals sent by mitochondria through retrograde communication (Liao and Butow 1993; Poyton and McEwen 1996; Liu and Butow 2006); thus the regulation of mitochondrial activity requires a bidirectional information flow (Cannino, Di Liegro et al. 2007).

Differential expression of mitochondrial encoded OxPhos mRNAs has been found among and within *Fundulus* populations (Oleksiak, Churchill et al. 2002), and these differences appear to be biologically significant (Oleksiak, Roach et al. 2005; Whitehead and Crawford 2006). Previous studies also found differentially expressed nuclear encoded OxPhos genes in *Fundulus* along the steep thermal cline and among populations exposed to pollution, which statistically explain metabolic variation (Oleksiak, Churchill et al. 2002; Oleksiak, Roach et al. 2005; Whitehead and Crawford 2006; Fisher and Oleksiak 2007).

1.3 Persistent Organic Pollutants

Persistent organic pollutants (POPs) are toxic organic compounds that are resistant to environmental degradation and adversely affect human health (Fisher, 1999; Arnot et al., 2011; Ruzzin 2012). Both polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) are POPs of major concern. PAHs are organic carbon compounds composed of fused aromatic ring structures (Jung et al., 2011), which are released into the environment primarily through incomplete organic matter combustion (Walker et al., 2005). They are a toxicologically important class of pollutants due to their carcinogenic or mutagenic effects (Srogi, 2007). PCBs, which were commercially manufactured and marketed in the U.S. from 1929 to 1977, are chemically stable

chemicals with high boiling points and low solubilities. Resistance to environmental degradation results in potential hazards, which affect natural organisms and human populations (Weaver, 1984).

POPs contribute significantly to human diseases and disrupt metabolic functions. POP exposure has been linked to carcinogenicity and mutagenicity in a variety of studies (Fisher, 1999; Li et al., 2006; Yu et al., 2010; Arnot et al., 2011; Ruzzin 2012; Lee et al., 2014). Both PAHs and PCBs could acquire carcinogenicity through the activation by enzymes, including the cytochrome P450 (CYP) 1 family, to generate highly reactive metabolites capable of attacking cellular DNA, which could be converted to the ultimate carcinogens (Preston BD 1984; Gillner, Bergman et al. 1985; Shimada and Fujii-Kuriyama 2004). Moreover, there is indication that POP exposure may have an effect on metabolic diseases, including type 2 diabetes, obesity, and energy metabolism (Lee et al., 2006; Lim et al., 2010; Airaksinen et al., 2011; KaramiMohajeri and Abdollahi 2011; Lee et al., 2011; Ruzzin, 2012). Mitochondria have also been reported as a target of POP toxicity, which could disrupt mitochondrial membrane potential, inhibit electron transfer, diminish ATP production, and elevate OxPhos uncoupling at the cellular level (Sivalingan, Yoshida et al. 1973; Li, Sioutas et al. 2002; Ko, Kim et al. 2004; Xia, Korge et al. 2004).

Both direct and indirect POP exposures alter gene expression profiles during toxicity. In a study testing PAH carcinogenic potency, acute exposure leads to altered gene expression of genes involved in various biological pathways including apoptosis, cholesterol biosynthesis, and fatty acid synthesis (Staal, van Herwijnen et al. 2006). POP-altered gene expression has also been detected in *F. heteroclitus*. In a study concerning

three *F. heteroclitus* populations inhabiting highly polluted Superfund sites, up to 17% of 384 metabolic genes were found to have evolved adaptive changes in gene expression (Fisher and Oleksiak 2007). Oleksiak 2008 reported differentially expressed genes in polluted populations that are involved in the oxidative phosphorylation pathway, indicating pollution may affect energy metabolism (Oleksiak 2008).

1.4 *Fundulus heteroclitus* as Study Organism

F. heteroclitus (or Atlantic Killifish) are small estuarine fish widely distributed along the eastern United States seaboard. This fish has wide distribution and local populations have limited migration (Burnett, Bain et al. 2007), which makes *F. heteroclitus* a valuable laboratory model for studying responses to environmental influences such as different salinities, temperatures, oxygen levels and even toxic chemicals (Burnett, Bain et al. 2007). Because of *F. heteroclitus'* ability to survive under extremely contaminated conditions, they are widely used for toxicology (Zhou, Rademacher et al. 1999; Zhou, Scali et al. 2001; Gonzalez, Roling et al. 2006) and genetic adaptation studies (Nacci, Coiro et al. 1999; Meyer and Di Giulio 2003; Nacci, Champlin et al. 2010). *F. heteroclitus* was one of the first fish species used as a model to study the links between pollution, immune response, and disease susceptibility (Fries 1986). In this dissertation, I examined OxPhos function, gene expression, and genomic variation in two *F. heteroclitus* populations inhabiting environments chronically polluted with POPs: one population from the Elizabeth River, VA, USA and a second population from New Bedford Harbor, MA, USA.

1.4.1 Elizabeth River *F. heteroclitus* population

The Atlantic Wood Industries Superfund site (AWI) along the Elizabeth River in Portsmouth, Virginia, is contaminated with extremely high concentrations of polycyclic aromatic hydrocarbons (PAHs) and metals (Vogelbein WK 2008; Wills, Jung et al. 2010) due to historical wood-treatment facility activity between 1926 and 1992. Total PAH concentrations of 383 ug/g dry sediment were reported in the Elizabeth River Superfund site (sites identified by the U. S. Environmental Protection Agency (EPA) that contain high levels of a variety of lipophilic, persistent and toxic contaminants and are worthy of remediation using Federal funds) by Vogelbein and Unger (2008) to the Virginia Department of Environmental Quality (Vogelbein WK 2008). It is noteworthy that collectively data from two reports by Vogelbein and Unger (2003; 2008) investigating the Elizabeth River system did not show widespread PAH level declines in this system over this time frame (Vogelbein WK 2003; Vogelbein WK 2008).

Wild-caught *F. heteroclitus* from this highly contaminated site are resistant to the acute toxicity (Ownby, Newman et al. 2002) and the cytochrome P4501A (CYP1A)-inducing activity of the sediments (Meyer and Di Giulio 2002). Both heritable (Nacci, Coiro et al. 1999; Meyer and Di Giulio 2002; Ownby, Newman et al. 2002; Meyer and Di Giulio 2003) and non-heritable (Meyer and Di Giulio 2002; Meyer and Di Giulio 2003) changes that could be related to pollution tolerance have been reported in this population as compared to *F. heteroclitus* from a nearby non-polluted reference site, King's Creek. *F. heteroclitus* from King's Creek, which has much lower sediment PAHs (< 1% of Elizabeth River concentrations) (Jung, Matson et al. 2011; Clark, Cooper et al. 2013), are genetically suited for use as a reference population for the Elizabeth River population

(Mulvey, Newman et al. 2002; Mulvey, Newman et al. 2003; Meyer, Volz et al. 2005). Potential trade-offs associated with adaptation to PAH-contamination, such as increased hepatic lesions and liver cancers (Vogelbein, Fournie et al. 1990) and sensitivity to hypoxia (Meyer and Di Giulio 2003), have also been reported in Elizabeth River *F. heteroclitus*.

1.4.2 New Bedford Harbor *F. heteroclitus* population

New Bedford Harbor (NBH), MA, is a federal Superfund site heavily contaminated with polychlorinated biphenyls (PCBs) and other halogenated aromatic hydrocarbons (HAHs) (Pruell, Norwood et al. 1990; Lake, McKinney et al. 1995) due to historical discharge of PCBs into the upper harbor as industrial waste from the 1940s to the 1970s (Nelson WG 1996). Those New Bedford Harbor contaminants are highly toxic, persistent, and extremely toxic to the early development of many fish species (Walker and Peterson 1991; Grimwood and Dobbs 1995). Within New Bedford Harbor, sediment PCB levels are as high as 22,666 ng/g dry weight (Nacci, Champlin et al. 2002).

However, despite this high contamination level, the *F. heteroclitus* population inhabiting New Bedford Harbor appears to be toxicity tolerant, existing in great abundance in New Bedford Harbor (Nacci, Champlin et al. 2002). Like the Elizabeth River fish, New Bedford Harbor *F. heteroclitus* exhibit reduced sensitivity to AHR agonists (Bello, Franks et al. 2001) and much higher POP tolerance as compared to individuals from nearby reference populations (Nacci, Kohan et al. 2002). There are indications that this tolerance is genetically inherited, supporting genetic adaptation (Nacci, Coiro et al. 1999; Nacci, Champlin et al. 2002). Scorton Creek, MA, is an uncontaminated site with a very low sediment PCB concentrations of 1 ng/g dry weight

(Nacci, Champlin et al. 2010) and has been used as a reference site for New Bedford Harbor (Powell, Bright et al. 2000; Hahn, Karchner et al. 2004).

1.5 Research Objective and Outline

Given that the detoxification process is metabolically costly, energy balance is crucial for the development of pollutant tolerance. The OxPhos pathway within mitochondria, which is the fundamental pathway for ATP production and supporting organism energy consumption, is potentially targeted by pollutant toxicity. Therefore, gaining a better understanding of how OxPhos metabolism is affected by environmental pollutants is an important research goal. The major objective of this research was to investigate the effects of chronic anthropogenic pollution on the OxPhos pathway and gene expression of hepatocytes from natural populations of the estuarine teleost, *F. heteroclitus* and to examine genotypic modulations induced by pollution. By examining alterations in OxPhos, gene expression, and genotypes that appear to be induced by anthropogenic stressors, this research provides insight into better understanding how natural populations tolerate and adapt to pollution from the bioenergetic point of view.

Chapter 2 describes the chronic and acute dosing effects of pollutant toxicity on OxPhos metabolism in the PAH contaminated, Elizabeth River *F. heteroclitus* population. Chronic pollution effects were estimated by comparing OxPhos metabolism in polluted *versus* clean reference populations. To evaluate whether polluted and reference populations respond similarly to acute toxicity, OxPhos metabolism was also quantified in fish dosed with a representative PAH and PCB.

Chapter 3 investigates the heritability of the OxPhos metabolic differences found in chapter 2. Thus, a laboratory-reared F3 generation of Elizabeth River *F. heteroclitus*

was compared to a laboratory-reared F1 generation from the reference population to see if OxPhos differences were consistent across generations. Elizabeth River F3 fish also were exposed to acute PAH dosing to determine the heritability of toxicity resistance.

Chapter 4 checks the generality of the OxPhos modulation patterns found in the Elizabeth River population by testing the chronic and acute dosing effect of pollutant toxicity on OxPhos metabolism from another *F. heteroclitus* population from New Bedford Harbor, a PCB contaminated site. This study used the same approach used for the Elizabeth River fish.

Chapter 5 examines gene expression modulations in polluted populations on the same individuals measured in chapters 2 and 4. Gene expression modulations were linked to OxPhos modulations to gain a better understanding of how pollution affects energy metabolism.

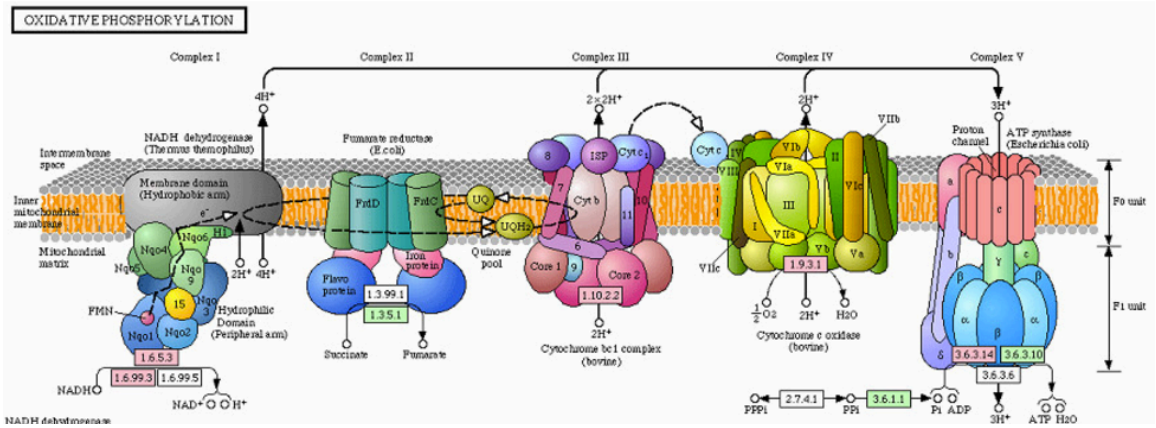
Chapter 6 identifies genotypic evidence of pollution adaptation by a genomic scan, which examined the genetic variation of thousands of markers derived from a genotyping-by-sequencing technique in *F. heteroclitus* inhabiting the strong pollution cline in New Bedford Harbor, MA, USA. This chapter aims to identify the genetic changes that may underlie the phenotypic changes due to chronic pollution exposure.

Finally, the findings of this dissertation as well as their implications are summarized in Chapter 7. Overall, OxPhos differences were examined in two independent, polluted populations and heritability of these changes was tested in laboratory-reared F3 generation fish. Gene expression changes were measured on the same fish to investigate their link to OxPhos changes. A genomic scan discerned genetic changes that potentially explain these phenotypic changes.

Table 1.1 Proteins encoded by mitochondrial or nuclear genomes for the five OxPhos enzyme complexes.

	Complex I: NADH Dehydrogenase	Complex II: Succinate Dehydrogenase	Complex III: Ubiquinol-cytochrome-c reductase	Complex IV: Cytochrome C oxidase	Complex V: ATP synthesis
Mitochondrial	7	0	1	3	2
Nuclear	38	4	10	10	14

Figure 1.1 OxPhos pathway. Five enzymatic complexes, electron transfer, and ATP synthesis on the OxPhos pathway (Granata, Zaza et al. 2009).



Chapter 2 Anthropogenic pollution effects on the oxidative phosphorylation pathway of hepatocytes from natural *Fundulus heteroclitus* populations

2.1 Summary

Persistent organic pollutants (POPs), including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), potentially target mitochondria and cause toxicity. I compared the effects of POPs on mitochondrial respiration by measuring oxidative phosphorylation (OxPhos) metabolism in hepatocytes isolated from lab-depurated *Fundulus heteroclitus* from a Superfund site contaminated with PAHs (Elizabeth River VA, USA) relative to OxPhos metabolism in individuals from a relatively clean, reference population (King's Creek VA, USA). In individuals from the polluted Elizabeth River population, OxPhos metabolism displayed lower LEAK and lower activities in complex III, complex IV, and E State, but higher activity in complex I compared to individuals from the reference King's Creek population. To test the supposition that these differences were due to or related to the chronic PAH contamination history of the Elizabeth River population, I compared the OxPhos functions of undosed individuals from the polluted and reference populations to individuals from these populations dosed with a PAH {benzo [a] pyrene (BaP)} or a PCB {PCB126 (3,3',4,4',5-pentachlorobiphenyl)}, respectively. Exposure to PAH or PCB affected OxPhos in the reference King's Creek population but had no detectable effects on the polluted Elizabeth River population. Thus, PAH exposure significantly increased LEAK and exposure to PCB126 significantly decreased State 3, E state and complex I activity in the reference King's Creek population. These data strongly implicate an

evolved tolerance in the Elizabeth River fish where dosed fish are not affected by PAH exposure and undosed fish show decreased LEAK and increased State 3 and E state.

2.2 Introductory Material[1]

Persistent organic pollutants (POPs) are some of the most prevalent pollutants because of their resistance to environment degradation and propensity to bioaccumulate (Fisher 1999; Arnot, Armitage et al. 2011; Ruzzin 2012). Both polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) are POPs of major concern. PAHs, which are released into the environment primarily through incomplete organic matter combustion (Walker, Dickhut et al. 2005), are organic carbon compounds composed of fused aromatic ring structures (Jung, Matson et al. 2011). They are a toxicologically important class of pollutants because some compounds have been identified as carcinogenic or mutagenic (Srogi 2007), and their environmental significance has been increasing partially due to the elevated rate of fossil fuel consumption (Van Metre, Mahler et al. 2000). PCBs, which were commercially manufactured and marketed in the U.S. from 1929 to 1977, are chemically stable, have high boiling points, low solubility, and their nonconductive nature cause them to persist in the environment and bioconcentrate. These traits create potential hazards that affect natural biota and human populations (Weaver 1984). Understanding the biochemical impact of PAHs and PCBs on natural populations provides insight into their toxicology

[1] POP, persistent organic pollutant; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; OxPhos, oxidative phosphorylation; BaP, benzo [a] pyrene; ANT, adenine nucleotide translocase; UCP, uncoupling protein; HRR, high-resolution respirometry; ER, Elizabeth River; KC, King's Creek; RCR, respiratory control ratio; UCR, uncoupling control ratio; QC, quality control.

and a greater understanding of their involvement in health and disease (Whitehead, Galvez et al. 2011).

POPs contribute significantly to human diseases: they are associated with cancers and mutagenesis and affect arteriosclerosis, intrauterine growth retardation and neurological development (Fisher 1999; Jones and de Voogt 1999; Li, Loganath et al. 2006; Porta, Puigdomenech et al. 2008; Lim, Cho et al. 2010; Arnot, Armitage et al. 2011; Ruzzin 2012). Initially, the primary health concerns about POP exposure focused on carcinogenicity and mutagenicity (Fisher 1999; Li, Loganath et al. 2006; Yu, Guo et al. 2010; Arnot, Armitage et al. 2011; Ruzzin 2012; Lee, Ra et al. 2014). More recently, POP exposure has been associated with metabolic diseases, including type 2 diabetes, obesity, and energy metabolism (Lee, Lee et al. 2006; Lim, Cho et al. 2010; Airaksinen, Rantakokko et al. 2011; Karami-Mohajeri and Abdollahi 2011; Lee, Lind et al. 2011; Ruzzin 2012). These associations link increased POP body concentrations with increased metabolic disorder incidences. However, there is little experimental evidence demonstrating that POPs directly affect the biochemical pathway responsible for most cellular energy production - the oxidative phosphorylation (OxPhos) pathway.

OxPhos is the metabolic pathway that produces most of the ATP in aerobic animals. This mitochondrial respiratory chain pathway consists of 89 proteins that form five multisubunit OxPhos enzyme complexes imbedded in the inner mitochondrial membrane (Hatefi 1985; Smeitink, van den Heuvel et al. 2001; Pagliarini and Rutter 2013). During OxPhos, complexes I and II accept reduction equivalents from NADH and $FADH_2$ respectively, and energy harvested from electron transfer drives respiratory complexes I, III, and IV to pump protons into the mitochondrial intermembrane space.

This pumping activity creates a proton gradient across the inner membrane that powers complex V to generate ATP (Hatefi 1985; Boyer 1997; Schultz and Chan 2001). Since protons can leak across the inner membrane thus relieving the proton gradient, OxPhos is considered incompletely coupled (Divakaruni and Brand 2011). Two protein families that influence proton leak are adenine nucleotide translocase (ANT) and uncoupling proteins (UPC) (Divakaruni and Brand 2011). Genetic variation in these proteins or their expression levels could alter proton leak and affect mitochondrial membrane potential.

Mitochondrial membrane potential loss, ATP production decreases, and mitochondrial morphology changes (Zhu, Li et al. 1995; Li, Sioutas et al. 2002; Ko, Kim et al. 2004; Xia, Korge et al. 2004) have been linked to one POP class, polycyclic aromatic hydrcarbons (PAHs). Exposure to another POP class, polychlorinated biphenyls (PCBs), has been reported to inhibit electron transfer, respiratory enzymes and mitochondrial respiration and increase OxPhos uncoupling (Pardini 1971; Sivalingan, Yoshida et al. 1973; Chesney and Allen 1974). Identifying the steps in the OxPhos pathway targeted by pollutants is essential to understanding the molecular basis of chronic pollutant toxicity, especially its involvement in metabolic health and disease.

I examined POP effects on OxPhos enzyme function in a population that has adapted to high POP concentrations compared to a reference "clean" population. A population of the salt marsh minnow, *Fundulus heteroclitus*, from the Elizabeth River, VA inhabits a Superfund site (an uncontrolled or abandoned site in the United States where hazardous waste is located) highly contaminated with PAHs (average ~200-400 μg/g sediments) and is resistant to the developmental toxicity of the sediments (Meyer and Di Giulio 2002; Ownby, Newman et al. 2002; Meyer and Di Giulio 2003;

Wassenberg and Di Giulio 2004; Burnett, Bain et al. 2007). In contrast, a nearby *F. heteroclitus* population from King's Creek, VA has been used as a genetically similar reference population. This reference population has much lower sediment PAH levels (< 0.4% of Elizabeth River PAH concentrations) (Jung, Matson et al. 2011; Clark, Cooper et al. 2013) and is sensitive to the toxicity of the polluted sediments from the Elizabeth River Superfund site (Meyer and Di Giulio 2002; Meyer and Di Giulio 2003; Burnett, Bain et al. 2007; Whitehead, Galvez et al. 2011). I tested the hypothesis that *F. heteroclitus* from a site chronically polluted with PAHs (Elizabeth River, VA) would have altered OxPhos metabolism as compared to *F. heteroclitus* from a nearby reference site (King's Creek, VA). I speculated that the polluted Elizabeth River population would compensate for chronic exposure to PAHs with altered OxPhos metabolism to cope with any disrupted membrane lipid bilayer dependent functions affecting ATP production.

To better understand potentially genetic effects as opposed to physiologically induced effects, I compared OxPhos enzyme function in fish that had been depurated in the laboratory for six months. To better understand physiologically induced POP effects on OxPhos functions, I dosed fish with two different POPs, benzo[a]pyrene (BaP) and polychlorinated biphenyl-126 (PCB-126). I dosed with two different POP classes because *F. heteroclitus'* adapted populations are resistant to a broad range of pollutants not found in their native habitats (Elskus, Monosson et al. 1999; Nacci, Coiro et al. 1999; Bello, Franks et al. 2001; Meyer and Di Giulio 2002; Clark and Di Giulio 2012). This dosing experiment allowed us to address two important questions: (*i*) Could PAH or PCB dosing induce acute, direct effects on the OxPhos functions of natural *F. heteroclitus*

populations? (*ii*) Did the polluted Elizabeth River population and reference King's Creek population respond similarly to PAH and PCB dosing?

2.3 Materials and Methods

2.3.1 Fish Husbandry and Treatments

Fundulus heteroclitus were collected from Elizabeth River, VA (36°48'26.20"N, 76°17'9.83"W, [EPA ID VAD990710410]) and a nearby reference site, King's Creek, VA (37°15'43.38"N, 76°29'4.57"W), by minnow traps in June 2012. Fish were depurated in re-circulating aquatic system tanks for 6 months with controlled temperature (20º C) and salinity (15 ppt). Fish were checked for health and fed daily (brine shrimp flake, blood meal flake, and Spirulina flake– FOD, Aquatic Biosystems).

Fish for the dosing experiment were similarly collected from Elizabeth River, VA and King's Creek, VA in May 2013. Fish were depurated in re-circulating aquatic system tanks for 4 weeks with controlled temperature (20º C) and salinity (15 ppt) and then dosed by intraperitoneal injection (IP injection) with either 50 mg/kg body weight PAH (BaP) or 10 mg/kg body weight PCB126 dissolved in corn oil with an injection volume of 5 μL/g body weight (Fig.2.1). Twenty-four hours later, fish hepatocytes were harvested and OxPhos functions were quantified *via* high resolution respirometry. Control or undosed groups for each pollutant were injected with corn oil only for 24 hours. Doses were based on previous studies (Willett, Steinberg et al. 1995; Karami, Christianus et al. 2011) and showed an effect on OxPhos functions in a preliminary time course experiment (data not shown). Experimental procedures were carried out following a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Miami.

I used BaP as a representative PAH in the dosing because BaP is one of the most intensively studied PAHs. Importantly, it is of high concentration in sediments and water from the Elizabeth River Superfund site (Vogelbein, Fournie et al. 1990; Bozinovic and Oleksiak 2010) and thus relevant for investigating the Elizabeth River population. I also used the PCB congener, 3,3',4,4',5-pentachlorobiphenyl (PCB126) as a representative PCB. This congener represents contaminants that are mediated through the aryl hydrocarbon receptor (AHR) pathway and such contaminants encompass major categories of toxic, organic anthropogenic pollutants (Nacci, Champlin et al. 2010).

2.3.2 Hepatocyte Isolation and Permeabilization

I used isolated liver hepatocytes because of the liver's importance in regulating many metabolic and physiological processes, particularly xenobiotic metabolism (Segner 1998). To measure OxPhos functions requires either 1) isolated mitochondria or 2) permeabilized cells so that substrate and inhibitor can be introduced to mitochondria. I used permeabilized hepatocytes rather than isolated mitochondria from the liver because permeabilized hepatocytes better reflect *in vivo* conditions: permeabilized hepatycytes provide relatively more intact outer mitochondrial membranes than isolated mitochondria (Nedergaard and Cannon 1979; Drahota, Krivakova et al. 2005; Phung, Saelid et al. 2011). Thus, the use of permeabilized hepatocytes better maintains the inter-mitochondrial contacts and mitochondria's interactions with other cytosolic structures, which are important for mitochondrial functional activity (Drahota, Krivakova et al. 2005).

Fish were sacrificed by cervical transection, and hepatocytes were harvested based on an *in situ* trypsin perfusion technique (Bello, Franks et al. 2001). Briefly, the

liver was minced in ice-cold Ca^{2++} free ringers (10 mls/liver) in a sterile petri dish with a sterile scalpel. Liver pieces were transferred without buffer into a 5 ml trypsin EDTA solution and incubated at room temperature for 20 minutes with occasional shaking. The suspension was filtered through four layers of sterile cheesecloth and then centrifuged at 100 x g for 5 minutes. Pelleted hepatocytes were resuspended in L15 media with 10% calf serum and centrifuged at 100 x g for another 5 minutes. The final hepatocyte pellet was resuspended in L15 media with 10% calf serum. Approximately $2\sim6 \times 10^6$ cells per liver were obtained with 95% viability as assessed by trypan blue exclusion with the use of a hemocytometer.

Hepatocyctes were diluted into Miro5 (respiration media: 0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6H2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1 g/l BSA, pH 7.1 adjusted with 5N KOH) at a density of 1.30 ± 0.11 million cells per ml for respiration measurements. Resuspended hepatocytes were transferred to respirometry chambers and allowed to equilibrate with atmospheric oxygen for 5 minutes. Digitonin was used to permeabilize the cells. Because excess digitonin can damage mitochondria, strictly optimized permeabilization conditions need to be determined for different cell types (Kuznetsov, Veksler et al. 2008). In our experiments, 20-30 μg digitonin per 1×10^6 cells was optimal for *F. heteroclitus* hepatocyte permeabilization. Thus, digitonin was added based on cell counts, and cells were incubated with the digitonin for 5 to 10 minutes prior to respirometry measurements.

2.3.3 High-resolution Respirometry

Hepatocytes metabolic function was quantified by high-resolution respirometry (HRR) with the OROBOROS Oxygraph-2k (OROBOROS instrument, Austria). HRR is

based on monitoring oxygen concentration in incubation medium in a closed chamber over time and measuring the rate the oxygen consumption (Pesta and Gnaiger 2012). Data acquisition and analysis were realized by the software DatLab (OROBOROS instrument, Austria). In our experiment, respiration was measured at 28°C, normal summer temperature for these populations. I chose this temperature because hepatocytes measured at 28°C were found to give robust measurements and oxygen consumption was relatively higher than when measured at 20°C. For depurated Elizabeth River and King's Creek populations, six individuals were measured for each population and each was measured in triplicate hepatocyte samples. In the dosing experiment, eight individuals were measured in each treatment group, and I made one measurement per individual.

2.3.4 OXPHOS Protocol

I quantified OxPhos measures as mean respiration rates in pmol O_2 s^{-1} ml^{-1} per 1×10^6 cells. The activities of the specific complexes in the electron transport chain were quantified by exposing hepatocytes to substrates and inhibitors (Table 2.2). Substrate and inhibitor concentrations were based on preliminary experiments to optimize the titration protocol. Resting respiration without ADP (State 2) and routine respiration (State 3, substrates and ADP) were measured; then proton leakiness (LEAK) and the contribution of different enzyme complexes were determined by the addition of specific inhibitors. Specifically, State 2 respiration (without ADP) was measured with the addition of pyruvate (5 mM), glutamate (10 mM), and succinate (10 mM). Routine respiration (State 3) was measured with the addition of adenosine diphosphate (ADP, 10 mM). Cytochrome C (Cyc, 10 uM) was added to check the integrity of the outer mitochondrial membranes.

Oligomycin (2 ug/ml) was added to block complex V by inhibiting ATP synthesis. Without ADP to ATP conversion, OxPhos is limited by the proton leak from the inner mitochondrial membrane. Thus, when complex V is blocked, the resulting respiration mainly reflects proton leak or LEAK respiration. LEAK is expressed as a ratio of respiration with oligomycin/State 3 respiration. "E-State" or maximal mitochondrial respiration is achieved when the proton gradient is disrupted by the uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, optimal concentration determined *via* titration). Rotenone (0.5 uM) was added to block complex I. Malonic acid (5 mM) was added to block complex II. Antimycin A (2.5 uM) was added to block complex III. Finally, N1,N1,N1,N1-tetramethyl-1,4-phenylene diamine (TMPD, 0.5 mM) and ascorbate (2 mM) were added to provide artificial substrates for complex IV.

2.3.5 Statistical Analyses

Statistical analyses were performed with Minitab 17 software. A t-test was used to compare the depurated polluted Elizabeth River and reference King's Creek population variances for each mitochondrial trait. For the dosing experiment, a two-way ANOVA (analysis of variance) was conducted to estimate the influences of population and dosage on OxPhos. Statistical significance was defined at $P< 0.05$. When there was a significant interaction among populations and dosing, differences between dosed and undosed fish were tested separately for each population using one-way ANOVA. Analysis of covariance (ANCOVA) was also conducted to explore the effect of body mass as a covariate on OxPhos. Mitochondrial traits that were affected by body mass were compared using residuals from regression with body mass for data analysis. For clarity, plots of mitochondrial traits used means and variance uncorrected for body mass.

2.4 Results

2.4.1 Body Mass

Average body masses ranged from 5.2 to 12.5 grams (Table 2.1). In depurated fish, body masses from the polluted Elizabeth River and reference King's Creek populations were similar ($P = 0.408$) and did not covary with any OxPhos measures (Table 2.2). In the PAH and PCB dosing experiment body mass was different among populations ($P < 0.05$). However, body mass was only a significant covariate in the PAH exposure having a significant effect on State 3 and E-State ($P < 0.05$). For the PAH exposures, the effect of body mass was removed by using the residuals from body mass vs. State 3 or E-state in all analyses. Differences among PAH and PCB are not reported because I treat these two experiments as separate since they were done a year apart.

2.4.2 OxPhos Determination

All OxPhos determinations were randomized among populations or population and dosing for the three separate experiments (depurated, PAH or PCB exposure). OxPhos functions are quantified by relative activities with the addition of substrates, or inhibition by poisons (Table 2.2). The most relevant measurements for this study are State 3 (routine metabolism with both substrates and ADP), LEAK (respiration limited by the H^+ leakage back into the mitochondrial matrix), E-state (maximum metabolism with the dissolution of the H^+ gradient) and measures of the enzyme complexes (Table 2.2).

OxPhos respiration with cytochrome c (Cyc) addition tests whether mitochondrial membranes were damaged during hepatocyte isolation and cell permeabilization. The effects of cytochrome c addition (Cyc/ State 3) were approximately 1 for all experiments (1.03, sem. 0.003). Thus respiration was unaffected by cytochrome c addition, indicating

the functional integrity of the outer mitochondrial membranes. RCR (State3/State2), which measures respiration dependency on ADP, was 4.19 (sem. 0.12) and was unaffected by any treatment except for exposure of King's Creek individuals to 10mg/kg PCB: RCR was 20% higher in undosed (3.84, sem. 0.12) compared to 10 mg PCB126/kg dosed fish (4.58, sem. 0.15).

