AN INTEGRATED BIOMARKER APPROACH FOR ASSESSING EXPOSURE AND EFFECTS OF ENDOCRINE DISRUPTORS AND OTHER CONTAMINANTS IN KILLIFISH (*FUNDULUS HETEROCLITUS*) FROM THE NEW YORK-NEW JERSEY HARBOR ESTUARY

by

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ABSTRACT OF THE THESIS

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A multi-tiered approach was used to evaluate killifish (*Fundulus heteroclitus*) health by examining a suite of biomarkers in killifish inhabiting the heavily industrialized Newark Bay and a reference population in Tuckerton, NJ. The general hypothesis of this study was that Newark Bay killifish would exhibit biomarker responses indicative of impaired health when compared to a reference population from Tuckerton, NJ. The biomarkers investigated included classical endpoints (histopathology, morphometrics, gonad maturation), hepatic mRNA expression (CYP1A, metallothionein, vitellogenin I), gonadal aromatase mRNA expression, hepatic protein levels (CYP1A and vitellogenin I) and chemical exposure analyses (bile PAHs). Newark Bay fish had significantly higher levels of bile PAHs compared to reference fish. Females had significantly higher concentrations of naphthalene, pyrene and benzo[a]pyrene (3, 6 and 4 fold higher, respectively, $p<0.05$). Males had significantly higher concentrations of pyrene (7-fold higher, $p<0.05$), and higher concentrations of naphthalene (4-fold higher, $p=0.06$) and

benzo[a]pyrene (9-fold higher, p=0.07). Histological lesions of the liver and pancreas in Newark Bay fish were similar to reference fish. Newark Bay fish had significantly higher expression of hepatic CYP1A for both males and females (7 and 3 fold higher, respectively, $p<0.05$) and CYP1A protein levels for both males and females (5 and 8 fold higher, $p<0.05$). Endocrine disruption in male gonads is demonstrated by a decreased gonad weight, altered testis development and decreased aromatase expression (3-fold, $p<0.05$), which indicates exposure to endocrine active compounds. Efforts to strip spawn