2.4.3 OxPhos Functions in Depurated Fish

OxPhos functions for E-state, LEAK, and the relative contribution of Complexes I, III and IV are significantly different between depurated fish from the polluted Elizabeth River and the reference King's Creek populations (Fig.2.2). Differences in OxPhos functions between populations are detailed below.

LEAK measures respiration that relies on the dissipation of the proton gradient without ATP production and is measured when Complex V (ATP synthase) is inhibited (Table 2.2). The lower the LEAK the more efficient or coupled mitochondria respiration is to ATP production. Fish from the reference King's Creek population had 40% higher LEAK than those from the polluted Elizabeth River population ($p = 0.0004$, Fig.2.2). Thus, the efficiency or coupling of the mitochondrial state is greater in the polluted Elizabeth River population.

E state measures the respiratory capacity without H^+ gradient inhibition. The maximum flux of this uncoupled state was obtained by titration of the ionophore uncoupler (FCCP). E-state was 20% lower for the polluted Elizabeth River population than for the reference King's Creek population ($p < 0.037$, Fig.2.2) showing that the maximum metabolic capacity is lower in the polluted Elizabeth River population.

I also compared the contributions of complexes I, II, III, and IV to OxPhos in fish from the polluted Elizabeth River and reference King's Creek populations. To estimate the relative contribution of these enzyme complexes to OxPhos, the activity of each complex was calculated as ratios to E state. No significant differences between populations were detected in complex II activity ($P = 0.897$). However, significant differences were found in complexes I, III, and IV activities (Fig.2.2). Compared to the reference King's Creek population, the polluted Elizabeth River population had 40% lower Complex III activity ($p = 0.008$), 50% lower complex IV activity ($p = 0.002$) and 20% higher complex I activity ($p = 0.010$).

2.4.4 OxPhos Functions with POP Exposure

Exposure to POPs altered OxPhos only in fish from the reference King's Creek population and did not induce any detectable effects on OxPhos in fish from the polluted Elizabeth River population.

A two-way ANOVA was used to assess the effects of population and POP exposure and showed significant population-specific exposure and interactions, which differ between PAH and PCB (Table 2.3). For PAH dosing, there were significant differences between populations for State 3, LEAK, E state, complex I, and complex IV (Table 2.3). Although the main effect of PAH dosing (dosed *versus* undosed across both populations) was not statistically significant for LEAK (Table 2.3), the interaction between population and PAH was highly significant.

Those main effects of population or population-PAH interaction are immediately apparent in Fig.2.3: populations are significantly different, but dosing only affects Kings Creek individuals, creating the significant interaction. For PCB dosing, a two-way

ANOVA showed significant main effects of dosing on State 3 and E state, a significant population-PCB interaction on LEAK, and a main effect of population on complex II (Table 2.3). Thus, as with PAH dosing, there is a significant interaction, with King's Creek fish showing an effect when exposed to PCB.

The interaction effects suggest that POPs may have an effect on OxPhos functions. To determine whether PAH exposure has a significant effect requires the application of a one-way ANOVA on the effect of PAH on each population separately. For the King's Creek population, a one-way ANOVA shows that PAH dosing induced a 30% increase in LEAK in King's Creek fish ($p = 0.043$, Fig.2.3). The one-way ANOVA comparing PCB dosed and undosed King's Creek fish shows that PCB dosing decreased State 3 by 30% ($p = 0.010$, Fig.2.3), E state by 25% ($p = 0.022$, Fig.2.3) and complex I activity by only 10% ($p = 0.033$, Fig.2.3), but increased LEAK by 30%, similar to the effect of PAH dosing on LEAK ($p= 0.060$, Fig.2.3).

In contrast to the effects of PAH and PCB dosing on OxPhos activity in the reference King's Creek population, I failed to detect any dosing effects of PAH or PCB on OxPhos activity in the polluted Elizabeth River population. For the one-way ANOVA between the undosed Elizabeth River fish and the PAH or PCB dosed Elizabeth River fish, no significant differences were detected for any OxPhos traits (Fig.2.3). These data suggest that the polluted Elizabeth River population was resistant to the acute effects of PAH and PCB toxicity on OxPhos.

I are treating the PAH and PCB exposures as different experiments (performed in different years) and are not comparing across these two conditions. However, one might expect that OxPhos measures of the controls for these two different experiments would

be consistent. Yet, for State 3 and E-State: the King's Creek controls are smaller for PAH control (0 exposure) than PCB control. For State 3, the difference among King's Creek controls was eliminated when body mass was controlled for (*i.e.,* using residuals from regression with body mass for comparison). For E-State, body mass did not explain the lower measures of E-State in the King's Creek PAH control. These inconsistencies do not alter the more important observation that the polluted Elizabeth River fish are insensitive to POP exposure.

2.5 Discussion

Many POP studies on natural fish populations have focused on reproductive and developmental effects (Nacci, Coiro et al. 1999; Ownby, Newman et al. 2002; Wassenberg and Di Giulio 2004; Nacci, Champlin et al. 2010) (Incardona, Vines et al. 2012), biomarkers of xenobiotic exposure such as CYP1A inducibility and DNA damage (Jewett, Dean et al. 2002; Jung, Matson et al. 2011; Raphael, Aude et al. 2014), xenobiotic metabolism *in vivo* (*e. g.,* the aryl hydrocarbon receptor pathway (Bello, Franks et al. 2001; Denison and Nagy 2003; Hahn, Karchner et al. 2004)), altered gene expression (Leaver, Diab et al. 2010) (Fisher and Oleksiak 2007; Bozinovic, Sit et al. 2011; Oleksiak, Karchner et al. 2011; Garcia, Shen et al. 2012; Bozinovic, Sit et al. 2013), and genotypic effects (Williams and Oleksiak 2008; Williams and Oleksiak 2011; Williams and Oleksiak 2011). Those studies investigated pollution's influences on natural populations at molecular, biological, and ecological levels and helped to elucidate evolved tolerance in polluted populations. However, few studies to date have looked at the effects of anthropogenic pollutants (*e. g.,* PCBs, PAHs) on mitochondrial OxPhos metabolism. Some studies in mammalian models have pointed out that PAHs might

target the mitochondria and could disrupt OxPhos ATP production (Zhu, Li et al. 1995; Li, Sioutas et al. 2002; Ko, Kim et al. 2004; Xia, Korge et al. 2004). Additionally, gene expression studies in polluted populations have shown altered OxPhos gene expression (Fisher and Oleksiak 2007; Oleksiak 2008; Pujolar, Marino et al. 2012). To study how pollution might affect OxPhos, I characterized PAH or PCB effects on OxPhos metabolism in natural populations. The OxPhos pathway is critical because it is responsible for aerobic ATP production. The data presented here indicate both that OxPhos is a POP target and that polluted Elizabeth River fish display resistance to the acute effects of POP exposure.

I examined OxPhos functions in the salt marsh minnow, *Fundulus heteroclitus*, from two populations: a clean reference population and a polluted population adapted to PAHs (Meyer and Di Giulio 2003; Williams and Oleksiak 2008; Nacci, Champlin et al. 2010; Clark and Di Giulio 2012; Clark, Bone et al. 2014). The differences between the populations (Table 2.4, rows 1, 2, and 6) and the insensitivity of the polluted Elizabeth River individuals to both PAH and PCB dosing (Table 2.4, rows 4 and 5) support an adaptive response in the Elizabeth River population.

An alternative to this adaptive explanation is a slow physiological response that takes more time than the 6 months during which fish were depurated in the laboratory. Arguing against this is the demonstration that, in fish, PAHs have 2 to 9 day half-lives (Niimi 1987). Growth plus the six month depuration period will remove >98% of the PAH body burden and bring levels down to those measured in clean populations (Nacci, Coiro et al. 1999). Thus, six months should be enough time to eliminate most PAHs (the dominant anthropogenic pollutants in the Elizabeth River), and should have allowed

sufficient time for remodeling of the inner-mitochondrial membrane. The data support this supposition. A range of PAH doses demonstrated a typical response curve (data not shown) in King's Creek fish. Thus one would assume that any remaining body burden in the Elizabeth River fish would have only a small effect. I investigated whether the differences between populations were due to physiological induction by treating individuals with a similar acute dose. If a residual POP body burden were responsible for the differences between populations, one would expect that dosing would cause the reference population to resemble the polluted population. Alternatively, if the differences were adaptive, the expectation is that the polluted Elizabeth River fish will be less sensitive to acute exposure and that there would be greater differences between populations. Only the King's Creek fish were sensitive to dosing, which enhanced population differences (Table 2.4, rows 6 and 7). Thus, I suggest that the data support an adaptive response.

This adaptive conclusion is supported by previous publications on F1 and F2 fish (Clark, Bone et al. 2014) and divergence in genetic markers that affect phenotypic differences (Williams and Oleksiak 2008; Williams and Oleksiak 2011). Although I cannot rule out early developmental or epigenetic effects, adaptation seems most parsimonious, and I will refer to these differences as adaptive for the remainder of the studies described.

2.5.1 Insights into POP Effects on OxPhos Functions

Among depurated fish, the polluted Elizabeth River population displayed lower LEAK and showed lower activities in complex III, complex IV, and E State and higher activity in complex I. State 3 (routine respiration) was not different, suggesting that the

polluted Elizabeth River population relies more heavily on complex I and this compensates for the lower complex III and IV activities.

Leak was lower in depurated Elizabeth River fish than in King's Creek fish (Figure 2, P=0.004), and this difference is larger when fish from both populations are dosed (Figure 3). Dosing only affects King's Creek fish where both PAH (P= 0.043) and PCB (P= 0.060) dosing induced an increase in LEAK in the reference King's Creek population. LEAK is regulated by adenine nucleotide translocase (ANT) and uncoupling proteins (UPC) (Divakaruni and Brand 2011), and is a normal phenomenon where oxygen is consumed without ADP to ATP conversion. Thus LEAK is the inefficient dissipation of the H^+ gradient (Nobes, Brown et al. 1990). PAHs and PCBs are lipophilic hydrocarbons, which may react with the membrane lipid bilayers and disrupt protein-lipid interactions making membranes "leakier" (Sikkema, de Bont et al. 1995). Decreased LEAK in the polluted Elizabeth River population strongly suggests that natural populations adapted to the environment by modifying the mitochondrial inner membrane and proteins responsible for LEAK.

PCB dosing significantly decreased State 3 (Fig.2.2, P= 0.010) and E state (P= 0.022) in the reference King's Creek fish but had no effect on the polluted Elizabeth River fish. This result in the reference King's Creek fish is consistent with previous research using isolated heart or liver mitochondria (Pardini 1971; Sivalingan, Yoshida et al. 1973; Chesney and Allen 1974). For instance, PCBs inhibited energy and electron transfer and caused an uncoupling effect with increasing chlorine content in rat liver mitochondria (Sivalingan, Yoshida et al. 1973). PCBs *in vitro* induced a marked inhibition of respiratory enzyme systems in heart mitochondria (Pardini 1971). Finally,

PCB addition to rat liver mitochondria *in vitro* inhibited OxPhos and respiration (Chesney and Allen 1974).

It is interesting that Elizabeth River fish, which are exposed to PAHs, are resistant to both PAH and PCBs. This is similar to the findings that PCB-resistant New Bedford Harbor fish also are resistant to PAHs (Nacci, Coiro et al. 1999; Bello, Franks et al. 2001), and PAH-resistant Elizabeth River fish also are resistant to PCBs (Meyer and Di Giulio 2002) and a variety of insecticides (Clark and Di Giulio 2012). One possible explanation is that exposure affects AHR or one of its downstream targets and thus changes in these targets provide resistance to a wide range of POPs.

The results presented herein showing POP effects on metabolism in the reference but not polluted fish populations may help explain similar studies of how POPs affect metabolism. The few studies examining POP effects on fish energetics (van Ginneken, Palstra et al. 2009; Cannas, Atzori et al. 2013) fail to reveal a general pattern due to the limitation of comparative assessments. PCB-contaminated European eels (*A. anguilla*) were characterized by lower aerobic metabolism than control individuals, indicating that PCB treatment decreased aerobic metabolism (van Ginneken, Palstra et al. 2009). Yet PCB contamination showed no effect on juvenile sole (*Solea solea*) aerobic metabolism (Cannas, Atzori et al. 2013). Differences in the type of pollutants tested and variety of the experimental approaches employed would lead to obstacles for comparing those studies (Cannas, Atzori et al. 2013). However, it is interesting to speculate that POP effects may be more consistent if the adaptive differences among populations are taken into consideration.

2.6 Conclusions

Overall, the acute dosing effects revealed two important insights: 1) OxPhos functions in the polluted Elizabeth River population were unaffected by POP dosage, whereas LEAK and other OxPhos functions in the relatively clean reference King's Creek population were affected by dosage. 2) OxPhos functions in the polluted Elizabeth River population were consistently different from OxPhos functions in the clean reference King's Creek population, regardless of whether or not they were dosed. These results suggest that PAHs and PCBs have acute, direct, toxic effects on OxPhos functions of natural *F. heteroclitus* populations and provide strong evidence for adaptation of OxPhos functions in the Elizabeth River population in response to chronic pollution. The chronic PAH contamination history of the Elizabeth River population may have led to a modification or improvement of the Elizabeth River population's OxPhos functions that allows the fish in this population to maintain their normal metabolic functions under polluted conditions. The observation that the polluted Elizabeth River population also showed resistance to PCB toxicity suggests that PAHs and PCBs share similar effects on OxPhos functions that are mitigated in the polluted Elizabeth River fish.

Table 2.1 Body mass. Mean body mass of *Fundulus heteroclitus* (± s.e.m.) in all treatment groups. 0PAH and 0PCB refer to the fish injected with corn oil (undosed control groups). 50PAH stands for 50 mg/kg PAH dosing and 10PCB stands for 10 mg/kg PCB dosing.

Body mass.	
Treatment Group	Body mass (g)
KC Depurated	11.35 ± 0.76
ER Deputated	12.45 ± 1.02
KC 0 PAH	5.19 ± 0.44
KC 50 PAH	5.83 ± 0.81
ER 0 PAH	7.50 ± 1.45
ER 50 PAH	8.03 ± 1.40
KC 0 PCB	7.27 ± 0.61
KC 10 PCB	7.53 ± 0.83
ER 0 PCB	9.02 ± 0.83
ER 10 PCB	8.86 ± 0.86

Table 2.2 OxPhos function expressed as mean respiration rates in pmol O_2 s^{-1} ml^{-1} per 1×10^6 cells.

Trait	Definition	Substrate or inhibitors
State 2	Respiration without ADP	Substrates: pyruvate, glutamate, and succinate
State 3	Routine Respiration	ADP plus substrates
RCR	Respiratory control Ratio	State 3 (ADP + substrates)/State 2 (substrates only)
QC	Quality Control	Cytochrome c / State 3
LEAK	LEAK	Oligomycin (ATP synthase inhibitor)/ State 3
E-state	Maximum or decoupled respiration	FCCP, proton gradient uncoupler
CI	Complex I contribution	{FCCP maximum activity – Rotenone (CI inhibitor)}(Rose, French et al. 2000)/ FCCP
CII	Complex II contribution	{Rotenone - Malonic acid (CII inhibitor)}/ FCCP
CIII	Complex III contribution	{Malonic acid– Antimycin A (CIII inhibitor)}/ FCCP
CIV	Complex IV contribution	{TMPD + ascorbate (electron donors)}/ FCCP

Table 2.3 Two-way ANOVA analysis of OxPhos traits as a function of population and PAH/ PCB exposure. State 3 and E state were calculated using residuals from regression with body mass. SS, sum of squares; MS, mean squares.

	PAH						PCB					
	Effect	df	SS	MS	F	P	Effect	df	SS	MS	F	P
State 3	Population	1	1184.7	1184.7	11.629	0.002 *	Population	1	95.1	95.1	1.572	0.221
	PAH	1	97.1	97.1	0.953	0.337	PCB	1	620.8	620.8	10.27	0.003 *
	Population × PAH	1	232.8	232.8	2.285	0.142	Population × PCB	1	26.9	26.9	0.445	0.51
	Residuals	28	2852.5	101.9			Residuals	27	1632.3	60.5		
LEAK	Population	1	0.141	0.141	59.946	1.96E-08 *	Population	1	0.002	0.002	0.767	0.389
	PAH	1	0.007	0.007	3.116	0.088 .	PCB	1	0.002	0.002	0.643	0.43
	Population × PAH	1	0.013	0.013	5.427	0.027 *	Population × PCB	1	0.011	0.011	4.336	0.047*
	Residuals	28	0.066	0.002			Residuals	27	0.068	0.003		
E state	Population	1	1118.8	1118.8	18.153	0.0002 *	Population	1	0.3	0.3	0.005	0.946
	PAH	1	137.3	137.3	2.228	0.147	PCB	1	442.7	442.7	7.075	0.013 *
	Population × PAH	1	212.9	212.9	3.454	0.074 .	Population × PCB	1	2.3	2.3	0.037	0.849
	Residuals	28	1725.6	61.6			Residuals	27	1689.4	62.6		
CI	Population	1	0.021	0.021	13.111	0.0012 *	Population	1	0.025	0.025	3.151	0.088 .
	PAH	1	0.0001	0.0001	0.06	0.808	PCB	1	0.001	0.001	0.143	0.709
	Population × PAH	1	0.0005	0.0005	0.284	0.598	Population × PCB	1	0.028	0.028	3.628	0.068 .
	Residuals	28	0.044	0.002			Residuals	26	0.203	0.008		
CII	Population	1	0.007	0.007	1.345	0.256	Population	1	0.033	0.033	6.011	0.021 *
	PAH	1	0	0	0.003	0.956	PCB	1	0.002	0.002	0.405	0.53
	Population × PAH	1	0.004	0.004	0.724	0.402	Population × PCB	1	0.016	0.016	2.894	0.1
	Residuals	28	0.136	0.005			Residuals	27	0.147	0.005		
CIII	Population	1	0.0001	1E-05	0.27	0.607	Population	1	7.40E-05	7.40E-05	0.363	0.552
	PAH	1	0.0004	0.0004	1.214	0.28	PCB	1	0	4.00E-08	0	0.99
	Population × PAH	1	1E-05	1E-05	0.023	0.88	Population × PCB	1	0	4.30E-05	0.213	0.648
	Residuals	28	0.008	0.0003			Residuals	27	0.005	2.03E-04		
CIV	Population	1	0.502	0.502	5.41	0.028 *	Population	1	0.105	0.10486	0.445	0.511
	PAH	1	0.08	0.08	0.867	0.36	PCB	1	0.152	0.15194	0.645	0.429
	Population × PAH	1	0.025	0.025	0.266	0.61	Population × PCB	1	0.009	0.00937	0.04	0.843
	Residuals	28	2.596	0.093			Residuals	25	5.888	0.23552		

Table 2.4 Summary of Significant Results. Rows 1, 6 and 7 summarize p-values of one-way ANOVA that compare: lab-depurated King's Creek (KC) with lab-depurated Elizabeth River (ER) *F. heteroclitus*, undosed KC with undosed ER in PAH exposure, and PAH dosed KC with PAH dosed ER. Rows 2 and 3 summarize the p-values of population effect and dosage effect (PAH or PCB) in PAH/PCB exposure using two-way ANOVA.

	STATE 3: Routine respiration	LEAK: H+ leakage	E-STATE: Maximum respiration	CI:NADH dehydrogenase	CII: Succinate dehydrogenase	CIII: Cytochrome b complex	CIV: Cytochrome C oxidase
1: Population effect depurated		0.0004 Ref>Poll	0.037 Ref>Poll	0.01 Poll > Ref		0.008 Ref > Poll	0.002 Ref > Poll
2: Difference between Populations (2-way)	PAH:0.002 Poll > Ref	PAH: >0.0001* Ref > Poll	PAH: >0.0002 Poll> Ref	PAH: 0.0012 Poll > Ref	PCB: 0.021 Poll > Ref		PAH: 0.028 Ref > Poll
3: Dosage effect (PAH or PCB) (2-way)	PCB: 0.003 0 > Dose	PAH: interact* PCB: interact*	PCB: 0.013 0 > Dose				
4: PAH effects for each population separately		KC: 0.043 Dose > 0					
5: PCB effects for each population separately	KC: 0.01 0 > Dose	KC: 0.06 Dose > 0	KC: 0.022 0 > Dose	KC: 0.033 0 > Dose			
6: Differences between populations when undosed (PAH exposure)	0.011 Poll > Ref	0.0004 Ref > Poll	0.002 Poll > Ref	0.013 Poll > Ref			
7: Differences between populations when dosed (PAH exposure).	PAH: 0:009 Poll > Ref	PAH: 0.0002 Ref > Poll	PAH: 0.019 Poll > Ref	PAH: 0.04 Poll > Ref			PAH:0.029 Ref > Poll
* Significant interactions; State 3 and E state were calculated using residuals from regression with body mass.							

Figure 2.1 Experiment design. a. Hepatocytes from the lab-depurated Elizabeth River (ER) and lab-depurated King's Creek (KC) populations were isolated for OxPhos functional comparisons. b. The polluted Elizabeth River (ER) population was compared to the reference King's Creek (KC) population when treated with PAH or PCB, with a corn oil control.

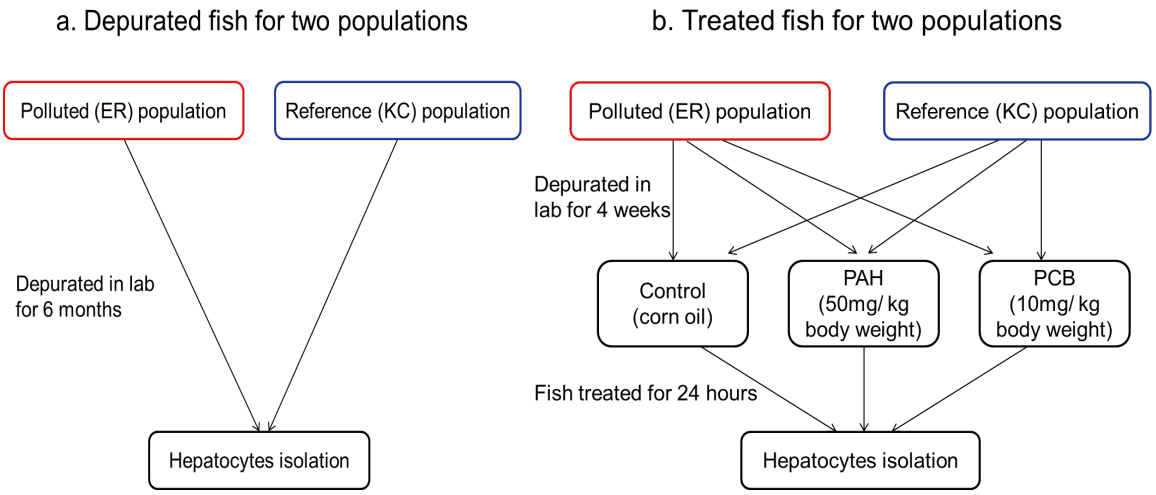


Figure 2.2 Depurated Fish for two populations. 95% confidence intervals (CI) for the means of the OxPhos traits are plotted for depurated fish from the reference King's Creek and polluted Elizabeth River populations. State 3 and E state are expressed as pmol s^{-1} ml^{-1} per 10^6 cells. LEAK, complex I, complex II, complex III, and complex IV are calculated as ratios. Only significant p-values between populations are marked.

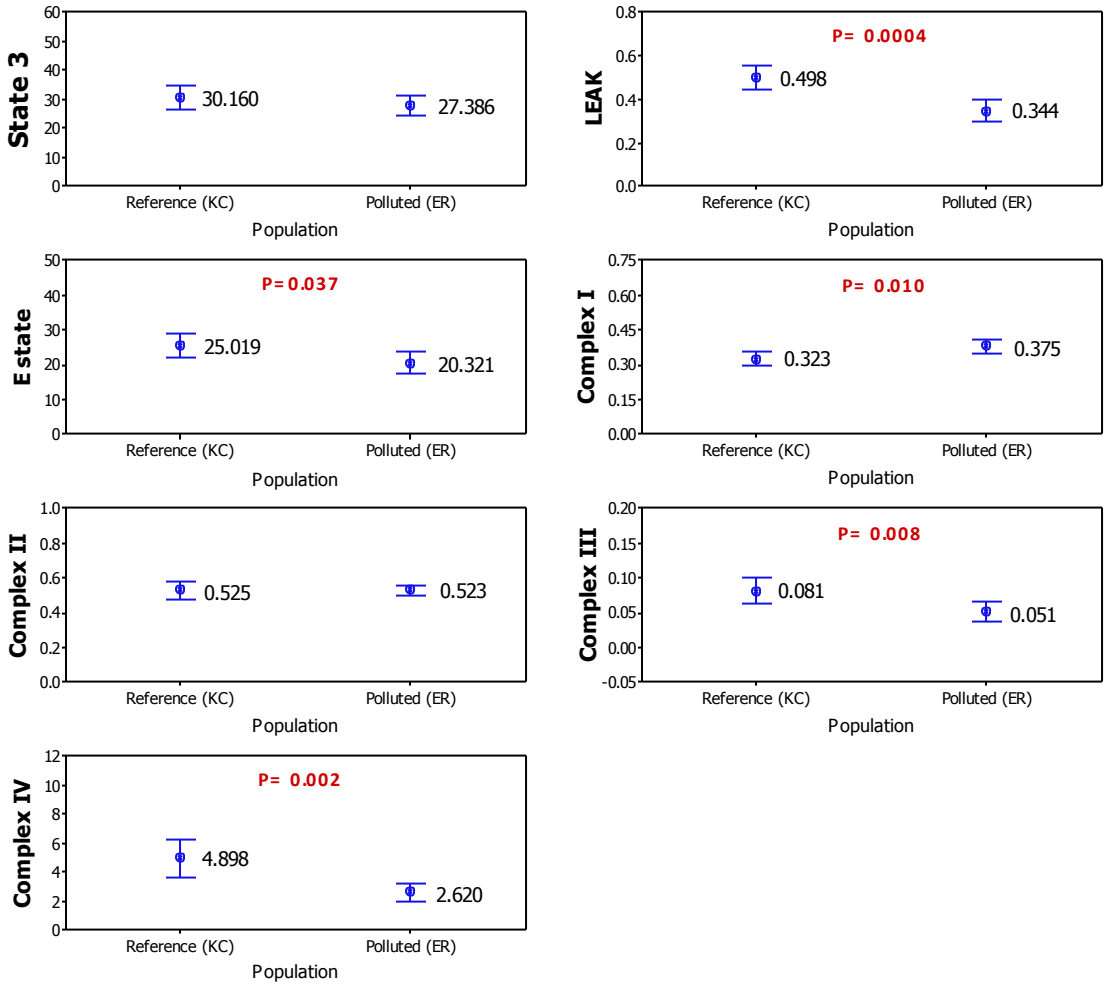
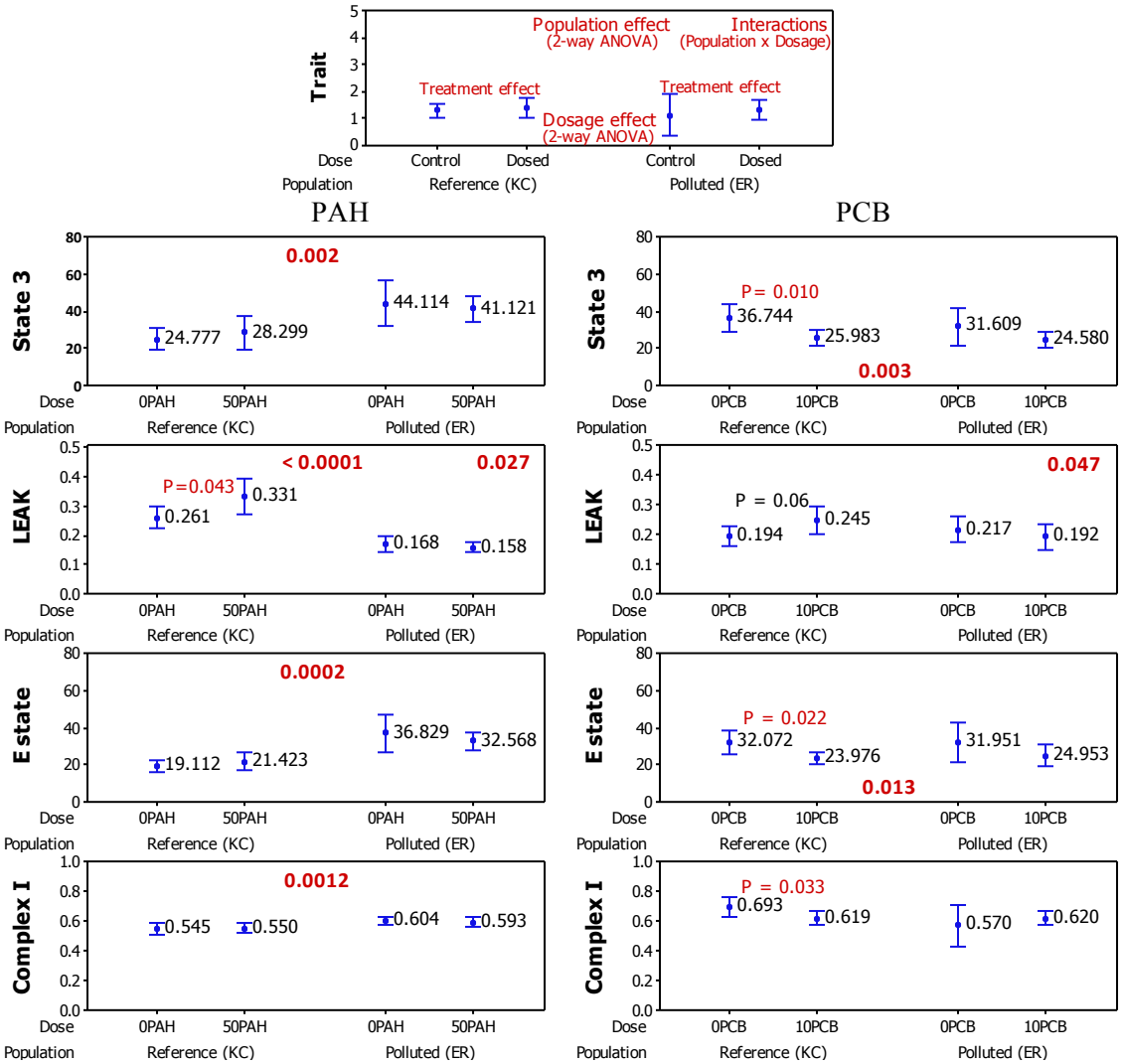
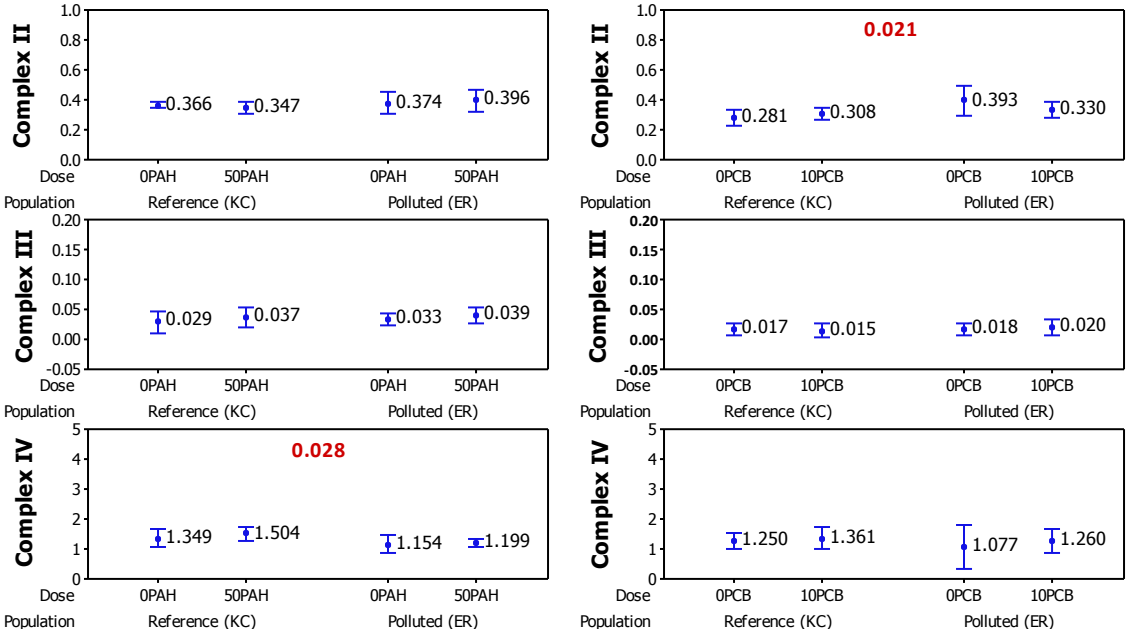


Figure 2.3 Population and dosage effects of POPs. The polluted Elizabeth River (ER) population was compared to the reference King's Creek (KC) population when dosed or undosed with PAH or PCB separately. Mean and 95% confidence intervals (CI) for OxPhos traits of KC and ER populations of *F. heteroclitus* were plotted. State 3 and E state are expressed as pmol s^{-1} ml^{-1} per 10^6 cells. LEAK, complex I, complex II, complex III, and complex IV are calculated as ratios. Only significant p-values are marked. P-values for the two-way ANOVA are displayed in center top (population effect), center bottom (dosage effect) and top left (interactions). State 3 and E state were compared using residuals from regression with body mass. Significant differences between treatments within each population are marked above means and CI. 50PAH stands for 50 mg/kg PAH dosing and 10PCB stands for 10 mg/kg PCB dosing.




Complex II
1.0
0.8
0.6
0.4
0.2
0.0
0.366
0.347
0.374
0.396
Dose
0PAH
50PAH
0PAH
50PAH
Population
Reference (KC)
Polluted (ER)
Complex II
0.021
0.281
0.308
0.393
0.330
Dose
0PCB
10PCB
0PCB
10PCB
Population
Reference (KC)
Polluted (ER)
Complex III
0.20
0.15
0.10
0.05
0.00
-0.05
0.029
0.037
0.033
0.039
Dose
0PAH
50PAH
0PAH
50PAH
Population
Reference (KC)
Polluted (ER)
Complex III
0.017
0.015
0.018
0.020
Dose
0PCB
10PCB
0PCB
10PCB
Population
Reference (KC)
Polluted (ER)
Complex IV
5
4
3
2
1
0
0.028
1.349
1.504
1.154
1.199
Dose
0PAH
50PAH
0PAH
50PAH
Population
Reference (KC)
Polluted (ER)
Complex IV
1.250
1.361
1.077
1.260
Dose
0PCB
10PCB
0PCB
10PCB
Population
Reference (KC)
Polluted (ER)

Chapter 3 Heritable oxidative phosphorylation differences in a pollutant resistant *Fundulus heteroclitus* population

3.1 Summary

Populations can adapt to stress including recent anthropogenic pollution. Our published data suggests heritable differences in hepatocyte oxidative phosphorylation (OxPhos) metabolism in field-caught killifish (*Fundulus heteroclitus)* from the highly polluted Elizabeth River, VA, USA, relative to fish from a nearby, relatively unpolluted reference site in King's Creek VA. Consistent with other studies showing that Elizabeth River killifish are resistant to some of the toxic effects of certain contaminants, OxPhos measurements in hepatocytes from field-caught King's Creek but not field-caught Elizabeth River killifish were altered by acute benzo [a] pyrene exposures. To more definitively test whether the enhanced OxPhos metabolism and toxicity resistance are heritable, I measured OxPhos metabolism in a laboratory-reared F3 generation from the Elizabeth River population *versus* a laboratory-reared F1 generation from the King's Creek population and compared these results to previous data from the field-caught fish. The F3 Elizabeth River fish compared to F1 King's Creek fish had significantly higher State 3 respiration (routine metabolism) and complex II activity, and significantly lower complex I activity. The consistently higher routine metabolism in the F3 and field-caught Elizabeth River fish *versus* F1 and field-caught King's Creek fish implies a heritable change in OxPhos function. The observation that LEAK, E-State, Complex I and Complex II were different in laboratory bred *versus* field-caught fish suggests that different physiological mechanisms produce the enhanced OxPhos differences. Finally, similar to field-caught Elizabeth River fish, acute benzo [a] pyrene exposure did not affect OxPhos function of the laboratory-reared F3 generation, supporting the heritability

of the toxicity resistance. Overall, these results suggest that the Elizabeth River population has evolved genetic changes in physiological homeostasis that enhance routine metabolism, and I speculate that these genetic changes interact with environmental factors altering the physiological mechanisms (*e.g.,* alter LEAK, Complex I, and electron transfer system capacity) used to achieve this enhanced metabolism.

3.2 Introductory Material

Populations exposed to environmental stress (*e.g.,* pollution) have the potential to adapt when stress i) remains constant over generations, ii) reduces individuals' fitness, and iii) limits survival or reproduction (Bijlsma and Loeschcke 2005). The successful individuals that function better under stress are more likely to survive and reproduce and the traits that enhance these traits will be inherited by future generations. Therefore, after generations, populations may be predominated by the selected types, and the resulting genetic structure change constitutes the process of adaptation (Futuyma 1986; Nacci, Coiro et al. 1999). However, differentiating adaptation from acclimation (the reversible physiological changes individuals make to cope with an altered environment) can be difficult in natural populations, when the responses to an altered environment can be due to both genetic adaptation and physiological acclimation. This appears to be the case with the saltmarsh minnow, *Fundulus heteroclitus,* inhabiting highly polluted environments. *Fundulus heteroclitus* from the Elizabeth River, VA, a site highly polluted with polycyclic aromatic hydrocarbons (PAHs) (Walker, Dickhut et al. 2004; Vogelbein WK 2008; Di Giulio and Clark 2015), exhibit both heritable (Nacci, Coiro et al. 1999; Meyer and Di Giulio 2002; Ownby, Newman et al. 2002; Meyer and Di Giulio 2003) and non-heritable (Meyer and Di Giulio 2002; Meyer and Di Giulio 2003) changes compared to *F.*

heteroclitus from a nearby non-polluted reference population, King's Creek. Total PAH concentrations of 383 ug/g dry sediment were reported in the Elizabeth River, by Vogelbein and Unger (2008) to the Virginia Department of Environmental Quality. Among those PAHs, concentrations of benzo [a] pyrene (BaP), a representative PAH, were 42 ug/g dry sediment (Vogelbein WK 2008). In contrast, King's Creek has much lower sediment PAHs, which were reported less that 1% of Elizabeth River concentrations (Jung, Matson et al. 2011; Clark, Cooper et al. 2013).

I recently examined oxidative phosphorylation (OxPhos) metabolism in field-caught Elizabeth River *F. heteroclitus*. The OxPhos pathway is responsible for cellular ATP production within mitochondria and consists of five multi-subunit enzyme complexes imbedded in the inner mitochondrial membrane (Hatefi 1985; Pagliarini and Rutter 2013). During OxPhos, electron transfer drives complexes I, III, and IV to pump protons into the mitochondrial intermembrane space, producing a proton gradient across the inner membrane (Hatefi 1985). Proton gradient dissipation through complex V drives ATP synthesis (Hatefi 1985; Boyer 1997; Schultz and Chan 2001). However, the electron transfer and ATP synthesis processes are considered incompletely coupled since protons can also leak across the inner membrane, thus relieving the proton gradient independently of complex V (Divakaruni and Brand 2011).

Anthropogenic pollutants have been reported to inhibit mitochondrial OxPhos functions, *e.g.* decrease ATP synthesis, inhibit electron transfer, and increase uncoupling efficiency (Sivalingan, Yoshida et al. 1973; Chesney and Allen 1974; Zhu, Li et al. 1995; Xia, Korge et al. 2004). Altered OxPhos gene expression was also detected in polluted *F. heteroclitus* populations (Fisher and Oleksiak 2007; Oleksiak 2008). Thus, altered

OxPhos functions in pollutant-exposed fish may reflect stress responses; however, altered OxPhos responses in populations chronically-exposed to pollutants might reflect adaptive responses. Considering its crucial role in energy production and cellular functions, understanding the interactions between OxPhos and anthropogenic pollutants will help clarify the molecular basis of population-level responses to chronic pollution exposure and aid in investigating metabolic diseases.

In chapter 2 I have shown that OxPhos function is altered in field-caught *F. heteroclitus* from the polluted Elizabeth River. These fish showed higher hepatocyte OxPhos metabolism, higher coupling efficiency, and greater resistance to BaP compared to fish from a nearby, clean, reference population inhabiting King's Creek, VA (Du, Crawford et al. 2015). To test the hypothesis that the altered OxPhos function in the Elizabeth River *F. heteroclitus* population is adaptive rather than a stress or acclimatory response associated with direct exposures, I compared OxPhos function in laboratory-reared, F3 *F. heteroclitus* from the polluted Elizabeth River population to OxPhos function in laboratory-reared, F1 *F. heteroclitus* from the reference King's Creek population. The retention of OxPhos traits in the 3rd generation Elizabeth River fish would eliminate epigenetic, developmental, and irreversible physiological effects, and thus would indicate that the OxPhos differences between the polluted and reference populations are heritable and most likely an evolutionary adaptation in response to anthropogenic pollution.

3.3 Materials and Methods

3.3.1 Fish Husbandry and Treatments

Fish used were a laboratory-raised, F3 generation of *Fundulus heteroclitus* that were collected from Elizabeth River, VA (Atlantic Wood Industries site) and a laboratory-raised F1 generation of *F. heteroclitus* collected from a nearby reference site, King's Creek, VA. The Elizabeth River fish were spawned in 2011, and the King's Creek were spawned in 2012 (Table 3.1). All fish were provided by the US Environmental Protection Agency (EPA), Office of Research and Development, Atlantic Ecology Division, Narragansett, RI, and shipped to University of Miami in March 2014. Then fish were acclimated in re-circulating aquatic system tanks for another four months with controlled temperature (20º C) and salinity (15 ppt).

Average weights (SD) are 8.96 (2.47) and 7.98 (3.12) grams for Elizabeth River F3 and King's Creek F1 fish, respectively (Table 3.1). Dose effects on isolated hepatocytes were determined following intraperitoneal (i.p.) injection, because it has been widely used in various dose exposures (Willett, Steinberg et al. 1995; Ishimaru, Takagi et al. 2009; Karami, Christianus et al. 2011), and more importantly it was reported as a more effective or efficient route of exposure in ecotoxicological studies as compared to other approaches (*e.g.* intramuscular injection or oral exposure) (Gerasimov, Franceschi et al. 2000; Gao, Li et al. 2011; Karami, Christianus et al. 2011). Therefore, before measuring hepatocyte specific OxPhos, Elizabeth River F3 fish were dosed by i.p. injection with 50 mg/kg body weight (198.2 umol/kg) benzo [a] pyrene dissolved in corn oil with an injection volume of 5 uL/g body weight for 24 hours. Control or undosed Elizabeth River F3 fish and King's Creek F1 fish were i.p. injected with corn oil only

with the same injection volume of 5 uL/g body weight for 24 hours. Eight fish were treated in each treatment group. Then fish hepatocytes were harvested and OxPhos function was quantified *via* high resolution respirometry. The BaP dose, 50 mg/kg body weight in the experiment, was the same dose used on field-caught Elizabeth River and King's Creek populations (Du, Crawford et al. 2015). I chose this dose based on literature values (Willett, Steinberg et al. 1995; Karami, Christianus et al. 2011) and a preliminary dose response experiment (data not shown) testing different BaP doses (0 mg/kg, 10 mg/kg, and 50 mg/kg body weight): 50 mg/kg was the most potent dose in introducing detectable OxPhos changes in hepatocytes isolated from a non-tolerant population. Experimental procedures were carried out following a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Miami.

3.3.2 Hepatocyte Isolation and Permeabilization

Hepatocytes were isolated as described (Du, Crawford et al. 2015). Half of the isolated hepatocyctes were saved for future gene expression analysis, and half were resuspended into Miro5 (respiration media: 0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6H2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1 g/l BSA, pH 7.1 adjusted with 5N KOH) and permeabilized with digitonin for OxPhos quantification (Du, Crawford et al. 2015).

3.3.3 High-resolution Respirometry

OxPhos hepatocyte function was quantified by high-resolution respirometry with the OROBOROS Oxygraph-2k (OROBOROS instrument, Austria) as described (Du, Crawford et al. 2015). Data were visualized and acquired by the software DatLab (OROBOROS instrument, Austria), and respiration was measured at 28°C, corresponding

to our previous study measuring field-caught fish. OxPhos metabolism was quantified as mean respiration rates in pmol O_2 s^{-1} ml^{-1} per million cells. Sequentially exposing hepatocytes to substrates and different inhibitors allows determinations of each complex activity in the electron transport chain (Table 2.2).

3.3.4 Statistical Analyses

To investigate both acute BaP exposure effects and how laboratory raised fish compare to field caught and acclimated fish, I combine the analyses of F3 Elizabeth River fish with a previous published BaP exposure in acclimated fish (Du, Crawford et al. 2015). Statistical analyses were performed in JMP Pro 12 (SAS, Cary NC). Although raw data are plotted, statistical analyses were done on ANCOVA data (analysis of covariance using body mass as a covariate). First, to estimate the population effect, generation effect, and population-generation interactions respectively on OxPhos (Table 3.3), the OxPhos data on laboratory-reared, undosed Elizabeth River F3 and King's Creek F1 fish were combined with published data on acclimated field-caught fish (Du, Crawford et al. 2015) for a two-way ANCOVA. “Acclimated field-caught fish” are field-caught fish acclimated in the laboratory for 4 weeks with controlled temperature, salinity, and diet to remove physiological effects induced by local environment or fish handling (Sidell, Wilson et al. 1973; Pottinger and Pickering 1992) and thus facilitate investigating chronic exposure effects.

Using this same data sets (laboratory-reared, undosed Elizabeth River F3 and King's Creek F1 fish and published data on acclimated field-caught (Du, Crawford et al. 2015)), a series of one-way ANCOVA were performed separately on BaP dosed *versus*

undosed Elizabeth River F3, field-caught Elizabeth River, and field-caught King's Creek fish (Fig.3.2). Statistical significance was defined at $P < 0.05$.

Regression analysis was performed in JMP Pro to analyze relationships between State 3 respiration and LEAK or different complexes. Using residuals from the regression with body mass should remove any covariance among OxPhos functions due to the shared body mass influence. Multiple regression analyses used JMP Pro 12 with forward stepping using minimum Bayesian information criterion (BIC) to choose the best model (Burnham and Anderson 2004). BIC is defined as follows:

$BIC = -2 \log Likelihood + k \ln(n)$

where k is the number of estimated parameters in the model and n is the number of observations in the data set (Schwarz 1978; Burnham and Anderson 2004).

3.4 Results and Discussion

To determine if differences between polluted and clean-reference populations are most likely heritable (Du, Crawford et al. 2015), I contrasted the differences among populations with and without acute BaP exposure in F3 fish from the polluted Elizabeth River population to F1 fish from the reference King's Creek population to earlier BaP exposure data on acclimated fish from these two populations (Du, Crawford et al. 2015). If OxPhos differences between the polluted and reference fish are retained in the 3rd generation, this would suggest that the differences are heritable and most likely an evolutionary adaptation in response to anthropogenic pollution because the use of 3rd generation fish would eliminate epigenetic, developmental, and irreversible physiological effects. Similarly, if the response to acute BaP exposure in F3 fish from the polluted

Elizabeth River were similar to acclimated fish, it would be indicative of a heritable response to PAHs in this population.

3.4.1 Mitochondrial Integrity and Quality Control

The respiratory control ratio (RCR) estimates respiration dependency on ADP; it is functionally related to the ratio of State3 to LEAK. Cytochrome c addition tests for mitochondrial membrane intactness. Average respiratory control ratios greater than 3 (Table 3.2) and lack of change upon cytochrome c addition (Table 3.2) of both F1 King's Creek fish and F3 Elizabeth River fish indicate mitochondrial membrane intactness after hepatocyte isolation and permeabilization (Table 3.2). Consistent with our previous data for acclimated, field-caught *F. heteroclitus* (Table 3.2), the F3 Elizabeth River fish exhibited a significantly higher RCR as compared to the F1 King's Creek fish (P=0.004). A high RCR implies that the mitochondria are more effective at ATP production and have low proton leak. Indeed, field caught and F3 fish from the Elizabeth River population have significantly higher State 3 respiration, which is a measure of ATP production, and lower leak than the field caught and F1 fish from the reference King's Creek population (Fig.3.1) although leak is only significantly different between the acclimated, field-caught fish.

3.4.2 Oxphos Comparison Between Polluted and Reference Populations

The two-way ANCOVA compares populations and generations with body mass as a covariate (Table 3.3). “Populations” are the polluted and reference population (Elizabeth River *versus* King’s Creek). Generations are between laboratory raised fish (F3 & F1) *versus* field caught acclimated fish (Table 3.3). The results of the two-way ANCOVA (Table 3.3) suggest that the population effect is the main source of variation in

State 3 respiration, LEAK, E state, and Complex II activity ($p < 0.05$; Table 3.3). Population differences explain most of the variations in those traits. Significant interactions of generation and population contribute to variability of E state and Complex I activity ($p < 0.05$; Table 3.3). Figure 1 illustrates the differences between populations for both laboratory raised and acclimated field-caught fish and p-values are for specific one-way ANCOVA between populations for F3/F1 or acclimated field-caught fish.

State 3 or routine respiration estimates ATP production depends on other OxPhos enzyme functions (Complexes I-V) and LEAK. Compared to the reference population, the polluted Elizabeth River population in both F3 and acclimated field-caught fish have significantly higher State 3 respiration ($p = 0.008$, $p = 0.013$, respectively; Fig.3.1). The interpretation of these data needs to be taken in context with the other experiment contrasting these two populations. Specifically, in a previous publication (Du, Crawford et al. 2015), the significant differences between the Elizabeth River and reference populations were restricted to the BaP dosed fish. For this study's other two experiments (depurated fish acclimated to a common environment for > 6 months, which should remove most if not all xenobiotic load, and PCB exposed fish—as a separate study with different control fish from PAH exposed fish) undosed fish show no significant population differences (Du, Crawford et al. 2015). As suggested in this earlier publication, this variation in State 3 most likely reflects the age of the fish, unknown environmental conditions and sample size. To address these inconsistencies, I combined data from all non-dosed fish from both populations. Similar to the two-way ANCOVA presented here (Table 3.3), fish from polluted Elizabeth River had significantly greater State 3 respiration than reference King's Creek fish ($p < 0.02$, 36.6 *versus* 28.3 pmol O_2 s^{-}

^{1} ml^{-1} per 1×10^{6} cells). These data suggest that the polluted Elizabeth River population has evolved adaptive enhancement in OxPhos capacity in response to its chronic pollutant exposure history. This enhanced OxPhos capacity may allow the chronically polluted Elizabeth River population to compensate for elevated metabolism needed for detoxification (Sousa, Mota et al. 2006).

State 3 respiration depends on LEAK. LEAK occurs because mitochondrial respiration is incompletely coupled to ATP synthesis, and thus protons can leak across the inner membrane of mitochondria without energizing ADP phosphorylation *via* Complex V (Divakaruni and Brand 2011). Therefore, lower LEAK indicates higher mitochondrial coupling efficiency. LEAK was significantly different between Elizabeth River and King's Creek ($p < 0.0015$) with no significant difference between laboratory-reared and acclimated field-caught fish or the interaction term (Table 3.3). Lower LEAK was detected in Elizabeth River fish compared to King's Creek fish; however, applying one-way ANCOVA to each generation separately, this was only significant for acclimated fish ($p = 0.002$) and was insignificant among laboratory-reared fish (ER F3 *versus* KC F1, $p = 0.1$). These complex results imply that both genetic and non-genetic factors contribute to the higher coupling efficiency of OxPhos in the polluted Elizabeth River population. In context with RCR values, where F3 polluted Elizabeth River fish RCR values were significantly larger, these data suggest that the Elizabeth River fish have a greater State 3 respiration and that State 3 respiration is more efficient: more ATP is produced per oxygen reduced to water.

State 3 respiration can be limited by the dissipation of the H+ gradient used by Complex V (ATP synthase). E state measures the fully uncoupled respiration rate and is

not limited by ATP synthase; thus the E state estimates the electron transfer system (ETS) capacity or the maximum OxPhos capacity. E state is significantly different between Elizabeth River and King's Creek fish ($p < 0.0022$), as is the interaction term (Table 3.3). The significant interaction term arises because differences between laboratory *versus* acclimated field-caught fish are dependent on population. This is illustrated in figure 1, where E state is not significantly different between F3 Elizabeth River and F1 King's Creek fish but is significantly different between Elizabeth River population *versus* the reference population for acclimate field-caught fish ($p = 0.005$; Fig.3.1). These results are surprising because Elizabeth River fish have higher State 3 respiration in both the F3 and field caught fish compared to those from the reference population. This suggests either a loss of genetic differences during laboratory breeding or long-term physiological or developmental effects on OxPhos capacity that reduces the difference between the polluted Elizabeth River and reference King's Creek populations.

State 3 respiration also depends on the different enzyme complexes. Complexes I and II activities, but not Complexes III and IV activities, have significant differences between fish from the Elizabeth River and King's Creek populations (Table 3.3). Complexes I and II use NADH and $FADH_2$, respectively, to provide the energy to initiate electron transport and H+ pumping. Complexes I and II are significantly different in F3 Elizabeth River fish compared to F1 King's Creek fish ($p < 0.02$ for both complexes; Fig.3.1). Yet for Complex I, there is a significant interaction (Table 3.3). This significant interaction illustrates that the Complex I differences between populations depends on whether laboratory or acclimated field-caught fish are compared. In F3 Elizabeth River fish, Complex I activity is significantly lower than F1 King's Creek fish, but in

acclimated field caught fish, Complex I activity is significantly higher in Elizabeth River fish ($p < 0.04$, Fig.3.1). That is, while significant in each comparison, the patterns are the opposite. Complex II activities were higher in both the F3 generation and acclimated field-caught Elizabeth River fish although the difference was only significant in F3 generation fish ($p=0.019$, Fig.3.1).

The two-way ANCOVA (Table 3.3), the one-way ANCOVA conducted within each generation separately (F3 or F1 *vs*. acclimated field fish; Fig.3.1) and combining all non-dosed data from an earlier publication ((Du, Crawford et al. 2015) $p < 0.02$) reinforce the idea that population differences in State 3 respiration exist between the Elizabeth River and King's Creek populations, and this OxPhos capacity difference is independent of generation. The enhanced State 3 or routine respiration in polluted and clean populations are due to changes in Complexes I and II activities. However, the enhanced State 3 metabolism in Elizabeth River fish changes from Complex I to Complex II for field caught *versus* F3 fish.

3.4.3 Oxphos Functions with Benzo [a] Pyrene Exposure

Similar to State 3, which was consistently different between Elizabeth River and King's Creek populations, acute BaP dosing had similar effects on F3 and acclimated field-caught fish from the polluted Elizabeth River (Fig.3.2). In all Elizabeth River fish, BaP dosing had no effect on any OxPhos measures. In contrast, the same BaP dose induced a significant increase in proton LEAK in the acclimated field-caught King's Creek population ($p < 0.05$; Fig.3.2). This proton LEAK increase in the BaP dosed, King's Creek population implies that BaP has an acute and direct toxic effect on natural *F. heteroclitus* populations *via* impairing their OxPhos coupling efficiency. However, the

consistent insensitivity in F3 and acclimated field Elizabeth River fish indicates that these fish have evolved tolerance to BaP induced LEAK.

LEAK dissipates the mitochondrial proton gradient without ATP production, and thus it is considered one of the important factors controlling cellular energy efficiency (Nobes, Brown et al. 1990). Therefore, I proposed that decreased LEAK in the polluted Elizabeth River population was likely an adaptive increase in coupling efficiency under chronic exposure (Du, Crawford et al. 2015). However, in F3 fish, while the insensitivity to acute BaP exposure was retained, the significant difference in LEAK between the polluted and reference populations was not (Fig.3.1). Thus, these data suggest that the polluted Elizabeth River fish have evolved modifications to the inner mitochondrial membrane functions that make it resilient to acute exposure.

3.4.4 Contribution of Each Complex to State 3

To explore which OxPhos functions alter State 3 metabolism, I regressed each parameter against State 3 for dosed and undosed Elizabeth River and King's Creek populations (Fig.3.3). To initiate this, I regressed all OxPhos parameters against body mass and used the residuals. Using residuals from the regression with body mass should remove any covariance among OxPhos functions due to the shared influence of body mass. Specifically I seek to define whether 1) the variation among fish in LEAK or any of the enzyme complexes explains the variation in State 3 respiration and 2) this covariance differs between populations or with dosing. For these analyses, acclimated and laboratory bred (F1 & F3) fish were pooled to enhance sample sizes and thus provide the necessary statistical power to identify significant patterns. Therefore, in the regression analysis there were 16 individuals in each group (KC Control, ER Control, and ER

Dosed) except for the KC Dosed group, which includes 8 individuals. A minimum of 10 to 15 observations per predictor variable will generally allow good parameter estimates while a smaller sample size may potentially lead to inflation of estimated R^2 of the linear regression (Babyak 2004). Thus, KC Dosed group regressions should be interpreted with caution.

For the reference King's Creek population, the variation in State 3 respiration is explained by the variation (R^2, which is the percentage of variation explained by the dependent variable, where an $R^2 > 0.2$ has a p-value < 0.1 for all but King's Creek Dosed, which has a smaller sample size) in LEAK, Complex II and Complex IV (Fig.3.3A). Notice that only the covariances with LEAK and Complex II are significant and Complex II is positive. These relationships between State 3 and LEAK, Complex II and Complex IV are similar with BaP dosing (Fig.3.3A) with $R^2 > 0.2$. However, for dosed King's Creek fish, none of these covariances are significant. For the undosed Elizabeth River fish, State 3 respiration increases with decreasing Complex II (Fig.3.3B). Surprisingly, with dosing, the variation in State 3 respiration is no longer related to Complex II activity and instead is related to Complex III activity (Fig.3.3B). Thus, without dosing, Complex II explains State 3 in both populations. Additionally, in the reference King's Creek population, LEAK and possibly Complex IV also explain State 3. The surprising difference is with BaP dosing: in the Elizabeth River fish, State 3 dependency changes from Complex II to Complex III.

These relationships are supported by multiple regressions using BIC maximum likelihood method (Burnham and Anderson 2004) to define the OxPhos factors that are related to State 3 respiration. For both populations, without BaP dosing, Complex II is the

first and most important factor, explaining 28% and 34% of the variation in State 3 for the Elizabeth River and King's Creek populations, respectively (Table 3.4). Yet in the reference King's Creek population, in addition to Complex II, Complex I and LEAK are important. When fish are dosed with BaP, State 3 in the King's Creek population is still dependent on Complexes I and II, but LEAK is no longer significant. However, dosed Elizabeth River fish are very different; only Complex III is entered into the regression model. That is, only Complex III significantly regresses with State 3. Using raw values and adding body mass as a covariate do not alter the regression but do reduce the overall p-value.

What these data suggest is that Elizabeth River fish have a greater State 3 than King's Creek fish due in part to the absence of a LEAK effect and greater reliance on Complex II. Surprisingly, even though State 3 remains higher in Elizabeth River fish than in King's Creek fish with dosing, the enzyme complexes responsible for this greater State 3 change with BaP exposure. With dosing, the greater State 3 in Elizabeth River fish is most reliant on Complex III, but in King's Creek fish Complex III is never significant, and Complex II remains most important (it has a greater R^2). The significance of these observations is that Elizabeth River fish have greater OxPhos respiration (State 3) with or without dosing, but the enzyme complexes affecting this enhanced metabolism differ depending on environment (*e.g.,* dosing).

3.5 Conclusion

The observation that State 3 respiration or routine metabolism is consistently higher in the F3 and field-caught Elizabeth River fish *versus* F1 and field-caught King's Creek fish indicates a heritable change in OxPhos that is most likely due to adaptive

evolution. Other studies examining resistance in *F. heteroclitus* from polluted environments support the hypothesis of genetic adaptation. For instance, laboratory-reared F1 and F2 *F. heteroclitus* from New Bedford Harbor (MA, USA), highly contaminated with polychlorinated biphenyls (PCBs), including dioxin like PCB congeners, indicated that this dioxin-like-compound resistance was genetically inherited and independent of non-genetic, maternal effects (Nacci, Coiro et al. 1999). Similarly, Elizabeth River *F. heteroclitus* embryos from field polluted fish showed similar resistance as F2 embryos to the teratogenic effects of Elizabeth River sediment, indicating that resistance was heritable through the F2 generation (Ownby, Newman et al. 2002). This was supported by a recent study by Clark, Bone, and Di Giulio (2014), which reported strong resistances of F1 and F2 Elizabeth River embryos to teratogenesis induced by several PAHs and PCB-126, suggesting certain aspects of the resistances were genetically inherited.

I detected consistently higher State 3 respiration between Elizabeth River and King's Creek *F. heterolitus,* independent of both BaP dosing and generation. Thus, in the context of OxPhos toxicity tolerance, I conclude that resistance in the Elizabeth River population is adaptive and genetically based. One caveat is that I cannot rule out the possibility that with a higher BaP dose, F3 Elizabeth River fish might display decreased resistance compared to field caught Elizabeth River fish. Yet, when I contrast the differences between populations, although State 3 is consistently higher in Elizabeth River fish with or without dosing, the enzyme complexes and LEAK, which functionally define State 3, are not consistently different. Specifically, LEAK, E-State, Complex I and Complex II in F3 *versus* acclimated, field caught fish are different. These differences

appear to offset each other because overall metabolism, State 3 respiration, is consistently higher in Elizabeth River fish. For E state, the lack of a difference in F3 fish from the Elizabeth River compared to F1 reference King's Creek fish suggests that the field polluted Elizabeth River population has elevated respiratory ETS capacity. Similarly, LEAK is only repressed in acclimated, field caught Elizabeth River fish and not in F3 fish. I suggest that an evolved genetic change in physiological homeostasis enhances routine metabolism but that physiological mechanisms that produce this enhanced metabolism (*e.g.,* altered LEAK, Complex I, ETS capacity) differ. That is, evolution has favored an enhanced metabolic set point (greater State 3 respiration), but I speculate that gene by environmental interactions, where subtle environmental factors (*e.g.,* lifetime diet, constant temperature, etc.) modulate the physiological responses. The generality of these responses might be testable using other pollutant-adapted killifish populations, such as those from PCB contaminated New Bedford Harbor.

Table 3.1 *Fundulus heteroclitus* in the experiment.

	Population Source	Year Spawned	Current Generation	Average Weight (SD)	Life Stages
KC lab-reared F1	King's Creek, VA	2012	F1	7.98 (3.12) g	Adult
ER lab-reared F3	Elizabeth River, VA	2011	F3	8.96 (2.47) g	Adult

Table 3.2 Respiratory control ratio and cytochrome c effect in permeabilized hepatocytes of laboratory-reared Elizabeth River (ER) F3, laboratory-reared King's Creek (KC) F1, and acclimated field-caught ER and KC *Fundulus heteroclitus* (mean ± s.e.m.).

	Lab-reared KC F1	Lab-reared ER F3	Acclimated field-caught KC	Acclimated field-caught ER
Respiratory Control Ratio	3.94 ± 0.19	4.85 ± 0.17	3.34 ± 0.16	4.88 ± 0.26
Cytochrome c effect	1.04 ± 0.01	1.03 ± 0.01	1.03 ± 0.01	1.02 ± 0.00

Table 3.3 Two-way ANCOVA with Population and Generation

	Term	Estimate	Std Error	t Ratio	Prob>|t|
State 3	Body mass	0.7505	0.4945	1.5200	0.1407
	Generation	3.4502	1.9608	1.7600	0.0898
	Pop	7.8149	1.9194	4.0700	0.0004
	Generation*Pop	3.2658	1.8138	1.8000	0.0830
LEAK	Body mass	-0.0033	0.0026	-1.2500	0.2211
	Generation	-0.0142	0.0104	-1.3600	0.1845
	Pop	-0.0362	0.0102	-3.5400	0.0015
	Generation*Pop	-0.0063	0.0097	-0.6600	0.5173
E state	Body mass	0.5299	0.4277	1.2400	0.2260
	Generation	0.5344	1.6959	0.3200	0.7551
	Pop	5.6102	1.6601	3.3800	0.0022
	Generation*Pop	4.6139	1.5688	2.9400	0.0066
CI	Body mass	0.0042	0.0024	1.7800	0.0866
	Generation	0.0110	0.0094	1.1700	0.2516
	Pop	-0.0073	0.0092	-0.8000	0.4312
	Generation*Pop	0.0317	0.0087	3.6500	0.0011
CII	Body mass	-0.0050	0.0031	-1.6200	0.1158
	Generation	-0.0192	0.0122	-1.5700	0.1270
	Pop	0.0288	0.0119	2.4200	0.0227
	Generation*Pop	-0.0192	0.0113	-1.7100	0.0994
CIII	Body mass	-0.0001	0.0010	-0.1500	0.8854
	Generation	0.0038	0.0038	0.9800	0.3375
	Pop	0.0064	0.0038	1.7100	0.0992
	Generation*Pop	-0.0042	0.0036	-1.1800	0.2486
CIV	Body mass	0.0015	0.0248	0.0600	0.9523
	Generation	-0.0314	0.0983	-0.3200	0.7522
	Pop	-0.1833	0.0962	-1.9100	0.0674
	Generation*Pop	0.0842	0.0909	0.9300	0.3628

Table 3.4 Multiple Regression. Factors listed in order of entry into BIC maximum likelihood model. R^2 for each factor is enclosed in parentheses.

	p-val	R2	1st Factor	2nd Factor	3rd Factor
ER Control	0.034	0.2828	CII (0.28)		
KC Control	0.0098	0.6216	CII (0.34)	CI (0.166)	LEAK (0.112)
ER Dosed	0.0377	0.2758	CIII (0.27)		
KC Dosed	0.0021	0.8777	CII (0.300)	CI (0.58)	

Figure 3.1 OxPhos comparison of laboratory-reared F3 Elizabeth River *F. heteroclitus versus* laboratory-reared F1 King's Creek *F. heteroclitus*, and acclimated field-caught Elizabeth River *F. heteroclitus versus* field-caught King's Creek *F. heteroclitus*. 95% confidence intervals (CI) for the means of the OxPhos traits were plotted. State 3 and E state measurements were expressed as mean respiration rates in pmol O_2 s^{-1} ml^{-1} per 1×10^{6} cells. LEAK and complex I-IV were calculated as ratios. OxPhos traits were compared using one-way ANCOVA with body mass as covariate. Only significant p-values ($p < 0.05$) are marked on the figure.

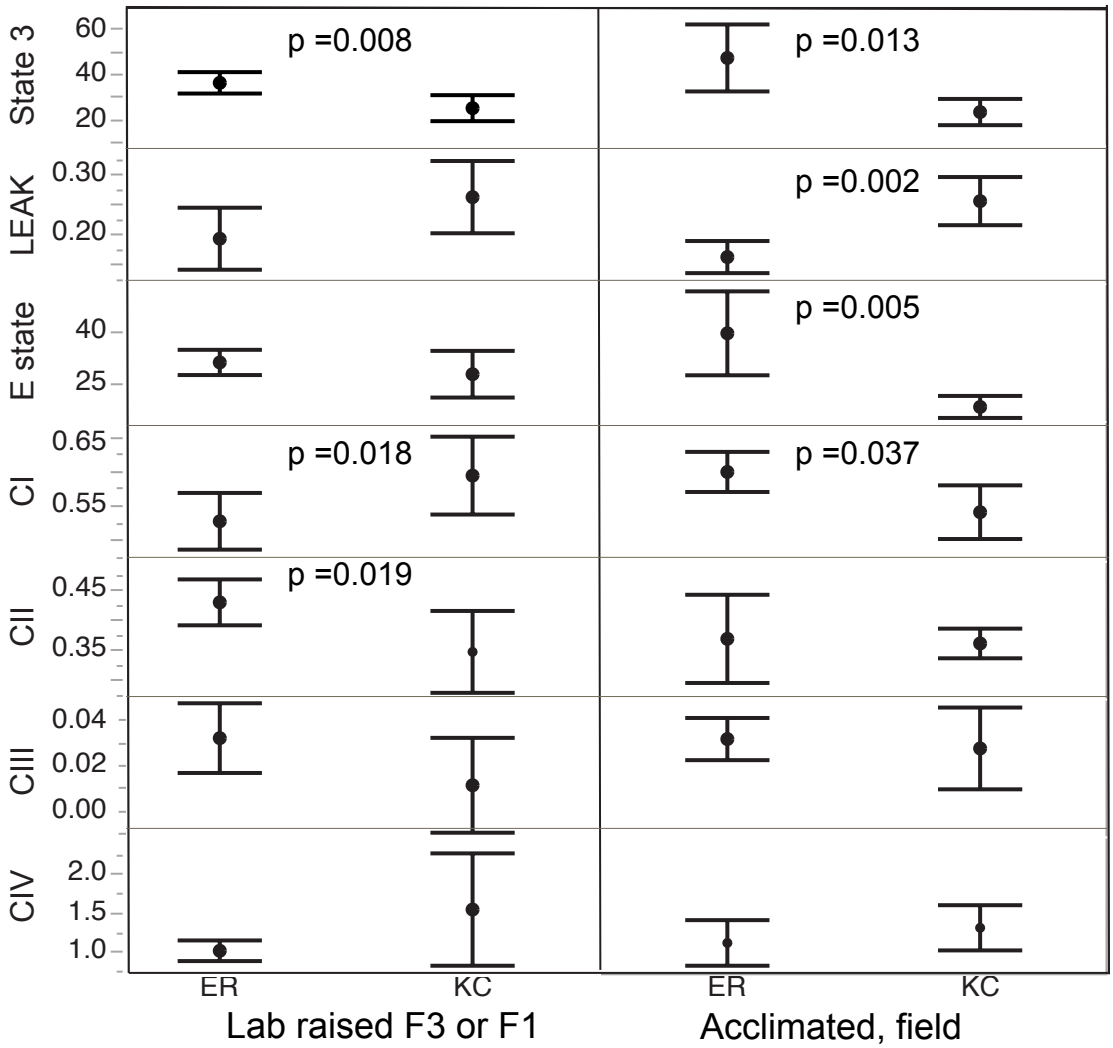


Figure 3.2 LEAK response to BaP exposure in polluted Elizabeth River *F. heteroclitus* and reference King's Creek *F. heteroclitus*. 95% confidence intervals (CI) for the means of LEAK was plotted for F3 Elizabeth River (ER), acclimated field-caught Elizabeth River, and acclimated field-caught King's Creek (KC) *F. heteroclitus*. LEAK was calculated as ratios of rates and thus is unitless. Dosage effects within each population separately were defined by one-way ANCOVA with body mass as covariate. Only significant p-value ($p < 0.05$) is marked.

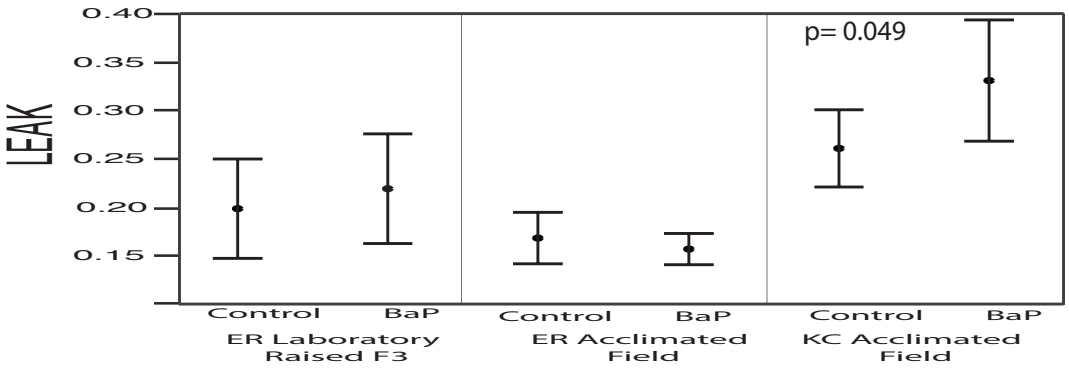
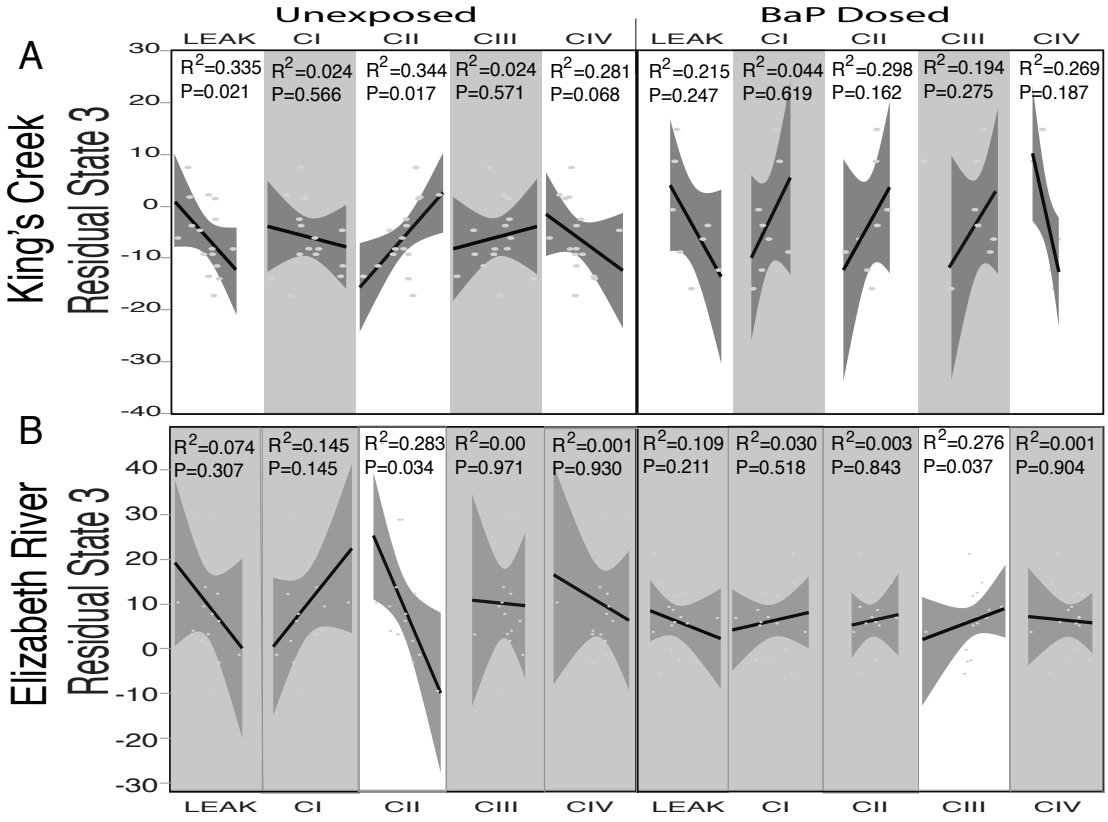


Figure 3.3 Regression analysis of residual State 3 *versus* residual LEAK and residual complexes. Residuals from body mass for OxPhos traits are used in the regression analyses. White regressions (versus grayed) have R2 > 0.2 (p <0.1 for all analyses except King's Creek, which has a smaller sample size).



Chapter 4 Altered mitochondrial energy metabolism in a PCB tolerant *Fundulus heteroclitus* population

4.1 Summary

Energy balance is a major concern for organisms developing pollution adaptation, as pollutant tolerance is bioenergetically costly. Yet, the bioenergetic consequences have not been well established for aquatic organisms chronically exposed to persistent organic pollutants (POPs). Exploring the link between energy metabolism, energy balance, and POP tolerance will facilitate the understanding of pollution adaptation. Therefore, I investigated mitochondrial energy metabolism in POP-tolerant killifish (*Fundulus heteroclitus)* from New Bedford Harbor by quantifying hepatocyte oxidative phosphorylation (OxPhos). Compared to reference individuals from a nearby population, the polluted New Bedford Harbor fish showed significantly higher respiratory control ratio (RCR), State 3 respiration (routine metabolism), proton LEAK, and complex IV activity, and lower complex II activity. The elevated RCR and routine respiration in New Bedford Harbor fish agrees with POP-tolerant population from Elizabeth River, VA in chapter 2, implying enhanced, adaptive mitochondrial metabolism in response to chronic pollution. The divergent changes in proton LEAK, complex II, and complex IV activity in New Bedford Harbor *versus* Elizabeth River populations suggest these natural populations' capacity to develop energy balance for stress tolerance in distinct ways. Similar to Elizabeth River fish, acute dosing with a model polycyclic aromatic hydrocarbon (PAH) or polychlorinated biphenyl (PCB) {benzo [a] pyrene (BaP) or PCB126 (3,3',4,4',5-pentachlorobiphenyl), respectively} did not affect New Bedford Harbor fish OxPhos function, indicating adaptive resistance to POP toxicity. Finally, no

significant OxPhos functional differences were found between undosed, field-collected New Bedford Harbor *F. heteroclitus* and undosed, laboratory-reared F3 generation fish, suggesting that OxPhos modulations in New Bedford Harbor *F. heteroclitus* are heritable.

4.2 Introductory Material

Combating pollutant toxicity is metabolically costly (Calow 1991), and this metabolic cost or energy balance needs to be considered to better understand how organisms tolerate and adapt to pollution. Focus on energy balance directly links physiological stress effects to organism fitness and thus facilitates predicting population consequences (Sokolova, Frederich et al. 2012). However, the bioenergetic consequences of pollutant toxicity in aquatic organisms have not been well established, especially for populations exposed to persistent organic pollutants (POPs). Although a variety of metal toxicity studies indicate that metal exposures can disrupt an organism's energy balance by increasing basal energy demand (*e.g.,* elevated cost of detoxification) (Calow 1991; Ivanina, Cherkasov et al. 2008), diminishing aerobic capacity (Sokolova and Lannig 2008), and even directly interfering with the ATP-producing pathways (Li, Xia et al. 2003; Sokolova, Sokolov et al. 2005), the limited studies investigating POP bioenergetic effects in aquatic populations have not revealed a general pattern (van Ginneken, Palstra et al. 2009; Marit and Weber 2012; Cannas, Atzori et al. 2013; Lucas, Bonnieux et al. 2016). While POPs have been reported to disrupt mitochondrial membrane potential, inhibit electron transfer, diminish ATP production, and elevate OxPhos uncoupling at the cellular level (Sivalingan, Yoshida et al. 1973; Li, Sioutas et al. 2002; Ko, Kim et al. 2004; Xia, Korge et al. 2004), the long-term bioenergetic response at the organism level has not been well established for natural populations with chronic POP exposure. Using

POP-adapted populations provides a good opportunity to explore the link between energy metabolism, energy balance, and POP tolerance, facilitating the understanding of the bioenergetic consequences of pollution adaptation.

One POP-adapted population of salt marsh minnow, *Fundulus heteroclitus,* inhabits New Bedford Harbor, MA, a site highly polluted with polychlorinated biphenyls (PCBs) (Nacci, Coiro et al. 1999; Nacci, Champlin et al. 2010). Within New Bedford Harbor, sediment PCB levels are as high as 22,666 ng/g dry weight due to past industrial discharge of PCBs (Weaver 1984; Nacci, Champlin et al. 2002). In contrast, Scorton Creek, a nearby site, which is often used as a reference site for New Bedford Harbor, has very low sediment PCB concentrations of 1 ng/g dry weight (Nacci, Champlin et al. 2010). *F. heteroclitus* from New Bedford Harbor exhibit much higher POP tolerance as compared to nearby reference individuals, and this tolerance is genetically inherited, supporting genetic adaptation (Nacci, Coiro et al. 1999; Nacci, Champlin et al. 2002; Nacci, Kohan et al. 2002). To investigate the link between toxicity tolerance and mitochondrial energy metabolism in this PCB-adapted population, I quantified oxidative phosphorylation (OxPhos).

The OxPhos pathway consists of five multi-subunit enzyme complexes imbedded in the inner mitochondrial membrane and is the main pathway for cellular ATP production (Hatefi 1985; Pagliarini and Rutter 2013). OxPhos enzyme complexes I, II, and III produce a proton gradient across the mitochondrial membrane by pumping protons into the mitochondrial intermembrane space (Hatefi 1985). This proton gradient powers ATP synthase to transform ADP to ATP *via* gradient dissipation (Hatefi 1985; Boyer 1997; Schultz and Chan 2001); at the same time, protons can also leak across the

inner membrane, relieving the proton gradient independently of ATP synthase. Therefore, the electron transfer and ATP synthesis processes are considered incompletely coupled and proton leakiness can affect OxPhos coupling efficiency (Divakaruni and Brand 2011). To gain a better understanding of the bioenergetic costs in the chronically polluted, New Bedford Harbor *F. heteroclitus* population, I quantified OxPhos in liver, the primary detoxification tissue.

4.3 Materials and Methods

4.3.1 Fish Collection

Fundulus heteroclitus were collected from New Bedford Harbor, MA (41°40'40"N, 70°54'58"W, [EPA ID MAD980731335]) and a nearby reference site, Scorton Creek, MA (41°43'21"N, 70°20'38"W), in October 2014 by the US Environmental Protection Agency (EPA), Office of Research and Development, Atlantic Ecology Division, Narragansett, RI, and shipped to University of Miami. Then fish were maintained in re-circulating aquatic system tanks for 4 weeks with controlled temperature (20º C) and salinity (15 ppt), to get rid of any possible physiological effects from local environment (*e.g.,* feeding, temperature fluctuation) and fish transportation (Sidell, Wilson et al. 1973; Pottinger and Pickering 1992).

Laboratory-raised fish used were a F3 generation of *F. heteroclitus* that were collected from New Bedford Harbor, MA (41°40'40"N, 70°54'58"W, [EPA ID MAD980731335]) and spawned in 2010. Fish were provided by the US Environmental Protection Agency (EPA), Office of Research and Development, Atlantic Ecology Division, Narragansett, RI, and shipped to University of Miami in April 2014.

Then fish were acclimated in re-circulating aquatic system tanks for another seven months with controlled temperature (20º C) and salinity (15 ppt).

The average *F. heteroclitus* weights (SD) were 8.36 (2.11), 8.50 (2.23), and 11.81 (2.75) grams for field-collected Scorton Creek, field-collected New Bedford Harbor, and laboratory-reared F3 generation of New Bedford Harbor, respectively. Considering the effectiveness and efficiency of exposure, I dosed fish via intraperitoneal (i.p.) injection. New Bedford Harbor and Scorton Creek fish were dosed *via* i.p. injection with either 50 mg/kg body weight (198.2 umol/kg) BaP or 10 mg/kg body weight (30.6 umol/kg) PCB126 dissolved in corn oil with an injection volume of 5 μL/g body weight for twenty-four hours, before OxPhos function measurements. Control or undosed groups from each population (Scorton Creek, field-collected and laboratory-reared F3 New Bedford Harbor) were injected with corn oil for 24 hours. Ten fish were treated in each treatment group. The BaP and PCB126 doses were the same doses applied in chapter 2 investigating another pollutant-adapted *F. heteroclitus* population from Elizabeth River, VA (Du, Crawford et al. 2015). In that study, these doses successfully induced detectable OxPhos changes in the clean-reference population but failed to exert an effect on the pollutant-adapted population. The generality of this pattern would be tested in this manuscript using the New Bedford Harbor population with the same dose.

4.3.2 Hepatocyte Isolation and Permeabilization

As described in greater detail in chapter 2, hepatocyte isolations were conducted based on an *in situ* trypsin perfusion technique (Bello, Franks et al. 2001), resulting in an average of 4×10^6 cells per liver. Half of the isolated hepatocyctes (~2×10^6 cells) were resuspended into Miro5 (respiration media: 0.5 mM EGTA, 3 mM MgCl2.6H2O, 60 mM

K-lactobionate, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, and 1 g/l BSA, pH 7.1 adjusted with 5N KOH) and permeabilized with digitonin (20-30 ug per million cells for 10 minutes) for OxPhos quantification (Du, Crawford et al. 2015). The other half was saved for future gene expression analysis.

4.3.3 OxPhos Protocol

OxPhos function was measured *via* high-resolution respirometry with the OROBOROS Oxygraph-2k (OROBOROS instrument, Austria). OxPhos traits measured at different steps of the OxPhos pathway were quantified by sequentially exposing permeabilized hepatocytes to specific substrates and inhibitors (Table 2.2) as described (Du, Crawford et al. 2015). OxPhos metabolism was quantified as mean respiration rates in pmol O_2 s^{-1} ml^{-1} per million cells. The most relevant traits include state 3 (routine respiration with substrates and ADP), LEAK (respiration limited by proton leakage back into the mitochondrial matrix), E state (maximum metabolism with the dissolution of the proton gradient), and complex enzymes' activities. Data visualization was realized by the software DatLab (OROBOROS instrument, Austria). OxPhos function was measured at 28°C, parallel to our previous study investigating the pollutant-adapted Elizabeth River population (Du, Crawford et al. 2015).

4.3.4 Statistical Analyses

Statistical analyses were performed in JMP Pro 12 (SAS, Cary NC). A t-test was conducted to compare undosed New Bedford Harbor and undosed Scorton Creek individuals to reveal OxPhos differences between polluted and clean populations. A two-way ANOVA (analysis of variance) was performed on dosed and undosed New Bedford Harbor and Scorton Creek OxPhos data to investigate the effect of population and dosing

on OxPhos function. To discover whether New Bedford Harbor and Scorton Creek populations respond differently upon pollutant exposure, a one-way ANOVA was conducted on each population separately comparing undosed, PAH dosed, and PCB dosed individuals. When an effect was found, a post hoc Tukey's HSD test was performed. To determine generation effect on OxPhos modulations in New Bedford Harbor *F. heteroclitus*, a t-test was performed to compare OxPhos functions in ten undosed New Bedford Harbor fish and ten undosed laboratory-reared F3 fish of New Bedford Harbor. For all above OxPhos analyses, residuals from regression with body mass were applied to get rid of potential body mass influences. For clarity, OxPhos trait plots used means and variances uncorrected for body mass. Statistical significance was defined at $P< 0.05$.

4.4 Results

4.4.1 Mitochondrial Integrity and Quality Control

The respiratory control ratio (RCR) is used as an index to evaluate functional mitochondrial integrity. It estimates respiration dependency on ADP (Table 2.2). RCRs from twenty-nine and twenty-eight individuals from the New Bedford Harbor and Scorton Creek populations respectively displayed well-coupled respiration (Table 4.1). Two-way ANOVA showed that population effect ($p = 0.009$) played a role on RCRs, but dosing ($p = 0.77$) or interaction ($p = 0.73$; for population x dosing) did not. Compared to the clean Scorton Creek population, the polluted New Bedford Harbor population displayed significantly higher RCRs (Table 4.1). To evaluate mitochondrial membrane intactness, oxygen consumption rates were compared before and after cytochrome c addition. The cytochrome c effect (cytochrome c / state 3) showed no change upon

cytochrome c addition (~ 1 for both populations; Table 4.1), supporting the functional integrity of outer mitochondrial membranes.

4.4.2 OxPhos Population and Dosing Effects

A simple t-test comparing ten undosed New Bedford Harbor and ten undosed Scorton Creek individuals revealed significant differences in proton LEAK (P = 0.050) and complex II activity (P = 0.019) between polluted and clean populations. Compared to the clean Scorton Creek population, the polluted New Bedford Harbor population displayed higher LEAK and lower complex II activity (Fig.2.1). To more comprehensively investigate OxPhos population and dosing effects, a two-way ANOVA was conducted on twenty-nine dosed and undosed New Bedford Harbor individuals and twenty-eight Scorton Creek individuals. Analyses of variance results are summarized in Table 4.2. Populations are the polluted and clean populations (New Bedford Harbor *versus* Scorton Creek). Dosing refers to PAH dosing and PCB dosing. For the oxygen fluxes measured at different OxPhos steps, population effect significantly influenced complex IV activity (P = 0.041) and dosing significantly affected proton LEAK (P = 0.030). Complex IV activity was higher in the polluted New Bedford Harbor population; dosing elevated LEAK in the clean Scorton Creek population (Fig.2.1). Population effect might also contribute to State 3 (P = 0.054) and complex II activity (0.086) variations. The polluted population had higher State 3 respiration (Fig.2.1). Interaction effect suggested that dosing might induce different responses on complex II activity (P = 0.058; POP x Dosing) in New Bedford Harbor and Scorton Creek populations.

To more specifically address whether polluted and clean populations responded to dosing in different patterns, a one-way ANOVA was conducted on each population

separately comparing undosed, PAH dosed, and PCB dosed OxPhos function. No OxPhos traits in the polluted New Bedford Harbor population were affected by either PAH or PCB dosing. On the contrary, in the clean Scorton Creek population, dosing significantly influenced LEAK ($P = 0.013$). Although both PAH and PCB dosing increased LEAK (Fig.4.1), the increase was significant only in PAH dosing based on post hoc Tukey's HSD test.

To sum up, there were significant differences in LEAK and complex II activity between the polluted New Bedford Harbor and clean Scorton Creek populations. Besides, population effect could also contribute to variations of State 3 respiration and complex IV activity. Neither PAH nor PCB dosing induced any detectable effects in the polluted New Bedford Harbor population. Yet, PAH dosing significantly elevated LEAK in the clean Scorton Creek population.

4.4.3 OxPhos Generation Effect

To determine if the observed OxPhos modulations in New Bedford Harbor *F. heteroclitus* were genetically based, OxPhos functions in ten undosed New Bedford Harbor fish were compared to OxPhos functions in ten undosed laboratory-reared F3 generation fish from New Bedford Harbor. A t-test was performed on each OxPhos trait separately, comparing generations. No significant differences were detected in any of the OxPhos traits ($P > 0.05$), indicating that OxPhos modulations due to chronic environmental pollutant exposure in New Bedford Harbor *F. heteroclitus* were heritable.

4.5 Discussion

It is well known that pollution can impair the fitness of natural populations in various ways, *e.g.,* triggering cancer (Shimada and Fujii-Kuriyama 2004; Srogi 2007),

causing genotoxicity (Rose, French et al. 2000; Jung, Matson et al. 2011), or inducing endocrine disruption (Sumpter and Johnson 2005). It is also well established that polluted populations can develop adaptation and resistance upon pollution exposure (Nacci, Coiro et al. 1999; Meyer and Di Giulio 2002; Ownby, Newman et al. 2002; Meyer and Di Giulio 2003). However, it is less well established how OxPhos, the fundamental pathway producing energy that supports other metabolic processes, is influenced by chronic pollution exposure. In our previous studies investigating another pollutant-adapted *F. heteroclitus* population from Elizabeth River, VA, a site highly contaminated with polycyclic aromatic hydrocarbons (PAHs), I observed changes in mitochondrial metabolic functions and resistance to BaP toxicity, and those patterns are persistent across generations, providing strong evidence for evolutionary adaptation in mitochondrial energy metabolism (Du, Crawford et al. 2015; Du, Crawford et al. 2016). The generality of those responses was tested in this study using an adapted population from PCB contaminated New Bedford Harbor.

In this study, I compared the OxPhos function between the polluted New Bedford Harbor and the clean Scorton's Creek populations and characterized their responses to PCB dosing to shed light on evolutionary adaptation in mitochondrial energy metabolism. Globally, I observed a population effect on respiratory control ratio (RCR), state 3 respiration, proton LEAK, and enzyme complexes II and IV activities. I also detected a resistance to acute pollutant toxicity in the polluted New Bedford Harbor population while the same dosing successfully triggered an upsurge in proton LEAK in the clean population. This evidence supports the idea that the PCB-adapted New Bedford Harbor

population has evolved OxPhos changes in mitochondrial function in response to chronic pollutant exposure.

Respiratory control ratio (RCR) is considered the best general measure of mitochondrial function in permealized cells; it represents the mitochondria's ability to respond to ADP by making ATP. RCR is controlled by a variety of factors including substrates oxidation, proton leakiness, and ATP turnover, and due to this complexity, any change in OxPhos would change RCR. Thus, RCR is one of the most important diagnostic features of mitochondria (Brand and Nicholls 2011). The polluted New Bedford Harbor population exhibited significantly higher RCRs as compared to the clean reference population ($p = 0.009$; Table 4.1). A higher RCR in the polluted population also has been reported in the PAH-adapted Elizabeth River population. The acclimated, field-caught Elizabeth River *F. heteroclitus* compared to reference, clean individuals displayed a significantly highly RCR, and this higher RCR was found to be heritable in the laboratory-reared, unexposed F3 generation fish, indicating that higher RCR was genetically inherited (Du, Crawford et al. 2016). A high RCR might indicate high substrate oxidation, high ATP turnover, or low proton LEAK. This suggested that the Elizabeth River POP-adapted population was more proficient in transforming ADP into ATP, which was indeed supported by observations of increased State 3 measurement.

State 3 or routine respiration is a measure of ATP production. Consistent with higher RCR, a higher State 3 respiration was detected in the polluted New Bedford Harbor population as compared to the clean population (Fig.4.1), and the two-way ANOVA demonstrated that population effect is the main source of variation in State 3 ($p = 0.054$, Table 4.2; Fig.4.1). This pattern of State 3 respiration agrees with measurements

in the polluted Elizabeth River population: compared to the reference population the polluted Elizabeth River population in both F3 and acclimated field-caught fish showed significantly higher State 3 respiration. The high State 3 measurements in both PAH and PCB polluted populations indicate that the POP-adapted populations have evolved enhanced OxPhos capacity in response to chronic exposure. This is of great importance for tolerance adaptation. The detoxification process is usually metabolically costly (Calow 1991). Limitations of energy acquisition lead to tradeoffs between stress tolerance and energy costs of fitness-related functions such as growth, development, and reproduction (Sokolova, Frederich et al. 2012). Therefore, I predict that the POP-polluted populations have evolved adaptive enhancement in OxPhos capacity to extend energy acquisition in order to successfully combat the toxicity while at the same time maintain regular fitness-related functions.

At the organism level, the balance is achieved by elevated respiration rates and cost for tolerance. At the cellular level, the equilibrium is primarily achieved by balancing ATP synthesis and proton LEAK. Both RCR and State 3 respiration can be potentially affected by LEAK. LEAK occurs because the OxPhos pathway is incompletely coupled and protons can leak across the inner mitochondrial membrane without ATP synthesis (Divakaruni and Brand 2011). POPs are lipophilic hydrocarbons, and thus they may affect mitochondria metabolism by potentially reacting with the mitochondrial membrane lipid bilayers, disrupting protein-lipid interactions and increasing proton leakiness (Sikkema, de Bont et al. 1995). Direct POP dosing has been linked to elevated proton leakiness or diminished mitochondrial coupling efficiency in previous studies (Sivalingan, Yoshida et al. 1973; Ko, Kim et al. 2004; Bonner 2006; Du,

Crawford et al. 2015). Similarly, in this manuscript acute POP dosing significantly increased LEAK in the clean, reference Scorton Creek population (Table 4.2; Fig.4.1). Furthermore, the polluted New Bedford Harbor population displayed higher proton LEAK than the reference Scorton Creek population (Fig.4.1), suggesting that chronic POP exposure toxicity targeted OxPhos proton leakiness. Yet, this pattern was different from observations in the PAH-polluted Elizabeth River population, which showed an adaptive lowered LEAK compared to its reference population. This discrepancy may indicate that although the PAH- and PCB- adapted populations share some convergent adaptive OxPhos changes in response to toxicity, *e.g.,* elevated RCR and enhanced State 3, they also have developed divergent strategies. Besides LEAK, complex II activity was lower and complex IV was higher in the polluted New Bedford Harbor population *versus* the reference population. These patterns are different from those in the Elizabeth River population. Those differences may arise from the fact that PAHs and PCBs target different proteins in the mitochondrial membranes or that populations in different areas have the potential to achieve tolerance in distinct ways. Although the polluted New Bedford Harbor fish had higher LEAK or lower coupling efficiency, they may compensate energy through other mechanisms, such as elevated RCR, enhanced OxPhos capacity, or increased complex IV activity.

Another important observation is that POP dosing did not induce any detectable OxPhos functional effects in the polluted New Bedford Harbor population, while POP dosing, specifically PAH dosing, significantly elevated LEAK in the reference Scorton Creek population (Table 4.2; Fig.4.1). The insensitivity in the New Bedford Harbor population suggests that these fish have evolved adaptive tolerance to POP induced

LEAK. In other words, although the New Bedford Harbor population has higher LEAK due to historically chronic PCB exposure, these fish, similar to the Elizabeth River fish, have developed higher tolerance to acute POP toxicity.

Finally, the OxPhos functional comparisons between undosed field-collected New Bedford Harbor *F. heteroclitus* and undosed laboratory-reared F3 generation fish revealed no significant differences. This highly indicates that OxPhos alterations due to chronic exposure to environmental pollutants are heritable and genetically based in New Bedford Harbor *F. heteroclitus*, supporting genetic adaptation. The same pattern of conserved OxPhos modulations across generations was reported in the PAH-adapted Elizabeth River population (Du, Crawford et al. 2016). This suggests that PAHs and PCBs, as different groups of pollutants, both contribute to genetic adaptation of OxPhos function by convergent adaptive OxPhos changes in response to toxicity, *e.g.*, elevated RCR and enhanced State 3, and also divergent changes such as proton LEAK and complex II and IV activities.

4.6 Conclusions

The consistent resistance to POP dosing in New Bedford Harbor and Elizabeth River populations implies evolved tolerance to POP toxicity in these POP-adapted populations. The consistently higher RCR and higher State 3 respiration in New Bedford Harbor fish *versus* Scorton Creek fish, similar to that seen in Elizabeth River fish *versus* King's Creek fish, indicate evolved OxPhos changes that are most likely due to pollution adaptation. The enhanced adaptive OxPhos changes could compensate for the extra energy need for toxicity tolerance. The divergent changes in coupling efficiency, complex II, and complex IV activity between New Bedford Harbor and Elizabeth River

populations may demonstrate each population's capacity to achieve energy balance and pollution tolerance in distinct ways. Finally, OxPhos observations from laboratory-reared F3 fish in both New Bedford Harbor and Elizabeth River population support genetic adaptation.

Table 4.1 Respiratory control ratio and cytochrome c effect in permeabilized hepatocytes of New Bedford Harbor and Scorton's Creek *Fundulus heteroclitus* (mean ± s.e.m.).

Respiratory control ratio and cytochrome c effect		
	New Bedford Harbor	Scorton's Creek
Respiratory Control Ratio	4.25 ± 0.19	3.50 ± 0.20
Cytochrome c effect	1.04 ± 0.02	1.02 ± 0.01

Table 4.2 Two-way ANOVA with Population (POP) and Dosing

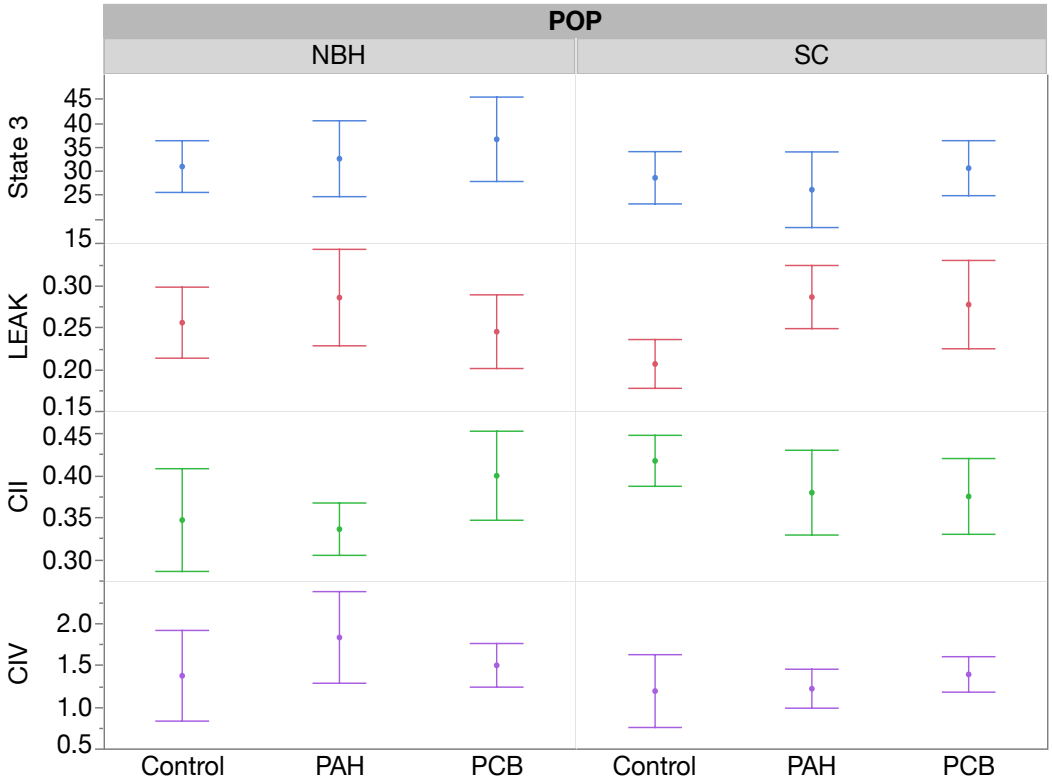
	Source	F Ratio	P values
State 3	POP	3.882	0.054*
	Dosing	1.392	0.258
	POP x Dosing	0.275	0.761
LEAK	POP	0.122	0.729
	Dosing	3.792	0.030**
	POP x Dosing	2.067	0.138
E state	POP	1.612	0.210
	Dosing	1.296	0.282
	POP x Dosing	0.395	0.676
CI	POP	0.125	0.725
	Dosing	0.014	0.987
	POP x Dosing	0.624	0.540
CII	POP	3.070	0.086*
	Dosing	1.032	0.364
	POP x Dosing	3.008	0.058*
CIII	POP	0.249	0.620
	Dosing	1.356	0.267
	POP x Dosing	0.860	0.429
CIV	POP	4.413	0.041**
	Dosing	0.971	0.386
	POP x Dosing	1.096	0.342

** $P < 0.05$

* $P < 0.1$

CI: Complex I; CII: Complex II; CIII: Complex III; CIV: Complex IV.

Figure 4.1 OxPhos comparison of undosed, PAH dosed, and PCB dosed New Bedford Harbor (NBH) and Scorton Creek (SC) *F. heteroclitus*. 95% confidence intervals (CI) for the means of the OxPhos traits were plotted. State 3 measurement was expressed as mean respiration rates in pmol O_2 s^{-1} ml^{-1} per 1×10^{6} cells. LEAK, complex II, and complex IV were calculated as ratios.



Chapter 5 Gene expression changes due to chronic and acute pollutant exposure and their link to mitochondrial energy metabolism

5.1 Summary

Populations of the teleost fish *Fundulus heteroclitus* inhabit and have adapted to highly polluted Superfund sites contaminated with persistent toxic chemicals. Chapter 2 and chapter 4 data suggests oxidative phosphorylation (OxPhos) function has evolved adaptive changes in response to chronic environmental contaminant exposure in two independent, polluted *F. heteroclitus* populations from New Bedford Harbor and Elizabeth River. To promote the understanding of OxPhos adaptation, gene expression on the same polluted fish that show OxPhos differences was measured. The goal was to discern gene expression alterations in polluted populations and link these alterations to OxPhos changes. I found approximately 3.4% of genes have potentially adaptive gene expression changes in these polluted fish. Genes with altered expression are enriched for functional clusters for stress responses and regulation of a variety of metabolic processes. Among these genes, five were shared in polluted New Bedford Harbor and Elizabeth River populations, which may indicate the presence of conserved responses to pollutant exposure. However, the fact that the majority of altered genes differ between polluted populations suggests that populations have evolved distinct strategies to cope with pollution. Genes that are significantly linked to OxPhos variations are involved in both a variety of energy-related metabolic processes and defense responses. These results suggest that pollution has a significant effect on mitochondrial energy metabolism by both directly modulating energy balance and indirectly elevating energy needs due to detoxification.

5.2 Introductory Material

It is well known that pollution can impair the fitness of natural populations in various ways (Rose, French et al. 2000; Sumpter and Johnson 2005; Jung, Matson et al. 2011). For instance, persistent organic pollutants (POPs), which are a major pollutant category of concern, are well known for their link to carcinogenicity and mutagenicity (Li, Loganath et al. 2006; Arnot, Armitage et al. 2011; Ruzzin 2012). Yet, it is also well established that populations exposed to environmental stress (*e.g.,* pollution) have the potential to adapt when stress remains constant over generations and traits that enhance successful individuals are inherited (Bijlsma and Loeschcke 2005). This appears to be the case with the saltmarsh minnow, *Fundulus heteroclitus* that inhabit and have adapted to highly polluted Superfund sites (Endler 1986; Nacci, Coiro et al. 1999; Meyer and Di Giulio 2002; Ownby, Newman et al. 2002; Meyer and Di Giulio 2003). These populations are exposed to some of the highest concentrations of aromatic hydrocarbon pollutants of any vertebrate species (Wirgin and Waldman 2004). Compelling evidence for adaptation in these populations include resistance to the acute toxicity (Nacci, Coiro et al. 1999; Ownby, Newman et al. 2002) and the cytochrome P4501A (CYP1A)-inducing activity of the sediments (Elskus, Monosson et al. 1999; Meyer and Di Giulio 2002). Additionally, both F1 and F2 generation embryos from Superfund sites at Elizabeth River and New Bedford Harbor are resistant to POP toxicity, suggesting that certain aspects of the resistance are genetically inherited (Nacci, Coiro et al. 1999; Clark, Bone et al. 2014). These populations also show potentially adaptive gene expression changes (Meyer, Nacci et al. 2002; Meyer, Wassenberg et al. 2003; Meyer, Volz et al. 2005; Oleksiak 2008).

Gene expression is often altered upon pollutant exposure and thus is considered a sensitive bioindicator of toxicity (Thomas, Rank et al. 2001; Hamadeh, Bushel et al. 2002). In a study concerning three POP contaminated *F. heteroclitus* populations, up to 17% of metabolic genes were found to have evolved adaptive gene expression changes (Fisher and Oleksiak 2007). Importantly, Oleksiak 2008 reported differentially expressed genes in polluted populations that are involved in the oxidative phosphorylation pathway, indicating pollution may potentially target energy metabolism in mitochondria (Oleksiak 2008).

Corresponding to gene expression observations, the oxidative phosphorylation pathway (OxPhos) within mitochondria have been proposed as a potential target of POP toxicity in a variety of physiological studies that report a link between POP exposure and OxPhos function deficiency, such as mitochondrial membrane potential loss, ATP production inhibition, and respiratory enzyme activity modification (Sivalingan, Yoshida et al. 1973; Zhu, Li et al. 1995; Li, Sioutas et al. 2002; Ko, Kim et al. 2004; Xia, Korge et al. 2004). Our published data quantifying the OxPhos function on the same set of fish measured in this manuscript, identified alterations in State 3 respiration (routine respiration), proton LEAK, and enzymatic complexes (Du, Crawford et al. 2015), indicating pollution's effect on fish mitochondrial energy metabolism.

To explore the link between, energy metabolism, gene expression, and pollutant toxicity, I measured gene expression in two independent, polluted *F. heteroclitus* populations from New Bedford Harbor and Elizabeth River, that have been chronically exposed to and adapted to extremely high POP concentrations (Nacci, Champlin et al. 2002; Vogelbein WK 2008; Clark, Cooper et al. 2013). The goal was to discern gene

expression alterations in polluted populations and link these alterations to OxPhos changes that were identified on the same set of fish (Du, Crawford et al. 2015). Quantification of gene expression will help us gain better understanding of how pollution affects energy metabolism.

5.3 Materials and Methods

5.3.1 Fish Collection

F. heteroclitus were collected from Elizabeth River, VA (36°48'26.20"N, 76°17'9.83"W, [EPA ID VAD990710410]) and a nearby reference site, King's Creek, VA (37°15'43.38"N, 76°29'4.57"W) by minnow traps in May 2013. *F. heteroclitus* also were collected from New Bedford Harbor, MA (41°40'40"N, 70°54'58"W, [EPA ID MAD980731335]) and a nearby reference site, Scorton Creek, MA (41°43'21"N, 70°20'38"W), in October 2014 by the US Environmental Protection Agency (EPA), Office of Research and Development, Atlantic Ecology Division, Narragansett, RI, and shipped to University of Miami. All fish were maintained in re-circulating aquatic system tanks for 4 weeks with controlled temperature (20º C) and salinity (15 ppt) before hepatocyte isolation and OxPhos function quantification, to get rid of any possible physiological effects from local environment (*e.g.,* feeding, temperature fluctuation) and fish transportation (Sidell, Wilson et al. 1973; Pottinger and Pickering 1992).

5.3.2 Fish Treatment and Hepatocyte Isolation

Four weeks after laboratory acclimation, fish were dosed via i.p. injection with either 50 mg/kg body weight (198.2 umol/kg) benzo [a] pyrene (BaP, a representative PAH) or 10 mg/kg body weight (30.6 umol/kg) 3,3',4,4',5-pentachlorobiphenyl (PCB126, a representative PCB) dissolved in corn oil with an injection volume of 5 µL/g

body weight for twenty-four hours, before OxPhos function quantification. Control or undosed groups for each population were injected with corn oil for 24 hours. Ten fish were treated in each treatment group. After 24 hours treatment, hepatocytes were isolated as described and half of the isolated hepatocyctes were used for measuring OxPhos function (Du, Crawford et al. 2015). The other half was saved for gene expression analysis.

5.3.3 RNA Isolation and cDNA Synthesis

mRNA from the subset of hepatocytes saved was isolated using a modified guanidinium thiocyanate buffer (Sacchi 2006). RNA quantity was determined by absorbance at 260nm; quality was checked by gel electrophoresis. To reduce the complexity of cDNAs from total RNA, I digested double stranded cDNAs synthesized with a biotin labelled NVdT primer. For each individual, these cDNAs were digested with AciI, Taq I, Hinp1 I, Hpa II, and HpyCH4 IV to produce a CG overhang and with CviQI 1, Mse I, and Xsp I to produce a TA overhang. The 3'-ends of the cDNAs were purified using streptavidin beads, and barcoded adaptors were ligated to these 3'-ends. Pooled individuals were sequenced using Illumina HiSeq technology. Transcripts with restriction enzyme recognition sites both too close to and too far from the polyA tail were missed.

5.3.4 Data Analysis

After sequencing, the sequencing adaptor and polyA tail were trimmed and low quality sequences removed. Next, sequences were sorted based on barcode and cut site. The barcode was removed, and the sequences aligned to the *F. heteroclitus* genome using Bowtie2 (Langmead 2012). BEDTools (IM 2010) was used to count the fragments

aligned to each locus. Count data was analyzed for differential expression using DESeq 2.0 (Love, Huber et al. 2014). DESeq 2.0 tests for differential expression that is based on a GLM model using the negative binomial distribution (Anders and Huber 2010; Anders, McCarthy et al. 2013) and is available as an R package. Initially, raw data counts were filtered to remove genes with less than 2 counts per individual. Then a multi-factor design concerning population effect, dosing effect, and their interaction (population x dosing) was performed in DESeq 2.0 on undosed, PAH and PCB dosed individuals from New Bedford Harbor *versus* Scorton Creek and from Elizabeth River *versus* King's Creek separately. Statistical significance was defined at a Benjamini-Hochberg adjusted p-value (BH adjusted p-values) < 0.05. Gene expression heatmaps and principal component plots were performed in DESeq 2.0. Principal component analysis on dosing affected genes, ANOVA test checking for significant principal components explaining dosing, and regression analysis against OxPhos were performed in JMP Pro 12 (SAS, Cary NC). Functional enrichment analysis was performed using GOrilla (Eden, Lipson et al. 2007).

5.4 Results

5.4.1 Differential Expression among Populations

To elucidate the effects of population, dosing, and their interaction (population x dosing) on gene expression, a multi-factor design in DESeq was performed on New Bedford Harbor *versus* Scorton Creek individuals and Elizabeth River *versus* King's Creek individuals separately (Table 5.1). Within the multi-factor design, population effect refers to the polluted *versus* reference population. Results show that the polluted populations have a similar number and similar percentage of differentially expressed

genes compared to their reference population. New Bedford Harbor population has 267 (or 3.38%) differentially expressed genes compared to Scorton Creek population at the adjusted p value of 0.05 (BH adjusted p-value). Elizabeth River has 223 (or 3.43%) genes that are differentially expressed from King's Creek. Yet, the up- and down-regulation patterns are slightly different. The percentage of down-regulated genes is ~1.7x higher in Elizabeth River (2.43%) compared to New Bedford Harbor (1.42%).

Functional enrichment analysis was performed on differentially expressed genes in polluted populations. Functional clusters for macromolecule biosynthetic process, aromatic compound biosynthetic process, response to stress, peptidyl-amino acid modification, regulation of cellular metabolic process, and regulation of biosynthetic process, are enriched in both polluted populations. Additionally, differentially expressed genes in New Bedford Harbor are also enriched for regulation of glucose metabolic process, signal transduction, regulation of GTPase activity, and regulation of cellular and biological processes.

Differentially expressed genes found in more than one polluted population may indicate shared solutions to deal with pollution or shared responses to stress (Oleksiak 2008). Overall, five genes, E3 ubiquitin-protein ligase HUWE1, F-box and WD repeat domain containing 12, fibulin-5, monocyte to macrophage differentiation-associated, and probable phospholipid-transporting ATPase IH, are significantly differentially expressed in both New Bedford Harbor and Elizabeth River population. E3 ubiquitin-protein ligase HUWE1, which mediates ubiquitination, regulates apoptosis, and ubiquitinates the p53/TP53 tumor suppressor gene, is down-regulated in both New Bedford Harbor and Elizabeth River population as compared to their respective reference populations. F-box

and WD repeat domain-containing 12 acts as a protein-ubiquitin ligase and is up-regulated in both polluted populations respectively. Yet, fibulin-5, monocyte to macrophage differentiation-associated, and probable phospholipid-transporting ATPase IH are less expressed in Elizabeth River as compared to King's Creek individuals, but more highly expressed in New Bedford Harbor as compared to Scorton Creek individuals. Fibulin-5 acts as s tumor suppressor in ovarian cancer by inhibiting the migration of cancer cells (Heo, Song et al. 2016). Monocyte to macrophage differentiation-associated modulates the tumor necrosis factor (TNF-α) production in macrophages (Liu, Zheng et al. 2012). Probable phospholipid-transporting ATPase IH plays a role in phospholipid translocation.

5.4.2 Gene Expression Modulation upon Dosing

Just as population effect reveals genes reflecting chronic pollution stress due to historical contamination, dosing effect identifies genes that are affected by acute PAH or PCB dosing (Table 5.1). In general, dosing affects a higher number (738 genes) and percentage (9.36%) of genes in the New Bedford Harbor *versus* Scorton Creek comparison; dosing affects 486 genes (7.47%) in the Elizabeth River *versus* King's Creek comparison. Genes differentially influenced by dosing in the polluted and reference populations are discerned by the interaction effect. Interaction results show that 598 genes and 391 genes display differential expressions upon acute dosing in polluted *versus* reference populations in New Bedford Harbor and Elizabeth River respectively.

Gene expression changes due to acute dosing are visualized by plotting the heatmaps of significantly PAH or PCB affected genes in each reference population (Fig.5.1). These figures show the presence of PAH- or PCB-specific gene expression

profiles in reference Scorton Creek and King's Creek populations. Analysis of this data reveals three important features: 1) approximately 70% of the affected genes are down-regulated or inhibited by PAH or PCB dosing, 2) the majority of the up-regulated genes in PAH dosing are also up-regulated in PCB dosing, and 3) these two patterns are consistent in the two reference populations.

Dosing induced gene expression differences are also visualized by principal component analysis. Principal component plots distinguish the PAH or PCB dosed groups from the undosed groups in the reference populations but not in the polluted populations (Fig.5.2). In Fig.5.2A, the first principal component separates the PCB dosed Scorton Creek individuals (turquoise) from the PAH dosed Scorton Creek individuals (dark blue), and the second principal component separates the undosed Scorton Creek individuals (pink) from PAH and PCB dosed Scorton Creek individuals. In Fig.5.2B, the first principal component supports a separation of undosed King's Creek individuals (pink) from PCB dosed King's Creek individuals (turquoise). Those principal component plots discriminate PAH or PCB groups in the clean reference populations, reinforcing the presence of PAH- or PCB-specific gene expression profiles.

5.4.3 Link to Mitochondrial Energy Metabolism

To investigate whether dosing affected genes explain OxPhos, I regressed statistically different principal components of gene expression against OxPhos traits that were influenced by either acute dosing or chronic contamination in our published data (Du, Crawford et al. 2015; Du, Crawford et al. 2016). Specifically, principal component analysis was performed on the 738 and 486 dosing significant genes (Table 5.1) in reference Scorton Creek and King's Creek individuals respectively. The top ten principal

components, which explain more than 50% of overall variation, were saved. A one-way ANOVA was performed on each principal component separately comparing undosed, PAH dosed, and PCB dosed groups to discern statistically significant principal components that were correlated with dosing. Finally, those significant principal components that explain gene expression variations upon dosing were regressed against exposure affected OxPhos traits from our previous results (Du, Crawford et al. 2015; Du, Crawford et al. 2016) to test for correlations. This will answer two important questions: 1) Do dosing influenced gene expression variations explain exposure affected OxPhos variations? 2) If they do, what genes are they?

For the reference King's Creek population, the first principal component of 486 dosing influenced genes is significant between treatments (undosed *versus* PAH/PCB dosed) by one-way ANOVA. Regression on thirty individuals from the King's Creek population shows that the variation in proton LEAK is explained by the variations in the first principal component (R^2, which is the percentage of variation explained by the dependent variable, where an $R^2 > 0.1$ has a p-value < 0.1, Fig.5.3A). Similarly, for the Scorton Creek population, the first, third, and sixth principal components of 738 dosing influenced genes differ significantly between treatments. When regressed to the OxPhos traits in twenty-eight individuals from the Scorton Creek population, the variations in proton LEAK and complex IV activity are related to the third principal component and the variation in State 3 respiration is correlated with the sixth principal component.

The top twenty genes explaining the largest possible variations in each of the above significant principal components are discerned by the eigenvectors and summarized in Table 5.2. A higher absolute value of the eigenvector indicates a higher

contribution to the variation. For the King's Creek population, given that the 1st principal component is significantly related to the OxPhos LEAK, the top twenty genes that explain the largest variations in the 1st principal component (Table 5.2) potentially clarify proton LEAK variations in the dosed King's Creek individuals. Similarly, for the Scorton Creek population, the top twenty genes describing the highest variations in the 3rd and 6th principal components (Table 5.2) help to interpret the variations in proton LEAK, complex II activity, and State 3 respiration respectively in the dosed Scorton Creek individuals. The top twenty genes that clarify variations in LEAK turn out to be different between Scorton Creek and King's Creek population. Additionally, the twenty genes explaining variations in different OxPhos traits within the same population (LEAK *versus* State 3 respiration in Scorton Creek) do not overlap either.

5.5 Discussion

The goal of this study was to better understand how natural populations tolerate and adapt to pollution. 3'-mRNASeq was used to characterize the gene expression patterns of *F. heteroclitus* populations inhabiting two distinct, highly POP contaminated sites, New Bedford Harbor and Elizabeth River, which have developed altered OxPhos function (Du, Crawford et al. 2015) as compared to relatively clean reference populations. Multiple-factor analysis of each site successfully differentiates genes with significant population effect (polluted *versus* reference) and captures genes significantly influenced by acute POP dosing. Additionally, regression analysis of the altered gene expression against the altered OxPhos function in the same *F. heteroclitus* individuals provides powerful statistical support for interpreting the phenotypic mitochondrial energy metabolism at the gene level. Finally, investigation of two polluted populations in

different locations helps to answer if populations with different contamination histories evolve convergent or divergent solutions to cope with stress.

The New Bedford Harbor population exhibits a similar percentage of differentially expressed genes (~3.4%) to the Elizabeth River population as compared to their respective reference populations. However, the proportion of down-regulated genes is ~1.7x higher in Elizabeth River (2.43%) compared to New Bedford Harbor (1.42%). This may reflect the differential biological consequences from different POPs. The Elizabeth River population is predominately contaminated with PAHs and metals while the New Bedford Harbor population is predominately contaminated with PCBs and metals. Another explanation is that these two polluted populations have responded differentially or evolved distinct strategies to chronic pollution exposure by differentially promoting or inhibiting a variety of genes. Indeed, this scenario is supported by the comparison of differentially expressed genes in New Bedford Harbor *versus* Elizabeth River. The majority of the 267 differentially expressed genes in New Bedford Harbor *versus* Scorton Creek populations do not overlap with the 223 differentially expressed genes in Elizabeth River *versus* King's Creek populations, although enrichment analysis suggests these genes play a role in similar biological processes. Actually, only five differentially expressed genes are shared between those two polluted populations. This pattern agrees with a previous study that used microarrays to investigate altered gene expressions due to chronic exposure, in which only eight differentially expressed genes are shared between the New Bedford Harbor and Elizabeth River populations (Oleksiak 2008).

Shared differentially expressed genes that are altered in the same direction in both the New Bedford Harbor and Elizabeth River populations suggest conserved responses or solutions to pollution. Two of the five shared, differentially expressed genes, E3 ubiquitin-protein ligase HUWE1 and F-box and WD repeat domain-containing 12 acts as protein-ubiquitin ligase, exhibit a conserved response: the former is less expressed and the latter is more highly expressed in both New Bedford Harbor and Elizabeth River populations as compared to their respective reference populations. The other three shared genes, fibulin-5, monocyte to macrophage differentiation-associated, and probable phospholipid-transporting ATPase IH, are altered in different directions in the New Bedford and Elizabeth River populations, which may indicate that those genes are more sensitive to stress or there are multiple ways to cope with pollution (Oleksiak 2008). Overall, the shared differentially expressed genes in polluted populations, either with a conserved response or high sensitivity to pollution, are mainly involved in biological processes from protein-ubiquitin, tumor suppression, to phospholipid translocation.

Gene expression profiles are altered during toxicity as either a direct or indirect result of toxicant exposure (Steinberg, Sturzenbaum et al. 2008). Acute POP dosing discerned PAH- or PCB-specific gene expression profiles in the reference Scorton Creek and King's Creek populations (Fig.5.1; Fig.5.2). Identifying genes modulated by direct (acute) POP dosing helps identify the metabolic processes or pathways that are affected by POP toxicity. Results show that genes affected by dosing are in involved in a variety of biological processes ranging from energy metabolism and protein regulation, to defense response to estrogens (Table 5.2). Specifically, phosphorylase kinase, gamma 1, putative adenosylhomocysteinase 3, and kelch-like protein 4 are responsible for protein

and glucose metabolism. Genes including OTU deubiquitinase 7A, coxsackie virus and adenovirus receptor and macrophage colony-stimulating factor 1 receptor 2 are involved in immune response. Additionally, DNA (cytosine-5-)-methyltransferase 3 alpha and programmed cell death protein 4-like play a role in apoptosis. Rho GTPase activating protein 35 is related with tumor suppressor. Featuring genes in response to toxic substances such as cytochrome P450 1A1 and cytochrome P450 2F2 are also affected in POP dosing. These observations agree with previous studies investigating POP dosing affected pathways, which report alterations in apoptosis (Staal, van Herwijnen et al. 2006), metabolic genes (Fisher and Oleksiak 2007; Oleksiak 2008), and cytochrome P450 activity (Ko, Kim et al. 2004).

Regression analysis of gene expression changes against OxPhos functional changes upon POP exposure discerns genes or pathways that are correlated or potentially contributing to OxPhos changes. The top twenty genes interpreting the highest variations of proton LEAK in the King's Creek population are mainly involved in biological processes including apoptosis, immune response, tumor suppressor, protein kinase activity, and aromatic compound responses (Table 5.2). For the Scorton Creek population, variations in proton LEAK and complex IV activity are significantly correlated with genes that play a role in protein biosynthesis and phosphorylation, immune response, cellular response to estrogen (DNA repair, CYP1A), transmembrane protein, and glucose homeostasis. Similarly, variation in State 3 or routine respiration is mainly governed by variations of genes involving in glycogen biosynthesis, cholesterol homeostasis, aromatic compound responses (DNA repair, CYP450 2F2), tumor suppressor, and G protein metabolism. Indeed, a variety of affected pathways in response to POP dosing such as

apoptosis, cholesterol, and lipid metabolism have been reported before (Staal, van Herwijnen et al. 2006). Yet, our results suggest significant genes involved in those pathways are correlated with mitochondrial energy metabolism. Although the genes interpreting LEAK variations are different between King's Creek and Scorton Creek populations, the major processes that those genes play a role in turn out to be the similar.

A striking group of the modulated genes that explain the largest possible variations in OxPhos function contains genes involved in energy storage and balance. Phosphorylase kinase, gamma 1 (PHKG1) encodes a crucial glycogenolytic regulatory enzyme that is fundamental for glycogen biosynthetic and catabolic processes. Low density lipoprotein receptor-related protein 5 (LRP5) plays a pivotal role in cholesterol homeostasis and lipid metabolism (Go and Mani 2012). Zinc finger and BTB domain containing 7C (ZBTB7C) contributes to positive regulation of fat cell differentiation (Jeon, Kim et al. 2012). 5-hydroxytryptamine receptor 2C is involved in glucose homeostasis (Giorgetti and Tecott 2004). Given that these genes are crucial in regulating glucose homeostasis and lipid metabolism, their link to OxPhos function suggests a vital role of energy balance in evaluating the bioenergetic consequences of pollutant toxicity. Interestingly, the idea that stress, *e.g.,* pollution, may have a significant effect on energy metabolism has been proposed in previous gene expression studies (Alexandre, Ansanay-Galeote et al. 2001; Oleksiak 2008). These studies suggest the polluted populations may have limited energy stores due to an extra energy cost due to coping with pollution (Oleksiak 2008). Our data support this hypothesis by illustrating that pollution may influence the energy stores by adjusting OxPhos metabolism to cover the extra energy cost due to copying with pollution. Indeed, enrichment analysis on differentially

expressed genes supports the link between energy metabolism and pollutant toxicity, by revealing functional clusters for regulation of cellular metabolic process and regulation of biosynthetic process. Additionally, differentially expressed genes involved in cholesterol biosynthesis due to chronic contamination or acute PAH dosing have also been reported in other studies (Staal, van Herwijnen et al. 2006; Oleksiak 2008), indicating that cholesterol metabolism may be targeted by POP toxicity.

Surprisingly, besides the altered gene expressions that may directly work on OxPhos function, *e.g.,* transmembrane proteins, glucose and cholesterol homeostasis, and protein regulation, a variety of genes that are crucial to organism survival upon exposure, including apoptosis, immune response, tumor suppressor, cellular response to estrogen (DNA repair), and other aromatic compound responses (CYP2F2), are significantly correlated with mitochondrial OxPhos. In other words, those fitness-crucial functions upon exposure potentially contribute to OxPhos function modulation. One possible explanation is that those defense functions are metabolically costly (Calow 1991) and thus indirectly affect OxPhos, the fundamental energy production pathway, to maintain their energy needs (*e.g.,* extra cost of detoxification). Energy balance is a major concern for organisms developing stress tolerance, as limitations of energy acquisition lead to tradeoffs between stress tolerance and energy costs of fitness-related functions such as growth and development (Sokolova, Frederich et al. 2012). The correlation between defense responses and OxPhos function reinforces the idea that energy balance may play a crucial role in pollution tolerance.

5.6 Conclusions

Shared, differentially expressed genes in polluted New Bedford Harbor and Elizabeth River populations may indicate the presence of conserved responses to pollutant exposure, while the fact that the majority of altered genes differ between polluted populations suggests that populations evolve distinct strategies to cope with pollution. Genes that are significantly linked to OxPhos variations are involved in both energy-related metabolic processes and defense responses. The most parsimonious explanation is that pollution has a significant effect on mitochondrial energy metabolism by both directly modulating energy balance and indirectly elevating energy needs due to detoxification.

Table 5.1 Multi-factor Analysis Result from DESeq 2.0

		Total	Up	Down	Total %	Up %	Down %
NBH *versus* SC	Population effect	267	155	112	3.38%	1.97%	1.42%
	Dosing effect	738	282	557	9.36%	3.58%	7.06%
	Population x Dosing	598			7.58%		
		Total	Up	Down	Total %	Up %	Down %
ER *versus* KC	Population effect	223	65	158	3.43%	1.00%	2.43%
	Dosing effect	486	169	388	7.47%	2.60%	5.96%
	Population x Dosing	391			6.01%		

* DESeq analysis Design = Population + Dosing + Population: Dosing
* DESeq results are calculated with BH adjusted p-values < 0.05.
* The reference population (KC or SC) is set as control in population effect.
* The undosed group is set as control in the dosing effect.
* Given that dosing effect includes both PAH and PCB dosing, the total number of significant genes with dosing effect are smaller than the sum of up- and down-regulated genes.

Table 5.2 Top twenty genes explaining the largest possible variations principal components

King's Creek	Scorton Creek	
1st Principal Component	**3rd Principal Component**	**6th Principal Component**
methyltransferase like 16	kin of IRRE like 2 (Drosophila)	nucleolar protein 4-like
heparan sulfate 2-O-sulfotransferase 1	dual specificity protein phosphatase 13 isoform B-like	DNA (cytosine-5-)-methyltransferase 3 alpha
synaptic vesicle glycoprotein 2A-like	coxsackie virus and adenovirus receptor	phosphorylase kinase, gamma 1
StAR-related lipid transfer (START) domain containing 13	polymerase (DNA directed) iota	ral guanine nucleotide dissociation stimulator-like 1
hemoglobin subunit alpha-A	cullin-associated and neddylation-dissociated 1	Rho GTPase activating protein 35
transcription factor MafB-like	ankyrin repeat, SAM and basic leucine zipper domain containing 1	low density lipoprotein receptor-related protein 5
mitogen-activated protein kinase kinase kinase 12-like	fibroblast growth factor receptor 1-A-like	cytochrome P450 2F2-like
CD209 antigen-like protein C	kelch-like protein 4	protein phosphatase 2, regulatory subunit B, alpha
OTU deubiquitinase 7A	band 4.1-like protein 1	putative adenosylhomocysteinase 3
programmed cell death protein 4-like	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	macrophage colony-stimulating factor 1 receptor 2
FYVE, RhoGEF and PH domain-containing protein 6-like	retinoic acid receptor alpha	epsin-1-like
aminopeptidase N-like	transmembrane protein 211-like	guanine nucleotide binding protein (G protein)
MAD1 mitotic arrest deficient-like 1 (yeast)	autism susceptibility gene 2 protein-like	calcium channel, voltage-dependent, L type, alpha 1F subunit
eva-1 homolog A (C. elegans)	SUN domain containing ossification factor	X-ray repair complementing defective repair in hamster cells 5 (double-strand-break rejoining)
chromodomain helicase DNA binding protein 5	cystathionine beta-synthase-like	zinc finger and BTB domain containing 7C
GRB10 interacting GYF protein 2	HECT, UBA and WWE domain containing 1	gamma-aminobutyric acid (GABA) A receptor
dynein beta chain, ciliary-like	mitogen-activated protein kinase kinase 2	tetraspanin-15-like
phosphatidylinositol 5-phosphate 4-kinase type-2 gamma-like	polycystic kidney disease 2 (autosomal dominant)	ribonuclease P/MRP 30kDa subunit
ubiquitination factor E4A	5-hydroxytryptamine receptor 2C-like	glutamate receptor, metabotropic 2
SNF related kinase	cytochrome P450 1A1	nuclear factor, erythroid 2-like 1

Figure 5.1 Heatmaps showing the expression data of genes significantly affected by dosing in reference populations. The data is of log2 normalized counts. Each row represents a single gene; each column stands for an individual. Colors refer to different gene expression levels: red represents high expression and dark blue represents low expression. (A) 290 genes affected by PAH dosing in Scorton Creek population. (B) 356 genes affected by PCB dosing in Scorton Creek population. (C) 168 genes affected by PAH dosing in King's Creek population. (D) 213 genes affected by PCB dosing in King's Creek population.

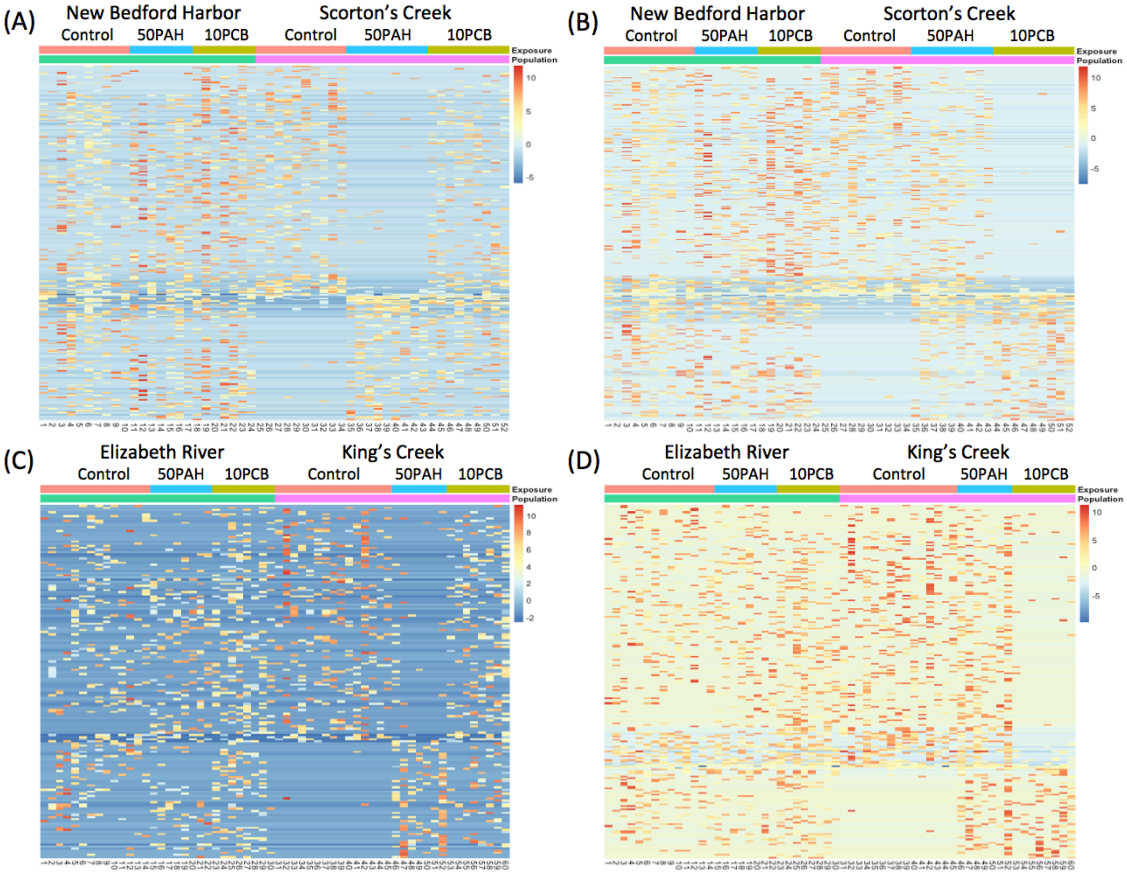


Figure 5.2 Principal component plot of dosing influenced genes in reference populations. (A) PCA plot of dosing influnced genes in Scorton Creek population. (B) PCA plot of dosing influenced genes in King's Creek population.

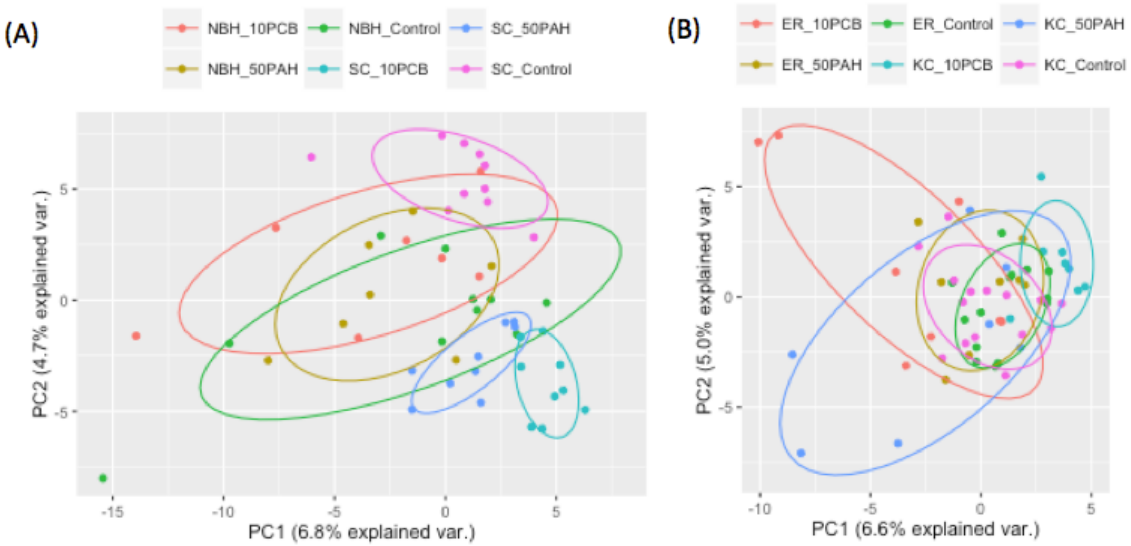
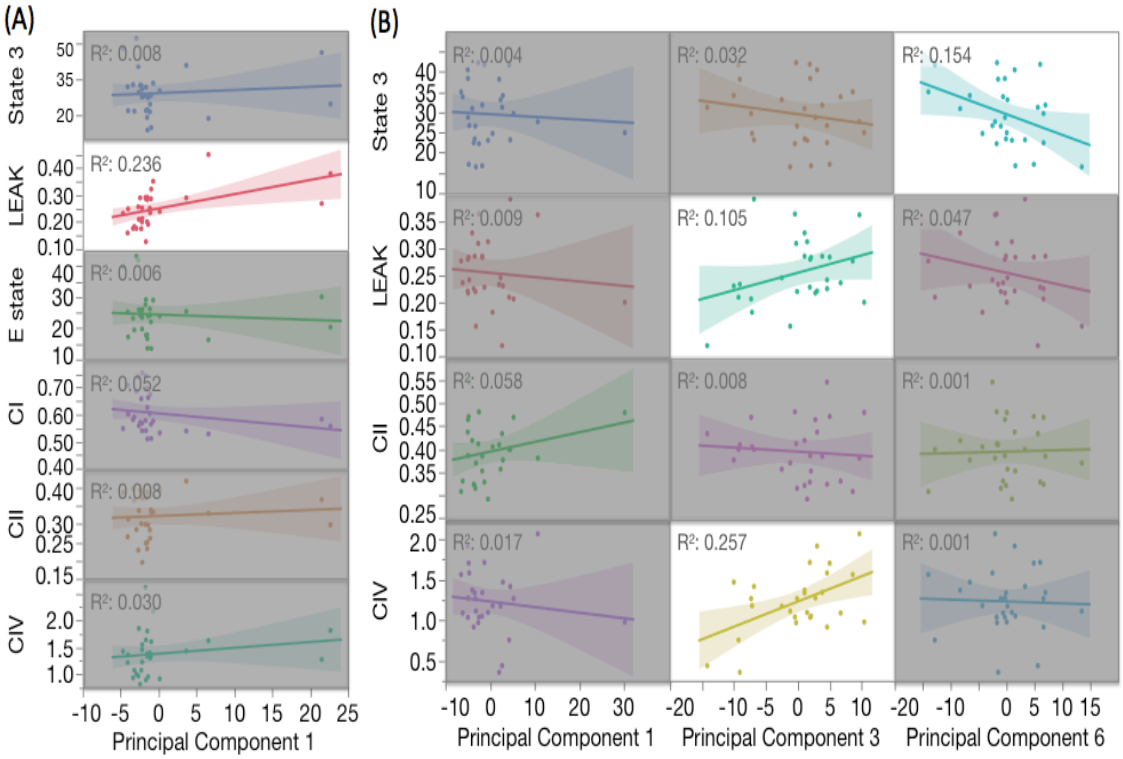


Figure 5.3 Regression analysis of principal components *versus* oxidative phosphorylation traits. White regressions (*versus* grayed) have R2 >0.1 (p <0.1). (A) King's creek population. (B) Scorton Creek population.



Chapter 6 A genotyping by sequencing study of *Fundulus heteroclitus* populations inhabiting a strong pollution cline in New Bedford Harbor

6.1 Summary

Chemical contaminations have the potential to affect natural populations' genetic structure, and some populations can adapt to chronic, chemical pollutant exposure. To better understand the genetic effects of chronic pollution and potential mechanisms underlying pollution resistance in natural populations, I examined the genetic variation of thousands of markers derived from genotyping-by-sequencing in *Fundulus heteroclitus* populations inhabiting a strong pollution cline in New Bedford Harbor, MA, USA. Outlier loci displaying high genetic variation successfully discerned a population genetic structure that parallels the geographic pollution cline. A direct test of correlation between outliers and environmental contamination was conducted in an environmental association study. Gene annotation revealed that these contamination-correlated outliers are functionally involved in diverse diseases, immune system response, and a variety of metabolic functions. Overall, these results suggest that identified outliers are most parsimoniously described as adaptive, and functionality of selected outliers supports adaptation.

6.2 Introductory Material

How populations adapt to a changing environment is a fundamental question in evolutionary biology and ecology, and signatures of adaptation can be identified using genome-wide scans of DNA polymorphisms to detect locus-specific signatures of positive selection (Nielsen 2005). Thus, candidate loci can be revealed by empirical tests to compare levels of variation to the genomic background (Beaumont and Nichols 1996;

Kelley, Madeoy et al. 2006). In this way, loci exceeding the expected differentiation will be identified as candidate outliers (Antao, Lopes et al. 2008; Excoffier, Hofer et al. 2009). In ecological studies, candidate outliers are of concern. On the one hand, the presence of outliers within the genome usually indicates that divergence is going on and that selection may be working on populations. On the other hand, outliers underlie biologically important variation. Positive selection shapes population genetic structure by working at the level of phenotypes; therefore, loci detected as outliers are likely to be functionally important (Vitti, Grossman et al. 2013).

An alternative approach to detect signatures of adaptation is to identify loci showing high correlation with environmental factors (Hancock, Witonsky et al. 2008; Coop, Witonsky et al. 2010; Frichot, Schoville et al. 2013). Genome-wide ecological association studies specifically test the link between allele frequencies and an environmental variable. As well as detecting selected loci, the environmental variable that plays the major role in selection can also be discerned. Such environmental factors encompass ecological variables such as salinity and temperature (Berg, Jentoft et al. 2015) and subsistence variables such as foraging and diet composition (Hancock, Witonsky et al. 2010). Spatial variation of these environmental variables contributes to spatially varying selection (Frichot and François 2015).

The strong pollution cline in New Bedford Harbor, MA, USA is a good ecological variable to investigate spatial selection. New Bedford Harbor (NBH), MA is a federal Superfund site heavily contaminated with polychlorinated biphenyls (PCBs) (Pruell, Norwood et al. 1990; Lake, McKinney et al. 1995) due to industrial discharge into the upper harbor, which has left a lasting impact (Weaver 1984; Nelson WG 1996). Within

the upper harbor, sediment PCB levels are as high as 22,666 ng/g dry weight at the pollution source and drop dramatically to 13 ng/g dry weight at the base of the harbor, creating a strong pollution cline (Nacci, Champlin et al. 2002). These PCBs are highly toxic, especially to the early development of many fish species (Walker and Peterson 1991; Grimwood and Dobbs 1995). Surprisingly, despite this high PCB contamination, the estuarine minnow, *Fundulus heteroclitus,* is abundant in NBH. The persistence of the non-dispersive *F. heteroclitus* population inhabiting this highly contaminated site strongly suggests genetic adaptation (Endler 1986; Burnett, Bain et al. 2007), and *F. heteroclitus* from NBH exhibit much higher toxic tolerance as compared to nearby reference individuals (Nacci, Coiro et al. 1999; Nacci, Champlin et al. 2002; Nacci, Kohan et al. 2002). Thus, the spatially distributed PCBs represent an intense selective pressure on the *F. heteroclitus* inhabiting the harbor and facilitate an ecological association study to investigate genomic regions selected by PCBs.

To investigate spatial selection in a natural population inhabiting a strong pollution cline, I examined the genetic variation of thousands of markers derived from genotyping-by-sequencing (Elshire, Glaubitz et al. 2011) in *F. heteroclitus* populations inhabiting NBH. I used both empirical tests to compare levels of variation to empirically neutral models of genomic background and tests to identify loci showing high correlation with environmental factors.

6.3 Materials and Methods

6.3.1 Sample Collection

Fundulus heteroclitus were collected from six populations from New Bedford Harbor, Massachusetts in the summer of 2013 using minnow traps. Five polluted

populations were from the PCB cline within the harbor and one clean population, m, was outside of the harbor (Table 6.1, Fig.6.1). Population nbh was collected near the original pollution source in the upper harbor, while population h was from the base of the harbor, which had the lowest PCB concentration Nacci et al. (2002). Because Mattapoisett (population m) is more distant from NBH than Hacker Street (population h, Table 6.1, Fig.6.1) and fish from population m displayed much lower resistance to PCB 126 than population h fish (Nacci, Champlin et al. 2002), population m was considered as the clean, reference population in our study.

6.3.2 GBS Library Preparation

Fin clips approximately $10mm^2$ in size were collected from 181 individuals (~30 per population) in the field and stored at 4°C in 270 ul of Chaos buffer (4.5M guanadinium thiocynate, 2% N-lauroylsarcosine, 50mM EDTA, 25mM Tris-HCl pH 7.5, 0.2% antifoam, 0.1M β-mercaptoethanol) prior to processing. Fish were released after being fin-clipped. Genomic DNA was isolated from these fin clips using silica columns (Ivanova, Dewaard et al. 2006). DNA quality was assessed *via* gel electrophoresis, and concentrations were quantified using Biotium AccuBlueTM Broad Range dsDNA Quantitative Solution according to manufacturer's instructions.

Genomic DNAs were prepared for library construction as described (Elshire, Glaubitz et al. 2011). Briefly, the complexity of the genomic DNA was reduced with a restriction enzyme *AseI* digest. Barcoded adaptors (0.4 pmol) were ligated to 50ng of gDNA per sample, and pooled individuals were sequenced on two Illumina HiSeq 2500 lanes with a 100bp single end read (Elim Biopharmaceuticals, Inc.). The GBS analysis

pipeline, TASSEL (Bradbury, Zhang et al. 2007), was used to map the sequences to the *F. heteroclitus* genome and call SNPs from the sequenced GBS library using Bowtie2.

6.3.3 Population Genetics and Outlier Detection

The SNP dataset from the Tassel pipeline was filtered through the TASSEL GUI for quality control purposes: loci with low minor allele frequency (<5%), loci with low coverage, and individuals with low coverage were removed. Additionally, Hardy-Weinberg equilibrium tests were conducted using Arlequin v3.5.1 to remove loci exceeding the expected heterozygosity ($p < 0.01$). All subsequent analyses used this filtered SNP dataset.

First, I calculated SNP allele frequencies and checked whether any allele frequencies corresponded to the pollution cline. Linear regression was conducted to detect significant correlations between SNP allele frequencies and PCB sediment concentrations.

Outlier detection was conducted using the FDIST2 approach (Beaumont and Nichols 1996) with LOSITAN (Antao, Lopes et al. 2008) and a hierarchical model with ARLEQUIN v.3.5 (Excoffier and Heckel 2006). LOSITAN permutated the data based on F_{ST} values in relation to individual SNP heterozygosities and identified outliers by comparing to the empirically neutral distribution. Using LOSITAN, I made pairwise comparisons between each of the populations collected within the harbor and the reference population m sequentially. I conducted 500k simulations for all pairwise comparisons and an adjusted P-value provided by LOSITAN was corrected with 1% FDR for defining statistical significance. ARLEQUIN analyses used a hierarchical model, in which demes exchange more migrants within groups than between groups (Antao, Lopes

et al. 2008). Using this model, populations were grouped based on the magnitude of geographic PCB concentrations and outliers were detected by permutation among the new groups.

Population genetic structure was inferred using STRUCTURE (Falush, Stephens et al. 2003) and DAPC (Jombart, Devillard et al. 2010). STRUCTURE analysis was run on outlier SNPs and neutral SNPs separately among six populations. I examined the number of genetic clusters K from 1 to 7, and for each number of K, I ran the simulation 5 times with a burn-in of 10k iterations and MKMC of 20k iterations. The Evanno method (Evanno, Regnaut et al. 2005) defining deltaK was used for inferring the best number of genetic clusters. Different from the Bayesian clustering method STRUCTURE, DAPC is a multivariate approach for discerning genetic clusters among populations. I applied outlier SNPs in DAPC analysis.

6.3.4 Environmental Association

Correlations between allele frequencies and the environmental factor, sediment PCB concentrations, were tested with BAYENV 2.0 (Coop, Witonsky et al. 2010; Günther and Coop 2013) using the Bayesian method and a latent factor mixed model (LFMM) algorithm implemented in LEA (Frichot, Schoville et al. 2013). BAYENV 2.0 first uses control SNPs to estimate a reference covariance matrix (null model) of how allele frequencies covary across populations. Second, it tests whether the correlation between allele frequencies at each SNP and an environmental variable is greater than expected given the covariance matrix (Coop, Witonsky et al. 2010). The statistic resulting is a Bayes factor (BF) for each SNP, which is calculated based on the ratio of the posterior probabilities between the two models. Data from the control SNPs was used to

provide an empirical null distribution of Bayes factors. The Bayes factor for the candidate SNPs was compared to the null distribution to judge its significance. A higher percentage of outliers in the tail of the BF distribution than the null model suggests the tail contains targets of positive selection (Hancock, Witonsky et al. 2010). A high BF supports that the environmental variable has an effect on the corresponding SNP.

In contrast to BAYENV 2.0, the latent factor mixed models (LFMMs) in LEA do not require a set of selectively neutral, unlinked loci from the genomic background as a prior. The LFMMs estimate the correlations between environmental and genetic variation while simultaneously inferring latent factors that reflect background population structure (Frichot, Schoville et al. 2013). The snmf function in LEA was used to estimate the optimal individual admixture coefficient from the genotypic matrix and to define the range of the number of latent factors to explore in lfmm analysis. A genomic inflation factor along with the distribution of adjusted P-values was used for inferring the best number of latent factors in lfmm for testing association between SNPs and the PCB concentration gradient.

6.3.5 SNP Annotation

To identify the function of outlier SNPs, I used BLAST to align the 64bp sequence tags of these SNPs from GBS against the *F. heteroclitus* genome (Altschul, Madden et al. 1997). Only matches with E values less than 1oE-5 were accepted. Functional enrichment analysis was performed using DAVID 6.7 (Huang, Sherman et al. 2008).

6.4 Results

6.4.1 SNP Discovery and Filtering

Genomic DNAs isolated from 181 individuals from 6 populations along a PCB cline were individually barcoded and used to create a reduced representation library for GBS (Elshire, Glaubitz et al. 2011). The GBS, TASSEL pipeline (Bradbury, Zhang et al. 2007) called 370,074 SNPs in the sequencing data by referring to the *Fundulus heteroclitus* genome. These 370,074 SNPs were covered by 120 million reads, with an average read depth of 300 reads/SNP. Then SNPs were filtered using Tassel 4 to remove individuals missing more than 40% of SNPs, SNPs occurring in less than 20% of individuals, SNPs with minor allele frequency < 5%, and SNPs for which Hardy-Weinberg equilibrium exceeded the expected heterozygosity. After filtering, 3,063 SNPs with an average read depth of 4,300 reads/SNP, and an average of 21 reads/SNP/individual were retained in the remaining 154 individuals for our data analysis. Sample size per location ranges from 20 to 30.

6.4.2 Major Allele Frequencies

The major SNP allele is defined by the allele with a frequency > 0.05 across all populations. First, I calculated the major allele frequencies of the 3,063 SNPs for each population and checked if any allele patterns exist among those populations on the pollution cline. As pollution concentration drops dramatically from site nbh to h on the cline, 29 SNPs display a consistent increases and 21 SNPs display a consistent decreases in major allele frequencies (Fig.6.2 a, b). Similarly, when considering m as the endpoint of the pollution cline 27 SNPs consistently increase and 37 SNPs consistently decrease in

allele frequency (Fig.6.2 c, d). I consider sites h and m separately because their PCB concentrations are very similar, and both are close to 0.

To statistically test the relationships between allele frequency and PCB pollution, I regressed the major allele frequencies of the 3,063 SNPs against the sediment PCB concentrations. Sixty-one SNPs showed positive regressions against PCB concentrations ($p < 0.05$, Fig.6.3a) and the average major allele frequencies for these SNPs ranged from 0.766 for m and 0.882 for nbh (Table 6.2A). In contrast, 105 SNPs showed negative regressions against sediment PCB concentrations ($p < 0.05$, Fig.6.3b), and the average major allele frequencies ranged from 0.699 to 0.855 (Table 6.2B). These significant regressions between alleles and PCB concentrations along with the consistent allele frequency patterns along the pollution cline indicate that frequencies of some alleles may be correlated with pollution. Pollution may favor or inhibit their frequency in the populations' genetic structure.

6.4.3 Outlier Detection

To identify the candidate loci that are potentially evolving by natural selection (*e.g.,* pollution), I conducted outlier tests using a F_{st}-outlier method based workbench, LOSITAN to infer molecular adaptation (Beaumont and Nichols 1996; Antao, Lopes et al. 2008). An empirical neutral F_{ST} value *versus* H_e (expected heterozygosity) distribution is used by LOSITAN to identify outlier loci that have unusually high or low F_{ST} values. The clean reference population m was sequentially compared to populations nbh, syc, p, f, and h on the pollution cline separately using LOSITAN. Each pairwise comparison was run in triplicate and I adjusted the p-values from LOSITAN with a conservative FDR of 1% (REF) to control for multiple comparisons. In the pairwise comparison of nbh (the

most polluted population) *vs*. reference population m, I found 224 SNPs identified as significant outliers by LOSITAN ($p < 0.01$), and with a 1% FDR correction, 155 of them were significant (Fig.6.4a). Similarly, with 1% FDR I identified 230 outlier SNPs in syc *vs.* m, 119 outlier SNPs in p *vs.* m, 104 outlier SNPs in f *vs.* m, and 113 outlier SNPs in h *vs.* m respectively (Fig.6.4b-e). The number of significant outlier with 1% FDR ranged from 3.3% to 7.5% of the 3,063 SNPs among the pairwise comparisons. In total, I have 531 outlier SNPs among all the pairwise comparisons to m. The mean F_{ST} value for those outlier SNPs is 0.086 with a maximum F_{ST} value of 0.56 (Fig.6.4). 32% of the outlier SNPs have F_{ST} values larger than 0.1, relative to 0.8% in the non-significant neutral SNPs. The mean F_{ST} value for non-significant neutral SNPs is 0.002.

Detecting loci under selection from F-statistics was performed with ARLEQUIN v.3.5 using a hierarchical model on the 3,063 SNPs. The hierarchical model was built based on the PCB concentrations along the cline. Population p and f have the same magnitude of PCBs thus I treated them as a single population. Population h and m were treated as different because individuals from h are more resistant to PCBs than those from m (Nacci, Champlin et al. 2002). Therefore, I ended up with 5 groups in the hierarchical model: nbh, syc, (p +f), h, and m. This hierarchical model identified 184 SNPs as selected loci by pollution with Fct p-value < 0.01. Of these, 66 SNPs (36%) were also detected as outliers in the LOSITAN analysis.

6.4.4 Population Genetic Structure

Genetic population structure was defined using a model-based Bayesian clustering approach, STRUCTURE (Pritchard, Stephens et al. 2000; Falush, Stephens et al. 2003), and a multivariate method, discriminant analysis of principal components

(DAPC) (Jombart, Devillard et al. 2010). STRUCTURE utilized 155 outliers from LOSITAN, nbh vs. m at a range of putative population clusters (K= 1 − 6), and the Evanno method (Evanno, Regnaut et al. 2005) finally found the best K was 2 as indicated by DeltaK (Fig.6.5a).

Using K =2 grouped the six populations into 2 genetic clusters (red and green, Fig.6.5c). One genetic cluster dominated the most polluted nbh and the other cluster dominated the reference population m. The other populations showed admixtures of both genetic clusters. The relative contribution of the green genetic cluster in each population corresponds to the PCB concentration along the pollution cline (Fig.6.5c; Table 6.1). STRUCTURE utilizing the union of 531 outliers from LOSITAN also grouped those individuals into 2 genetic clusters (Fig.6.5b). While all individuals were admixtures of both clusters, the reference population m was overwhelmed by the green cluster and remarkably different from the other polluted populations (Fig.6.5d). Different from that, STRUCTURE applying 1,000 neutral SNPs showed two clusters were evenly distributed in all populations; thus no population structure could be identified with neutral loci (Fig.6.5e).

Inference of population genetic structure was also realized using a multivariate method, DAPC. DAPC partitions genetic variation into an among-group and a within-group component, yielding principal components of genetic variations that attempt to summarize the among-group differentiation while overlooking the within-group differentiation (Jombart, Devillard et al. 2010). Using the 155 LOSITAN outlier SNPs from nbh *vs*. m, DAPC showed that the first discriminant function explained the majority of the variation (indicated by eigenvalue) and separated the most polluted nbh from the

reference m (Fig.6.6a). More interestingly, the six populations were distributed along the major axis in a pattern consistent with the PCB concentrations along the cline. The second discriminant function separated reference population m from other polluted populations. DAPC analysis utilizing the union of the 531 LOSITAN outliers revealed a similar genetic structure pattern (Fig.6.6b). The first discriminant function supported a separation between the reference population m and the other polluted populations. The second discriminant function revealed a population structure cline reflecting the geographic PCB cline.

6.4.5 Environmental Association

6.4.5.1 Bayenv 2.0

In contrast to the outlier tests (LOSITAN & ARLEQUIN), BAYENV 2.0 and LEA specifically test the correlation between SNPs and environmental PCB concentrations. These methods correct for demography and provide more direct evidence of PCBs' effects on population genetic structure.

To implement BAYENV 2.0, I first estimated the covariance matrix (null model) using 588 neutral, unlinked, control SNPs. The set of neutral, unlinked control SNPs was built by excluding any SNPs inferred as outliers using LOSITAN or Arelquin above and any SNPs linked to the outlier SNPs. BAYENV 2.0 was run for 500,000 Markov chain Monte Carlo (MCMC) iterations and 1,000 covariance matrices were made (output every 500^{th} iteration). Considering variation across the draws and the convergence of MCMC in the posterior stage, the reference covariance matrix was created using an average of the final 200 covariance matrices. Repeating the process produced very similar results. Next, all 3,063 SNPs were tested for correlation with sediment PCB concentrations using

200,000 MCMC iterations. To control for the run-to-run variability of the BAYENV algorithm (Blair, Granka et al. 2014), 20 independent runs were carried out. For each SNP, the median log10 Bayes factor from 20 runs was taken as its final log10 (BF) for environmental PCB concentrations.

I compared the distributions of log10 (BF) between neutral loci and LOSITAN outliers. Compared to neutral loci, the outliers displayed a log10 (BF) distribution more skewed to the right with a higher frequency of large log10 (BF) values (Fig.6.7a). One approach I used to interpret the Bayes factor data was using the distribution of log10 (BF) from neutral loci as an empirical distribution and statistical significance for the SNP of interest was assessed against this empirical distribution (Hancock, Witonsky et al. 2008; Coop, Witonsky et al. 2010). I found 133 outlier SNPs from LOSITAN were in the 5% of the empirical null distribution. At the 0.01 significance level, 100 of them remained significant (Fig.6.7a). The observation that a large number of outliers fell in the extreme tail (<0.01) of the empirical null distribution suggests that those outliers may be targets of positive selection.

In addition to comparison to the empirical distribution, another way to elucidate the BAYENV result is to target SNPs with large Bayes factors. In our results, I found 9 outlier SNPs showing median log10 (BF) > 1, which were considered “strong evidence” for selection (Berg, Jentoft et al. 2015). Those nine genes were tops hit for the PCB correlation in our analysis and they were distributed on five different scaffolds of the genome (Fig.6.7b).

6.4.5.2 LEA

The R function snmf in package LEA inferred K=2 as the optimal individual admixture coefficients; therefore I computed locus-specific z-scores and P-values for numbers of latent factors ranging from K=1 to K=7 using lfmm function. For each value of K, the lfmm algorithm was run 10 times using 5,000 cycles following a period of 5,000 cycles for burn-in. As a result, K=2 with a genomic inflation factor of 0.83189 (close to 1), was decided as the best number of latent factors based on the analysis of histograms of adjusted P-values. Using 2 latent factors, the lfmm function produced a list of 46 candidate SNPs correlated with environmental PCB concentrations (Fig.6.7c), representing 1.5% of the total 3,063 SNPs. Of these 46 SNPs, three were also inferred as associated SNPs in BAYENV association study; they were S323_153774, S9880_365667, and S10094_1034480. Eight were found on the same scaffolds with candidate SNPs identified in BAYENV ($p < 0.01$). SNPs located on the same scaffolds are very close to each other (~20 bps) (Table 6.3).

6.4.6 Annotation: Genes or Genomic Regions under Selection

To annotate the outlier SNPs, I used BLAST to align the 64bp sequence tags of these SNPs from GBS against the *F. heteroclitus* genome (Altschul, Madden et al. 1997). Functional clusters for various diseases (*e.g.,* cardiovascular, aging), phosphoprotein, cytoplasm, glycoprotein, alternative splicing, and ATP-binding are enriched in the 100 significantly correlated outliers ($p < 0.01$) from the Bayesian analysis. Specifically, a variety of genes targeted by PCBs were involved in pathophysiology (Table 6.4a). The first major group was cancer and tumor related genes, including estrogen receptor 1, spindlin 1 (SPIN1), and deleted in bladder cancer protein 1 (DBC1). These genes play a

role in various cancers and tumorgenesis. For instance, estrogen receptor 1 (ESR1) that was identified in multiple outlier SNPs is correlated with negative regulation of gene expression (Yoffou, Edjekouane et al. 2011) and breast cancer development (Holst, Stahl et al. 2007). Besides cancer development genes, diverse tumor suppressor genes were also targeted in outliers, which include glioma tumor suppressor candidate region gene 1 (GLTSCR1), dual specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A), and calmodulin binding transcription activator 1 (CAMTA1).

The second group of pathophysiology genes was involved in apoptotic process. This group includes pituitary tumor-transforming gene 1 (PTTG1), dual specificity tyrosine-(Y)-phosphorylation regulated kinase 2 (DYRK2), and unc-13 homolog B (UNC13B). The third group contained genes related to cellular response to DNA damage stimulus, such as MACROD2 and bromodomain adjacent to zinc finger domain 1B (BAZ1B). Besides disease related genes, genes playing a role in defense response and immune system were also revealed in outlier SNPs. This encompasses cysteine-rich secretory protein 3 (CRISP3)(Udby, Calafat et al. 2002) and interleukin 31 receptor A (Il31ra).

Apart from pathophysiology genes, outlier SNPs play a role in in a variety of metabolic genes (Table 6.4b). These genes are involved in various metabolic functions, including energy metabolism (hexokinases, aldehyde dehydrogenase 8 family, member A1), fatty acid synthesis (3-hydroxy-3-methylglutaryl-CoA synthase 1, ELOVL fatty acid elongase 5), protein synthesis (ribosomal mitochondrial proteins), and enzymatic activities (calcium/calmodulin-dependent protein kinase II). Other annotated genes were involved in assorted biological functions, including nervous system development (*e.g.,*

cadherin 20, type), signaling pathways (e.g., 1-phosphatidylinositol 4,5-bisphosphate, GTPase or receptors), reproductive system development (*e.g.,* Pleckstrin domain-containing family A member 5), and cardiac growth (*e.g.,* calmodulin binding transcription activator 2).

Significantly correlated SNPs from LEA were annotated and their functions turned out to be similar to Bayesian associated SNPs. Glioma tumor suppressor candidate region gene 1 (GLTSCR1) and glucose 1,6-bisphosphate synthase found above were also called in LEA correlated SNPs. Pentraxin 3 (PTX3), junctional adhesion molecule C, ephrin type-A receptor 6, and interleukin-6 receptor subunit beta fragment were called in LEA, and they all play a role in immune system (Matthews, Schuster et al. 2003; Luo, Yu et al. 2004; Zimmerli, Lee et al. 2009; Job, Bottazzi et al. 2013).

6.4.7 Comparison to Gene Expression

I compared the genes identified in the current genomic scan study (100 significantly correlated outliers in Bayesian analysis; $p < 0.01$) to the significant genes identified in the gene expression study in chapter 5. Four genes were shared in those two independent studies: protein polybromo-1 (PBRM1), calmodulin-binding transcription activator 1 (CAMTA1), bromodomain adjacent to zinc finger domain protein 1A (BAZ1A), and nuclear factor erythroid 2-related factor 1 (NFE2L1). PBRM1 mutations contribute to cancer development by involving in chromatin regulation (Varela, Tarpey et al. 2011), and CAMTA1 may act as a tumor suppressor (Barbashina, Salazar et al. 2005). Both BAZ1A and NFE2L1 are mainly responsible for transcriptional regulation, and NFE2L1 may also contribute to anatomical structure morphogenesis. Shared genes strongly suggest those genes are targeted by POP contamination. Overall, these genes

play a significant role in cancer and tumor development and transcriptional regulation. POPs have been identified as carcinogenic and mutagenic (Srogi 2007), and high prevalence of liver cancers have been reported in *F,heteroclitus* from POP contaminated sites (Vogelbein, Fournie et al. 1990). Shared carcinogenic and tumor genes in genomic markers and gene expression highlight the fundamental role of POP carcinogenic effects in adapted fish.

Although only four significant genes were exactly the same between these two studies, the biological pathways that significant genes are involved in turned out to be very similar. Gene expression in chapter 5 revealed that approximately 3.4% of genes showed potentially adaptive changes in polluted fish and that those altered genes were enriched for functional clusters for stress responses (*e.g.* diseases, immune responses, responses to chemical stimulus) and regulation of a variety of metabolic processes. Correspondingly, in the genomic scan study functional annotation and enrichment analysis suggest significant outliers are mainly involved in cancer and tumor development, apoptosis, immune system, and various metabolic processes. In a word, genomic scan functional results agree well with gene expression findings. The similar biological pathways identified in the gene expression and genomic scan analyses strongly suggest that stress response pathways and regulation of a wide range of metabolic pathways are crucial for POP adaptation development.

6.5 Discussion

To better understand the genetic effects of chronic pollution exposure and potential mechanisms underlying pollution adaptation in natural populations, I examined the genetic variation of thousands of markers derived from genotyping-by-sequencing in

F. heteroclitus populations inhabiting a strong pollution cline in New Bedford Harbor, MA to detect locus-specific signatures of positive selection. The presence of outlier loci within the genome usually indicates divergence is going on. Association studies can help determine the selective force causing the observed genetic divergence. Importantly, outliers are often correlated to biologically important variation. Gene annotation reveals the functionality of outliers and thus facilitates understanding pollution adaptation.

6.5.1 Genetic Evidence of Pollution Adaptation

Given that some alleles exhibit consistently increasing or decreasing patterns along the pollution cline (Fig.6.2), regression analysis was first applied to provide statistical evidence proving the correlations between certain alleles and sediment PCB concentrations (Fig.6.3). The significant regressions provide some of the most intuitive evidence that PCBs play a role on some alleles and thus may potentially affect population genetic structure.

Outlier detection was conducted to identify locus-specific signatures of positive selection among 3,063 SNPs. Using the FDIST2 approach, 3.3% to 7.5% of the 3,063 SNPs were revealed as significant outliers with 1% FDR correction ($p < 10^{-4}$), among the pairwise comparisons of populations along the pollution cline *versus* a nearby reference population, m. With such conservative p-values, permutations of similar SNPs were unlikely to happen randomly. In addition, these outlier SNPs have higher mean F_{ST} values (0.086) than the non-significant neutral SNPs (0.002). These indicate that the majority of 3,063 SNPs exhibits no genetic differences (mean F_{ST} value = 0.002) with insignificant p-values ($p < 0.01$; Fig.6.4) between polluted and reference populations. Thus overall these populations are not isolated and still are “one” population in general.

Moreover, compared to the majority of the 3,063 SNPs, the outlier SNPs underlie significantly larger genetic divergence (higher F_{ST} values) among polluted and reference populations. The observation that 36% of outlier SNPs inferred by the hierarchical model in ARLEQUIN v.3.5 overlaps with FDIST2 outliers increases the credence of these adaptive candidates (revealed by different models).

Both the Bayesian clustering method, STRUCTURE and the multivariate approach, DAPC successfully discerned population genetic structures among the six populations using outlier SNPs (Fig.6.5, Fig.6.6). Yet with neutral SNPs, no genetic structure was detected in either of them. This indicates that compared to the neutral SNPs, outlier SNPs are informative in revealing genetic structures that potentially result from selection pressure. More interestingly, genetic structures revealed by both approaches exhibit a genetic structure cline that corresponds to the geographic PCB cline in harbor. This suggests the genetic structure discerned by outlier SNPs is very likely due to geographic PCB concentrations.

Although outlier detection discovered hundreds of outlier SNPs that exhibited high genetic divergence, and a parallel population genetic structure corresponding to geographic PCB concentrations was revealed, I could not definitively conclude that environmental PCBs were the determining selective force causing the observed genetic differentiation. To better address the causative selective force, I used ecological association studies and gene annotation. Using BAYENV, 100 outlier SNPs, 3.3% of 3,063 SNPs, were found significantly associated with environmental PCB concentrations ($p < 0.01$). Compared to neutral SNPs, these 100 SNPs, as outliers, exhibited higher genetic differentiation and at the same time were strongly correlated with PCB

concentrations. Similarly, LEA identified 46 candidate SNPs correlated with environmental PCB concentrations. These data strongly support the idea that environmental PCB concentrations impacted genetic structure. These results provide genetic evidence to prove selection pressure from PCBs is working on *F. heteroclitus* populations and that adaptation is going on.

Methodological Comparisons

Three overlapping SNPs were significantly correlated with PCB concentrations in both BAYENV and LEA (Table 6.3). The first SNP is associated with microtubule-actin cross-linking factor 1, which facilitates actin-microtubule interactions and couples the microtubule network to cellular junction. The second gene is voltage-dependent N-type calcium channel subunit alpha-1B. It is responsible for controlling neurotransmitter release from neurons. The third gene is interleukin-31 receptor subunit alpha that is involved in defense response and negative control of apoptosis (Ghilardi, Li et al. 2002). The LEA correlated SNP that was located close to BAYENV SNP on the same scaffold turned out encoding the same gene (Table 6.3). SNPs located on scaffold 2521 encode melanophilin that facilitate intracellular protein transport. SNPs on scaffold 9986 are Glioma tumor suppressor candidate region gene 1. SNPs on scaffold 10104 encode bromodomain adjacent to zinc finger domain protein 1B (BAZ1B) that is related to DNA repair. Although other LEA correlated SNPs were different from Bayesian associated SNPs, annotation results showed that correlated SNPs from these two approaches were responsible for similar biological processes. SNPs from both LEA and BAYENV were associated with tumor development, metabolic functions, and immune system response. This indicates that significant markers identified by different approaches, LEA and

BAYENV, are not necessarily the same, yet the biological functions that those markers are responsible for are very consistent. The functional convergence of different markers targeted by different methods highly suggests these biological functions are being selected for by PCB contamination.

6.5.2 Functionality Indicates Adaptation to Pollution

6.5.2.1 Outlier SNPs Associated with Diseases and Immune System

As selection shapes genotypes at the level of phenotype, genetic markers being selected for are anticipated to exhibit functional relevance (Vitti, Grossman et al. 2013). Thus, functionality is often associated with signatures of selection. Genomic regions related to phenotypic fitness are more likely to be targeted by selection. A variety of genetic markers identified in selection studies were found linked to diseases or immune system in humans (Hancock, Witonsky et al. 2008; Fumagalli, Sironi et al. 2011). For instance, pathogens in infectious diseases were considered as one of the main selective pressures for human evolution (Fumagalli, Sironi et al. 2011).

I detected a couple of genes involved in immune response in our outlier SNPs. The link between immune system and pollution has long been studied in *Fundulus* (Fries 1986). Physiological studies investigating pollution effects discovered a decrease in immune function (Fries 1986; Rice and Xiang 2000). Yet, modification of certain immune parameters upon pollutant exposure has also been reported (Roszell and Anderson 1996). These seemingly contradicting results point to the needs to evaluate innate and adaptive features of immune system. In the selected outlier SNPs, SNPs encoding cysteine-rich secretory protein 3 (CRISP3) and interleukin 31 receptor A (Il31ra) were responsible for defense response to other organisms (Udby, Calafat et al.

2002; Perrigoue, Zaph et al. 2009). Ingestion and metabolism of xenobiotics (*e.g.*, PCBs) may contribute to selection of those defense genes. For instance, PCBs can covalently bind to genomic DNA and cause DNA damage (Jung, Matson et al. 2011). O-acetyl-ADP-ribose deacetylase (MACROD2) that facilitates cellular response to DNA damage stimulus was selected by PCBs as outliers. Other selected genes like pentraxin 3 (PTX3) and interleukin-6 receptor subunit beta fragment were involved in the innate immune system (Matthews, Schuster et al. 2003; Job, Bottazzi et al. 2013). Junctional adhesion molecule C was reported responsible for adaptive humoral immune response against pathogens (Zimmerli, Lee et al. 2009). Since PCBs impair individual fitness (Jones and de Voogt 1999), the observation that immune-related SNPs were selected by PCBs may potentially serve as evidence supporting adaptation. Future studies investigating how those immune genes work upon pollutant exposure may help evaluate the functional consequences of immune system changes in *Fundulus* adapted to PCBs.

F. heteroclitus inhabiting a site highly polluted with polycyclic aromatic hydrocarbons (PAHs) exhibited a high prevalence of liver diseases including neoplasms and hepatocellular carcinoma (Vogelbein, Fournie et al. 1990; Vogelbein WK 2008), suggesting a strong correlation between pollutant exposure and toxicopathic liver disease. Our results provide genetic support for these phenotypic studies. Gene annotation identified various genes involved in cancer and tumor development and a couple of suppressor genes. Targeting these genes is important for three reasons. First, it proves the power of our approaches. Our study successfully targeted markers that were associated with functions affected by PCBs. For instance, estrogen receptor 1 was identified in the outliers and it is well known for being acted upon by PCBs (Jansen, Cooke et al. 1993;

Connor, Ramamoorthy et al. 1997). Further, estrogenic compounds have been shown to differentially impact estrogen signaling pathways, including estrogen receptor 1, in NBH fish relative to nearby reference fish (Greytak and Callard 2007; Greytak, Tarrant et al. 2010). Second, cancer genes including estrogen receptor 1, spindlin 1, and deleted in bladder cancer protein 1 were shown to be selected by PCBs in our study, providing genetic evidence for a cause-effect relationship between PCBs and carcinogenesis. Last, given that PCBs trigger tumors and fish inhabiting the polluted site are being selected for, detection of tumor suppressor genes (*e.g.,* glioma tumor suppressor candidate region gene 1) may indicate *F. heteroclitus* exposed to PCBs have developed corresponding strategies against tumor development to maintain their fitness. This may serve as another evidence supporting adaptation.

6.5.2.2 Outlier SNPs Associated with Metabolic Functions

Pollutant exposure has been associated with metabolic disorders, including type 2 diabetes and obesity, (Lee, Lee et al. 2006; Lim, Cho et al. 2010; Airaksinen, Rantakokko et al. 2011; Karami-Mohajeri and Abdollahi 2011; Lee, Lind et al. 2011; Ruzzin 2012), energy metabolism (*e.g.,* decreasing aerobic metabolism) (van Ginneken, Palstra et al. 2009), and changes in oxidative phosphorylation (Du, Crawford et al. 2015). A gene expression study concerning three polluted *F. heteroclitus* populations reported up to 17% of 384 metabolic genes with evolved adaptive gene expression changes, and these genes are involved in various metabolic functions including oxidative phosphorylation (Fisher and Oleksiak 2007; Oleksiak 2008). These physiology and gene expression results suggest pollutants have an influence on organism metabolic functions and that

populations adapted to pollutants may develop adaptive metabolic changes (Fisher and Oleksiak 2007; Du, Crawford et al. 2015).

Correspondingly, our genome-wide scan revealed various metabolic genes selected by PCBs. Genes that play a role in lipid metabolism and fatty acid biosynthesis (e.g., 3-hydroxy-3-methylglutaryl-CoA synthase 1, and ELOVL fatty acid elongase 5 (ELOVL5) may be fundamental in certain biological processes, energy metabolism, or metabolic disorders. Selected genes encoding hexokinases and NAD+ protein (aldehyde dehydrogenase 8 family, member A1) are responsible for glucose phosphorylation and NAD activity, thus contributing to energy metabolism and the oxidative phosphorylation pathway. Interestingly, another member of NAD proteins, aldehyde dehydrogenase 7 family member A1, was also reported to exhibit adaptive gene expression changes (Fisher and Oleksiak 2007), corresponding to our results. In a word, the selected metabolic genes play a role in various biological functions that are affected by pollutants, which reinforces the idea those outlier SNPs are selected by PCBs and are adaptive. Uncovering the genetic markers selected by PCBs will provide important insight into the genetic basis for metabolic changes and help better understanding pollutants' effects on organisms.

6.6 Conclusions

I used a genotyping-by-sequencing technique to investigate natural populations inhabiting a strong pollution cline and detect locus-specific signatures of positive selection. Given that major allele frequencies along the cline exhibited a correlation to geographic PCB concentrations, I conducted a genome wide scan and identified a list of outliers underlying high genetic variation using pairwise comparisons. Application of

those outliers successfully discerned population genetic structure paralleling geographic PCBs, and association studies reinforced the correlation between outlier SNPs and PCBs. Gene annotation revealed those outliers were mainly responsible for diseases, immune system response, and various metabolic functions. Finally, I conclude that identified outliers are most parsimoniously described as adaptive and that functionality of selected outliers supports adaptation.

Table 6.1 Collection sites, approximate shoreline distances from NBH, and surficial sediment polychlorinated biphenyl (PCB) concentrations for *F. heteroclitus* collection sites. Sediment PCB concentrations were retrieved from Nacci et al. (2002).

Site code	Population sources	Distance from NBH (km)	Sediment PCBs (ng/g dry wt)
nbh	NBH Superfund Site	0	22,666
syc	Sycamore Street	3	3,762
p	Pilgrim Avenue	5	874
f	Fairhaven Launch	5	541
h	Hacker Street	10	13
m	Mattapoisett	30	27

Table 6.2 Major allele frequencies for alleles significant regressed to sediment PCB concentrations.

A. Major allele frequencies for alleles positively regressed to PCB concentrations						
	nbh	syc	p	f	h	m
Average	0.882	0.781	0.782	0.770	0.768	0.766
Min	0.614	0.417	0.521	0.458	0.480	0.411
Max	1.000	0.967	0.981	0.942	0.938	0.950
B. Major allele frequencies for alleles negatively regressed to PCB concentrations						
	nbh	syc	p	f	h	m
Average	0.699	0.824	0.843	0.855	0.854	0.843
Min	0.306	0.472	0.521	0.500	0.542	0.500
Max	0.938	1.000	1.000	1.000	1.000	0.980

Table 6.3 SNPs overlap or located on the same scaffold identified by BAYENV 2.0 and LEA.

	BAYENV 2.0		LEA	
SNPs overlap	Scaffold	SNP location	Scaffold	SNP location
	323	153774	323	153774
	9880	365667	9880	365667
	10094	1034480	10094	1034480
SNPs close	Scaffold	SNP location	Scaffold	SNP location
	2521	17085	2521	17065
			2521	17066
			2521	17079
			2521	17094
	9880	365667	9880	365683
	9986	1107087	9986	1245382
	10104	490976	10104	490937
			10104	490959

Table 6.4 Gene annotation

a) Pathophysiology genes	
Estrogen receptor 1 (ESR1)	Breast cancer development (Holst, Stahl et al. 2007); Mutations of ESR1 found associated with the resistance of breast cancer treatment (Robinson, Wu et al. 2013; Jeselsohn, Buchwalter et al. 2015)
Spindlin 1 (SPIN1)	Related to cancer development via Wnt signaling pathway (Fuguo, Qiang et al. 2006; Wang, Zeng et al. 2012)
Deleted in bladder cancer protein 1 (DBC1)	Involved in the process of bladder tumorigenesis (Wright, Messing et al. 2002)
	Associated with breast and lung cancer (Kim and Sung 2010)
Glioma tumor suppressor candidate region gene 1 (GLTSCR1)	Tumor suppressor gene
Dual specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A)	Tumor suppressor gene (Liu, Liu et al. 2014)
calmodulin binding transcription activator 1 (CAMTA1)	Tumor suppressor gene (Schraivogel, Weinmann et al. 2011)
Pituitary tumor-transforming gene 1 (PTTG1)	Negatively regulated intrinsic apoptotic signaling pathway by p53 (Read, Seed et al. 2014)
Dual specificity tyrosine-(Y)-phosphorylation regulated kinase 2 (DYRK2)	Directly phosphorylated p53 and resulted in apoptotic cell death in response to DNA damagev (Taira, Nihira et al. 2007)
Unc-13 homolog B (UNC13B)	Apoptotic process (Song, Ailenberg et al. 1999)
MACROD2	Related to cellular response to DNA damage stimulus (Jankevicius, Hassler et al. 2013)
Bromodomain adjacent to zinc finger domain 1B (BAZ1B)	Related to cellular response to DNA damage stimulus (Xiao, Li et al. 2009)
Cysteine-rich secretory protein 3 (CRISP3)	Defense response and immune system (Udby, Calafat et al. 2002)
Interleukin 31 receptor A (Il31ra)	Defense response and immune system (Perrigoue, Zaph et al. 2009)

(Continued)

b) Metabolic genes	
Hexokinases (HK1)	Phosphorylate glucose to produce glucose-6-phosphate, which serves as the first step in most glucose metabolism pathways
Glucose 1,6-bisphosphate synthase	Plays a role in glucose metabolic process and phosphorylation
Aldehyde dehydrogenase 8 family, member A1 (ALDH8A1)	Belongs to NAD+ protein family and is responsible for aldehyde dehydrogenase (NAD) activity
3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1)	Operating in lipic metabolic process (Rokosz, Boulton et al. 1994)
ELOVL fatty acid elongase 5 (ELOVL5)	Encodes a multi-pass membrane protein and is involved in long-chain fatty acid biosynthesis
Ribosomal mitochondrial proteins (MRP)	Encoded by nuclear genes and help in protein synthesis within the mitochondrion
Calcium/calmodulin-dependent protein kinase II (CaMKII)	Involved in protein phosphorylation and protein serine/ threonine kinase activity (Morrison, Murakami et al. 2000); regulating lots of biological functions, such as energy metabolism and cell cycle.
Cadherin 20, type	Development of nervous system
1-phosphatidylinositol 4,5-bisphosphate, GTPase or receptors	Signaling pathways
Pleckstrin domain-containing family A member 5	Reproductive system development
calmodulin binding transcription activator 2	Cardiac growth

Figure 6.1 Sampling locations along the pollution cline. Samples were collected from six locations New Bedford Harbor, MA with decreasing surficial sediment PCB concentrations from nbh to h in.

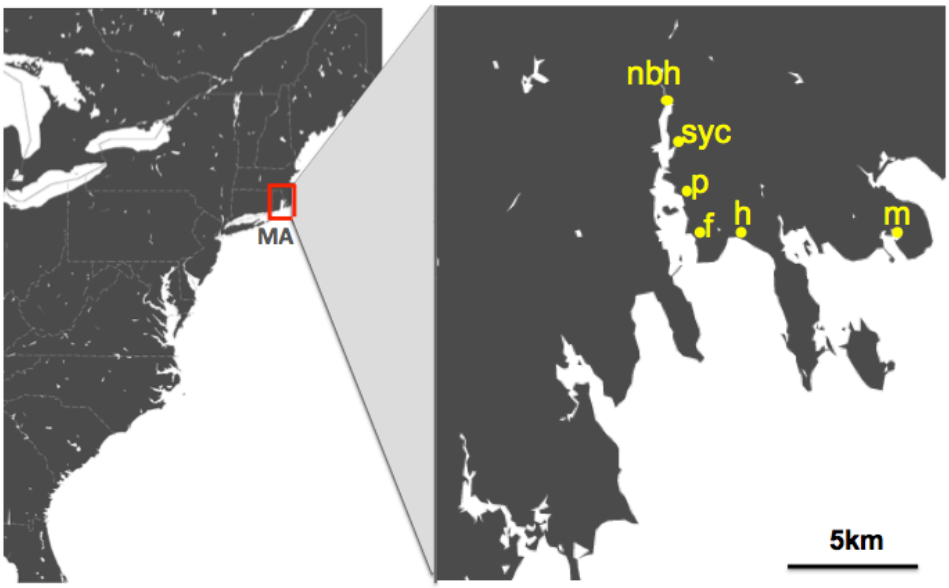


Figure 6.2 Allele frequency patterns along the pollution cline. Allele frequencies consistently increase (a) or decrease (b) as pollution drops from site nbh to h on the cline. Allele frequencies consistently increase (a) or decrease (b) as pollution drops from site nbh to m on the cline.

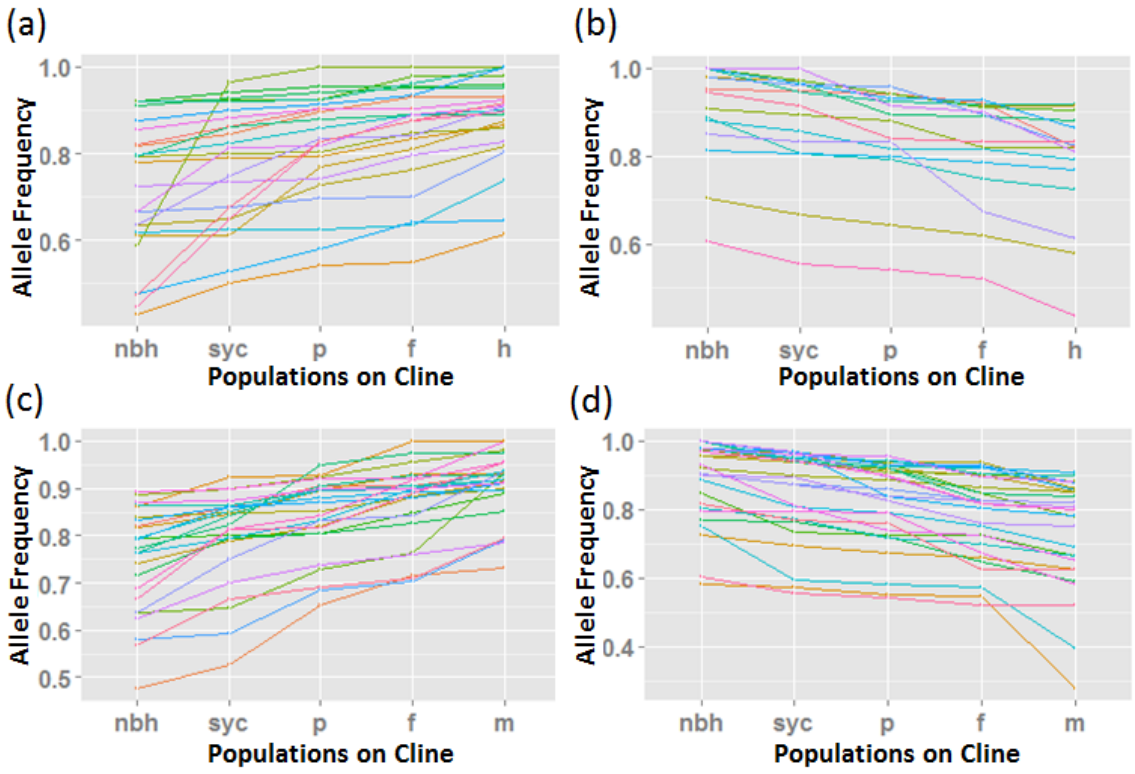


Figure 6.3 Positive (a) and negative (b) regression plots of allele frequency *versus* sediment PCB concentrations. All regressions plotted are significant with $p < 0.05$.

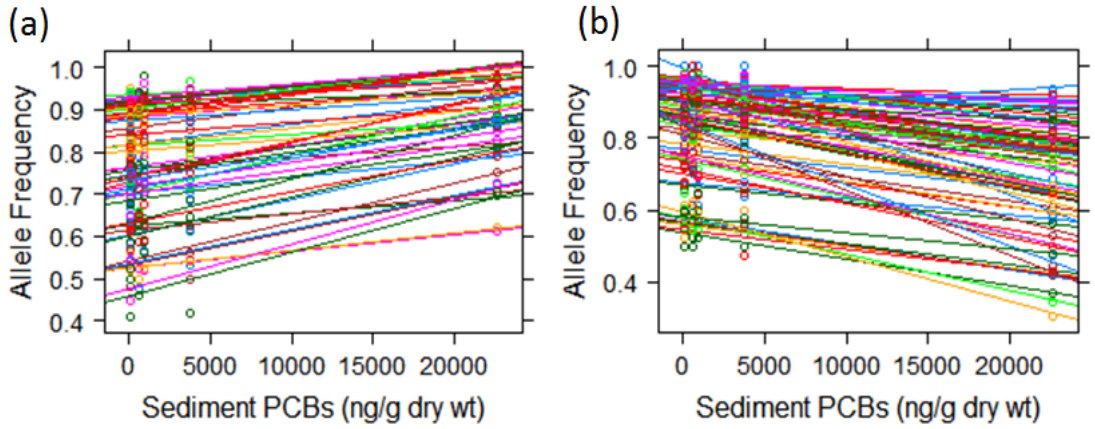


Figure 6.4 Plot of F_{ST} values and corresponding p-values in pairwise comparisons by LOSITAN. Black values are neutral, green values are significant ($p < 0.01$), and red values are significant with 1% FDR correction. Histograms on top show F_{ST} value distributions and histograms on the side show p-value distributions.

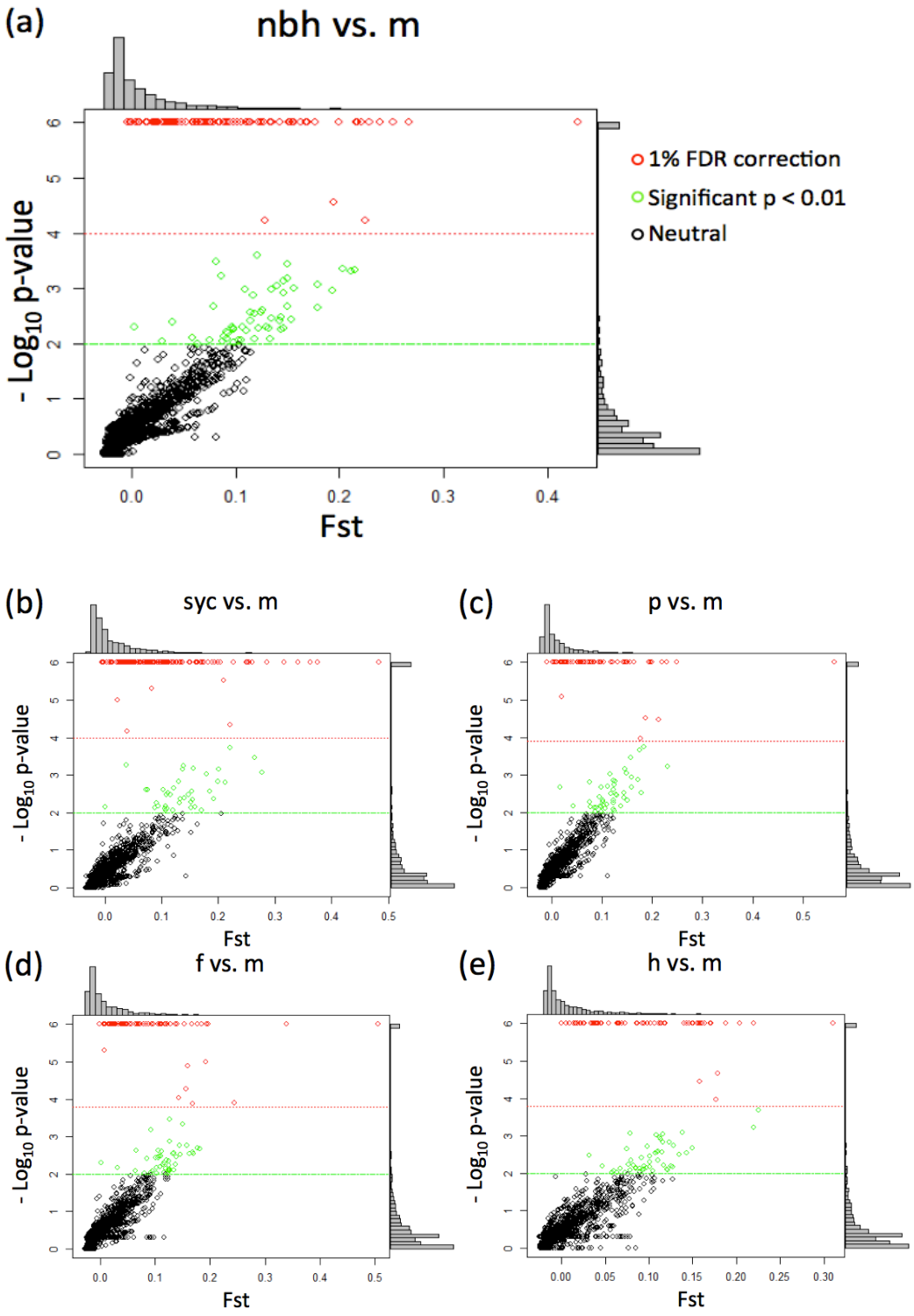


Figure 6.5 STRUCTURE plots of six populations along the pollution cline. (a) DeltaK plot of 155 outlier SNPs from nbh *vs*. m (LOSITAN). (b) DeltaK plot of the union of 531 outlier SNPs from LOSITAN. (c) STRUCTURE plot utilizing 155 outlier SNPs from nbh vs.m (LOSITAN). (d) STRUCTURE plot utilizing the union of 531 outlier SNPs from LOSITAN. (e) STRUCTURE plot utilizing 1,000 neutral SNPs. Each individual is represented by a thin vertical line, which is partitioned into two colored genetic clusters according to modeled admixture proportions.

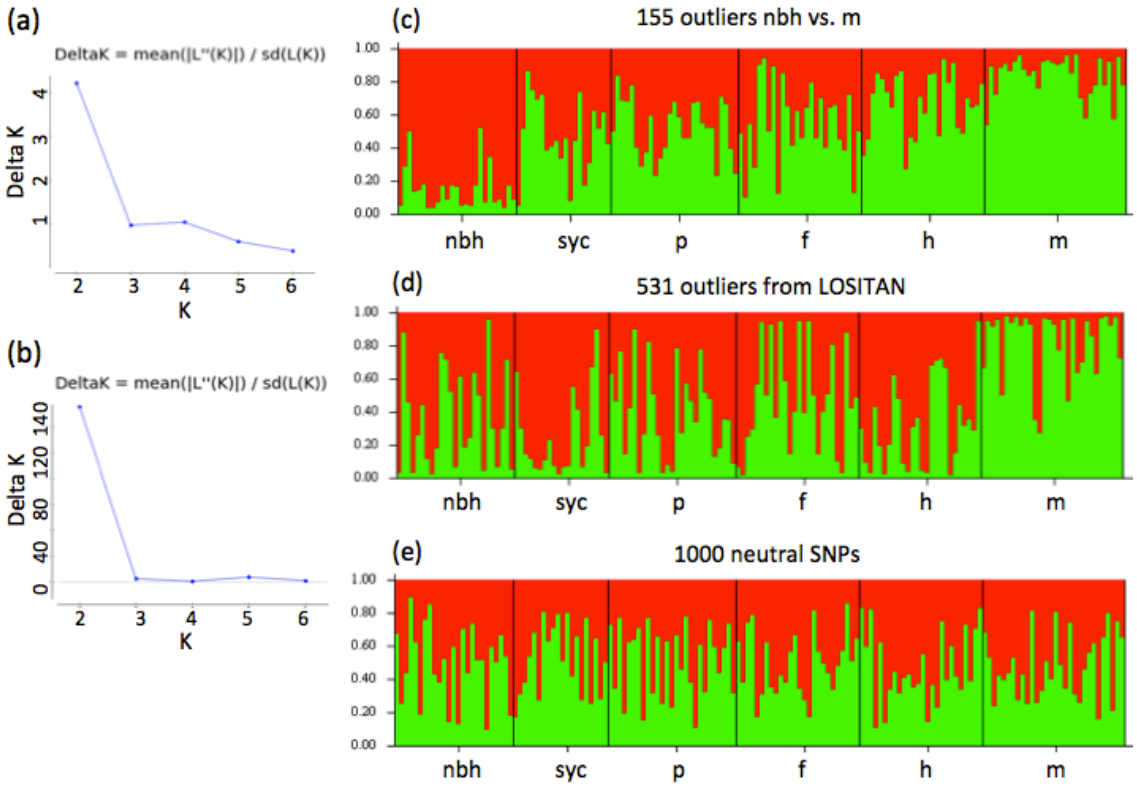


Figure 6.6 Scatterplots of DAPC for six populations along the pollution cline. (a) Scatterplot of DAPC using 155 outliers from nbh vs. m (LOSITAN). (b) Scatterplot of DAPC using the union of 531 outliers from LOSITAN. The scatterplots show the first two principle components of the genetic variations. Dots represent individuals and populations are shown by different colors and inertia ellipses. The relative eigenvalues of the first (horizontal) and second (vertical) principal components are shown at bottom right.

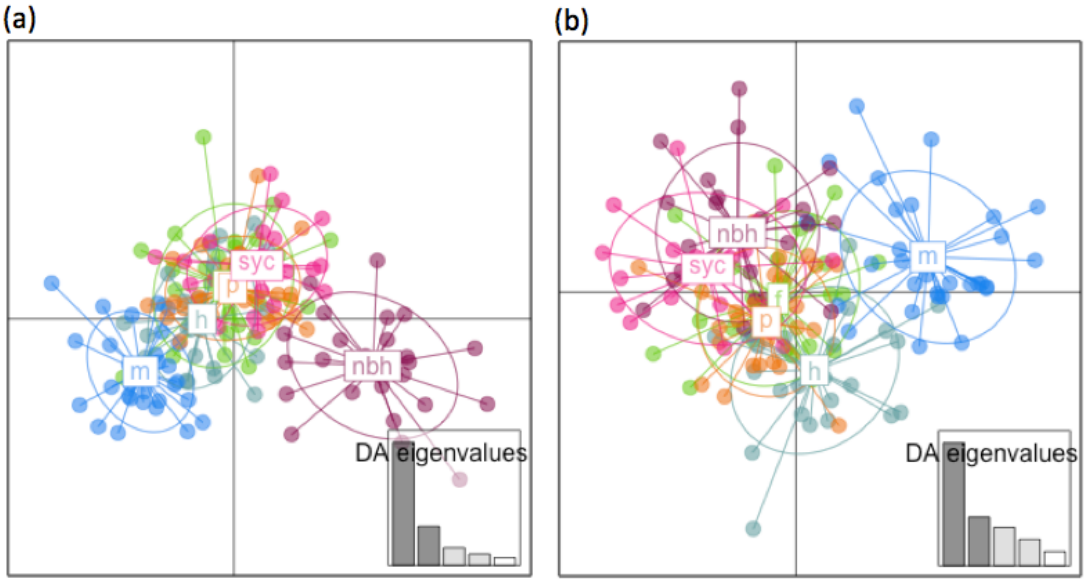
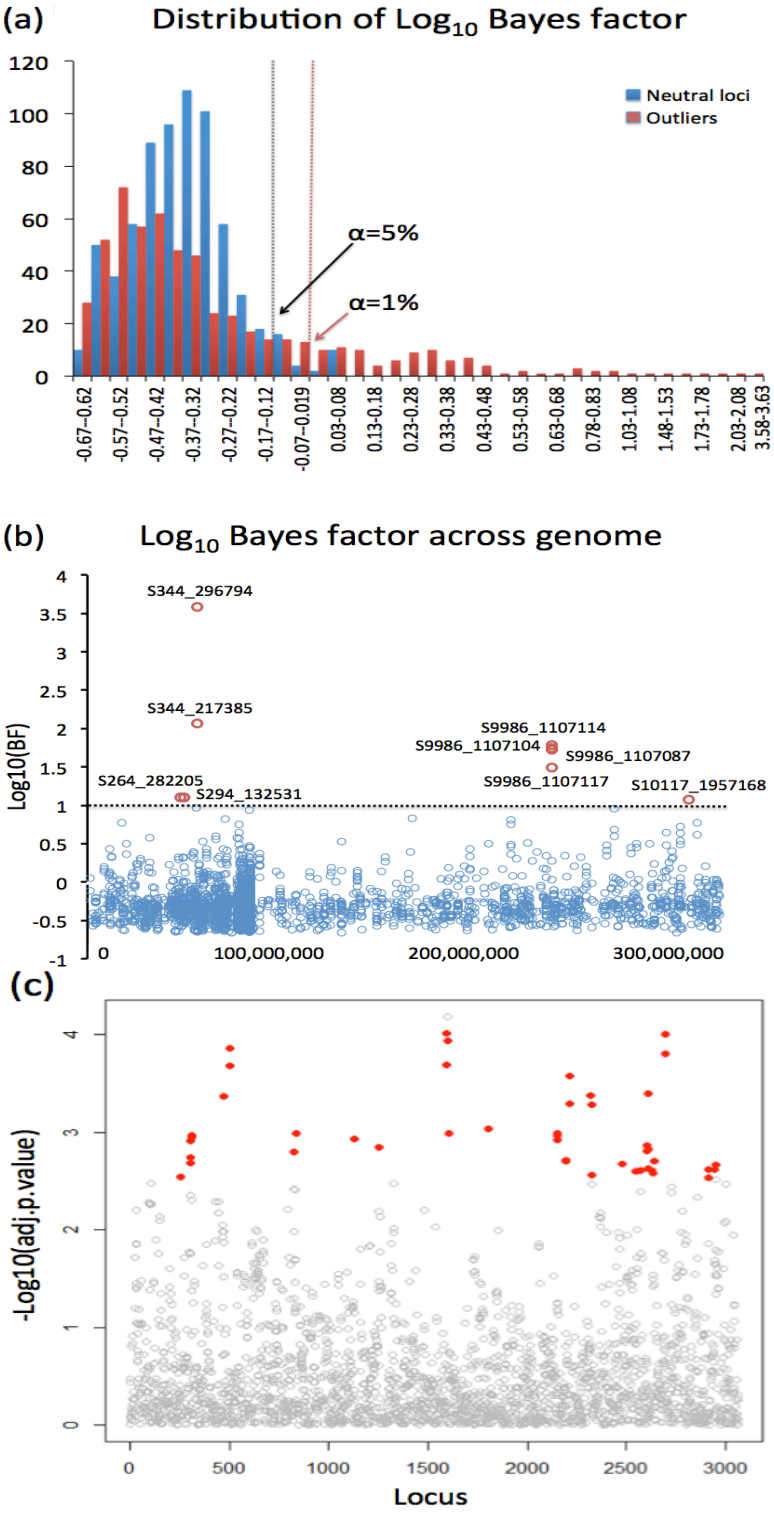


Figure 6.7 Environmental association. (a) Distribution of Log10 Bayes factor for neutral SNPs and outlier SNPs. (b) Distribution of Log10 Bayes factor across the genome. Loci are named by scaffold (S) and position along scaffold. (c) Candidate SNPs identified by LEA. Grey represent neutral SNPs; candidate SNPs associated with PCBs are highlighted in red.



Chapter 7 Conclusions

The observations that energy balance is a fundamental concern for organisms developing pollutant tolerance and that the OxPhos pathway, the biochemical pathway for most cellular energy production, is targeted by acute pollutant toxicity was the impetus for this dissertation. To gain a better understanding of how natural populations tolerate and adapt to pollution from the bioenergetic standpoint, the goals of this dissertation were to describe modulations of OxPhos metabolism and gene expression in pollutant tolerant *F. heteroclitus* populations and to identify evidence of genetic adaptation to pollution.

To determine if OxPhos function was modulated by chronic environmental pollutant exposure in natural *F. heteroclitus* populations, this investigation of OxPhos began with a comparison of OxPhos metabolism between polluted and clean reference populations, with and without exposure to acute pollutant dosing (Chapter 2). This characterization of OxPhos differences in polluted *versus* reference populations provides the groundwork for future chapters by demonstrating the presence of pollution adaptation in the OxPhos pathway. To further confirm that OxPhos modulation is genetically adaptive, laboratory-reared *F. heteroclitus* were examined in Chapter 3 to check for consistency with observed OxPhos modulations in field-collected fish. In chapter 4, the generality of OxPhos adaptation pattern was tested in a polluted *F. heteroclitus* population chronically exposed to another group of pollutants. The next step was to determine if gene expression changes were linked to OxPhos metabolic modulations by measuring gene expression of the same individuals measured for OxPhos. Finally, to

identify genotypic evidence of genetic adaptation, genetic variation at thousands of markers was examined in one of the tolerant *F. heteroclitus* populations inhabiting a strong pollution cline.

Chapter 2 measured OxPhos metabolism in *F. heteroclitus* from Elizabeth River, VA. This population is chronically exposed to polycyclic aromatic hydrocarbons (PAHs). The results suggest that the Elizabeth River population has developed significant changes in various OxPhos functions, including LEAK, State 3, E state, and enzyme complexes compared to the reference King's Creek population. Although acute PAH dosing has been shown to lead to mitochondrial functional deficiency, such as disrupted mitochondrial membrane potential and inhibited electron transfer at the cellular level (Sivalingan, Yoshida et al. 1973; Li, Sioutas et al. 2002; Ko, Kim et al. 2004; Xia, Korge et al. 2004), this study, by examining a pollution-tolerant population, demonstrates the chronic, evolutionary effects of PAH contamination on OxPhos metabolism. Indeed, given that acute PAH dosing tends to diminish ATP production and elevate OxPhos uncoupling (Ko, Kim et al. 2004; Xia, Korge et al. 2004), the enhanced routine respiration (State 3) and suppressed proton LEAK (thus higher coupling efficiency) in the polluted Elizabeth River population highly suggest a modification or improvement of this population's OxPhos functions that allows the fish to maintain their normal metabolic functions under polluted conditions.

In addition to the observed OxPhos population differences, differences in the response to acute POP toxicity were also detected in the Elizabeth River *versus* reference population. Acute PAH and PCB exposure affected OxPhos in the reference population but had no detectable effects on the polluted Elizabeth River population. This

insensitivity of OxPhos in Elizabeth River *F. heteroclitus*, corresponding to previously reported resistance to the teratogenic effects (Ownby, Newman et al. 2002) and the cytochrome P4501A (CYP1A)-inducing activity of the sediments (Meyer and Di Giulio 2002), supports an evolved tolerance to the acute effects of POP toxicity in the polluted fish. The observation that the polluted Elizabeth River population showed resistance to PCB toxicity also suggests that PAHs and PCBs may have similar effects on the evolution of OxPhos functions.

Both heritable (Nacci, Coiro et al. 1999; Meyer and Di Giulio 2002; Ownby, Newman et al. 2002; Meyer and Di Giulio 2003) and non-heritable (Meyer and Di Giulio 2002; Meyer and Di Giulio 2003) changes that could be related to pollution adaptation have been reported in Elizabeth River *F. heteroclitus* as compared to individuals from the reference site. To determine whether the observed OxPhos modulations and tolerance to acute POP toxicity in Elizabeth River *F. heteroclitus* is genetically based, OxPhos metabolism was measured in a laboratory-reared F3 generation of Elizabeth River *F. heteroclitus* and compared to OxPhos metabolism in a laboratory-reared F1 generation from the reference King's Creek population. When the responses to an altered environment can be due to both genetic adaptation and physiological acclimation, differentiating adaptation from acclimation (the reversible physiological changes individuals make to cope with an altered environment) in natural populations can be difficult. The use of 3rd generation fish would eliminate epigenetic, developmental, and irreversible physiological effects, and thus facilitate examining genetically based changes.

The F3 Elizabeth River fish compared to F1 King's Creek fish showed significantly higher State 3 respiration (routine metabolism) and complex II activity, and significantly lower complex I activity. Importantly, the consistently higher routine metabolism in the F3 and field-caught Elizabeth River fish *versus* F1 and field-caught King's Creek fish highly suggests a heritable change in OxPhos function that is most likely due to adaptive evolution. Unexpectedly, LEAK, E-State, Complex I and Complex II were different in laboratory bred *versus* field-caught fish. This may suggest that different physiological mechanisms produce the enhanced OxPhos differences. Additionally, OxPhos function of the laboratory-reared F3 generation displayed resistance to acute benzo [a] pyrene exposure that was found in the field-caught Elizabeth River fish, supporting the heritability of the toxicity resistance. Overall, the consistency of enhanced OxPhos metabolism and toxicity resistance across generations imply that the Elizabeth River *F. heteroclitus* has evolved genetic changes in physiological homeostasis that enhance routine metabolism. This result agrees with other studies examining resistance in *F. heteroclitus* from polluted environments that support the hypothesis of genetic adaptation. For instance, Elizabeth River *F. heteroclitus* embryos from field polluted fish showed similar resistance as F2 embryos to the teratogenic effects of Elizabeth River sediment, indicating that resistance was heritable through the F2 generation (Ownby, Newman et al. 2002).

Independent *Fundulus* populations chronically exposed to different groups of pollutants provide the opportunity to investigate adaptive responses to pollution evolved in a complex natural environment (Fisher and Oleksiak 2007). *F. heteroclitus* from Elizabeth River, which were predominantly contaminated PAHs in the sediments,

showed resistance to both PAH and PCB toxicity in Chapter 2. A recent study by Clark, Bone, and Di Giulio (2014) also reported strong resistance to teratogenesis induced by both PAHs and PCB-126 in F1 and F2 Elizabeth River embryos. These observations suggest PAHs and PCBs may share similar evolutionary effects in the adaptive development of exposed populations. Indeed, a study examining gene expression patterns in three independent, pollutant Superfund populations exposed to a variety of pollutants revealed both convergent and divergent changes in gene expression in response to environmental contamination (Fisher and Oleksiak 2007). Therefore, to more comprehensively understand the OxPhos modulation pattern in response to chronic pollution, I quantified OxPhos function with and without acute dosing in a PCB-adapted *F. heteroclitus* population from New Bedford Harbor (Chapter 4). Similar to the Elizabeth river population, significant OxPhos functional differences were also detected in the New Bedford Harbor *versus* reference Scorton's Creek population, proving that OxPhos metabolism has been targeted by pollution in independent populations. Similarly, neither PAH nor PCB acute dosing affected OxPhos function of New Bedford Harbor fish, indicating adaptive resistance to POP toxicity.

As discussed in the introduction, combating pollutant toxicity is metabolically costly (Calow 1991), and this metabolic cost or energy balance needs to be considered to better understand how organisms tolerate and adapt to pollution. Energy acquisition limitation leads to tradeoffs between the metabolic cost of stress tolerance and the energy costs of fitness-related functions such as growth, development, and reproduction (Sokolova, Frederich et al. 2012). Therefore, investigating bioenergetic consequences of environmental contamination will promote predicting population-level consequences.

Understanding OxPhos metabolism potentially contributes to interpretation of population adaptation to pollution. Importantly, both New Bedford Harbor and Elizabeth River fish showed elevated respiratory control ratio (RCR) and (State 3) routine respiration. RCR, which represents mitochondrial ability to respond to ADP by making ATP, is one of the most important diagnostic features of mitochondria (Brand and Nicholls 2011). A high RCR might indicate high substrate oxidation, high ATP turnover, or low proton LEAK. This suggested that the adapted populations were more proficient in transforming ADP into ATP, which was indeed supported by observations of State 3 measurement. State 3 or routine respiration is a measure of ATP production. Consistent with higher RCR, elevated State 3 respiration was detected in the polluted New Bedford Harbor and Elizabeth River fish. The high State 3 measurements in both PAH and PCB polluted populations indicate that the adapted populations have evolved enhanced OxPhos capacity in response to chronic exposure. This is of great importance for tolerance adaptation. I predict that the polluted populations have evolved adaptive enhancements in OxPhos capacity to extend energy acquisition in order to successfully combat the toxicity while at the same time maintain regular fitness-related functions. These OxPhos modulations are heritable as evidenced by data from laboratory F3 generations from both Elizabeth River and New Bedford Harbor fish. Additionally, this enhanced OxPhos capacity is conserved in independent, polluted populations, while the divergent changes in proton LEAK, complex II, and complex IV activity in New Bedford Harbor *versus* Elizabeth River population may suggest natural populations' capacity to realize energy balance for stress tolerance in distinct ways.

To promote the understanding OxPhos changes due to chronic pollution exposure, gene expression was measured for examining potential pathways or processes contributing to OxPhos changes. Gene expression is often altered upon toxicant exposure and thus is considered as a sensitive bioindicator of pollutant toxicity (Thomas, Rank et al. 2001; Hamadeh, Bushel et al. 2002). Toxicant-altered gene expression has been reported in a variety of studies in *F. heteroclitus* (Meyer, Nacci et al. 2002; Meyer, Wassenberg et al. 2003; Meyer, Volz et al. 2005). Recently, pollutant exposure was linked to gene expression changes on metabolic genes. In a study concerning three POPs contaminated populations of *F. heteroclitus*, up to 17% metabolic genes were found to have evolved adaptive changes in gene expression (Fisher and Oleksiak 2007). Importantly, Oleksiak 2008 reported differentially expressed genes in polluted populations that are involved in the oxidative phosphorylation pathway, indicating pollution may potentially target energy metabolisms in mitochondria (Oleksiak 2008). Therefore, in Chapter 5 I aimed to measure gene expression changes in the same *F. heteroclitus* individuals that showed OxPhos differences between polluted and reference populations in Chapters 2 and 4, and link these gene expression changes to OxPhos changes to better understand how pollution affects energy metabolism changes.

Gene expression changes were regressed against OxPhos changes upon POP exposure to discern genes or pathways that are correlated or potentially contributing to OxPhos changes. Although not identical, the top twenty genes interpreting the highest variations of proton LEAK in King's Creek and Scorton Creek populations respectively are involved in similar processes including transmembrane proteins, apoptosis, immune response, protein metabolism, aromatic compound responses, and glucose homeostasis.

Variation in State 3 or routine respiration is mainly governed by variations of genes involving in glycogen biosynthesis, cholesterol homeostasis, transmembrane proteins, aromatic compound responses (DNA repair, CYP450 2F2), tumor suppressor, and G protein metabolism. Some of the affected pathways upon POP dosing such as apoptosis, cholesterol, and lipid metabolism have been reported before (Staal, van Herwijnen et al. 2006). Yet, our results suggest significant genes involved in those pathways potentially affect mitochondrial energy metabolism. Indeed, gene expression differences between polluted and reference populations also support the evidence that pollution has a significant effect on metabolism. Importantly, enrichment analysis suggests functional clusters for regulation of cellular metabolic process and regulation of biosynthetic process are enriched in both polluted populations.

A striking group of the modulated genes that are linked to OxPhos function play a role in glucose homeostasis and lipid metabolism, suggesting a vital role of energy storage and balance in OxPhos upon pollutant exposure. Actually, a previous gene expression study demonstrated that the polluted populations may have limited energy stores due to an extra energy cost due to coping with pollution (Oleksiak 2008). Our data support this hypothesis by illustrating that pollution may influence the energy stores by adjusting OxPhos metabolism to cover the extra energy cost due to coping with pollution. Unexpectedly, besides the altered gene expression patterns that may directly affect OxPhos function, *e.g.,* transmembrane proteins, glucose and cholesterol homeostasis, and protein regulation, a variety of genes that are crucial to organism fitness upon exposure, including apoptosis, immune response, tumor suppressor, cellular response to estrogen (DNA repair), and other aromatic compound responses (CYP2F2), are also significantly

correlated with mitochondrial OxPhos. One possible explanation is that those defense functions are metabolically costly and thus indirectly affect OxPhos, the fundamental energy production pathway, to maintain their energy needs (*e.g.,* extra cost of detoxification). The correlation between defense responses and OxPhos function reinforces the idea that energy balance may play a crucial role in pollution tolerance.

Finally, with the observed phenotypic changes in OxPhos and gene expression in polluted populations, the genetic changes that may underlie the phenotypic changes due to chronic pollution exposure were investigated. Identifying signatures of adaptation was achieved by examining the genetic variation of thousands of markers derived from genotyping-by-sequencing in *F. heteroclitus* inhabiting the strong pollution cline in New Bedford Harbor, MA, USA. Positive selection shapes population genetic structure by working at the level of phenotypes; therefore, loci detected as outliers are likely to be functionally important (Vitti, Grossman et al. 2013). A list of outliers underlying high genetic variation successfully discerned population genetic structure paralleling geographic PCBs. This indicates that outlier SNPs are informative in revealing genetic structures that potentially result from geographic PCB concentrations. Association studies, which proved the correlation between outlier SNPs and geographic PCBs, strongly support the idea that environmental PCB concentrations impacted genetic structure. These results provide genetic evidences to prove selection pressure from PCBs is working on *F. heteroclitus* populations and that adaptation is going on.

Functionality is often associated with signatures of selection. As selection shapes genotypes at the level of phenotype, genetic markers being selected for are anticipated to exhibit functional relevance (Vitti, Grossman et al. 2013). *F. heteroclitus* inhabiting

highly contaminated sites exhibited a high prevalence of liver diseases including neoplasms and hepatocellular carcinoma (Vogelbein, Fournie et al. 1990; Vogelbein WK 2008), suggesting a strong correlation between pollutant exposure and toxicopathic liver disease. Our results provide genetic evidence for these phenotypic studies. Gene annotation identified various genes involved in cancer and tumor development (*e.g.*, estrogen receptor 1, spindlin 1), immune response (*e.g.*, cysteine-rich secretory protein 3 (CRISP3), interleukin 31 receptor A (Il31ra)), and a couple of suppressor genes (*e.g.*, glioma tumor suppressor candidate region gene 1 (GLTSCR1), dual specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A)). Targeting these genes is important for three reasons. First, it proves the power of our approaches. For instance, estrogenic compounds have been shown to differentially impact estrogen signaling pathways, including estrogen receptor 1, in New Bedford Harbor fish relative to nearby reference fish (Greytak and Callard 2007; Greytak, Tarrant et al. 2010). Second, detection of cancer genes provides genetic evidence for a cause-effect relationship between PCBs and carcinogenesis. Last, detection of immune and tumor suppressor genes may indicate polluted *F. heteroclitus* have modulated immune responses and developed corresponding strategies against tumor development to maintain their fitness. This may serve as functional evidence supporting adaptation.

The ultimate goal of was to identify genetic changes that may underlie the observed phenotypic changes. Gene annotation revealed various metabolic genes selected by PCBs, which potentially contribute to energy balance and OxPhos metabolism. Genes that play a role in lipid metabolism and fatty acid biosynthesis (*e.g.,* 3-hydroxy-3-methylglutaryl-CoA synthase 1, and ELOVL fatty acid elongase 5 (ELOVL5)) are

selected by pollution. This corresponds to gene expression results, in which genes involved in lipid metabolism are affected by exposure and at the same time significantly linked to OxPhos function. These results highly suggest that lipid metabolism is targeted by pollution and fundamental for tolerance adaptation by contributing to changes in OxPhos and energy balance. Additionally, selected genes encoding hexokinases and NAD+ protein (aldehyde dehydrogenase 8 family, member A1) are responsible for glucose phosphorylation and NAD activity, thus directly contributing to energy metabolism and oxidative phosphorylation pathway. Interestingly, another member of NAD proteins, aldehyde dehydrogenase 7 family member A1 was also reported exhibiting adaptive changes in gene expression study (Fisher and Oleksiak 2007), corresponding to our results. In a word, the selected metabolic genes play a role in energy metabolism that is affected by pollutants, which reinforces the idea that those outlier SNPs of functionality are selected by PCBs and are adaptive. Uncovering the genetic markers selected by PCBs will provide important insight into the genetic basis for metabolic changes and help better understanding metabolic changes due to chronic pollution exposure. Finally, I conclude that identified outliers are most parsimoniously described as adaptive and that functionality of selected outliers supports adaptation.

Summary

This work establishes both that OxPhos function in polluted *F. heteroclitus* has developed adaptive changes in response to chronic pollution and that energy metabolism is fundamental for tolerance development. Enhanced OxPhos functions (*e.g.*, elevated respiratory control ratio, elevated routine respiration) and resistance to acute POP dosing are detected in independent, polluted *F. heteroclitus* populations. Those OxPhos changes

are heritable to the laboratory-reared F3 generation fish. Gene expression analysis discerns genes that are involved in energy metabolism and defense responses may potentially contribute to OxPhos changes upon exposure. The fundamental role of mitochondrial energy metabolism in pollution adaptation is reinforced by functionality of genotypic markers significantly correlated to environmental pollutants. The findings in this thesis contribute to the understanding of bioenergetic consequences of environmental pollutants. Further studies should focus whether such changes affect organism level fitness in populations.

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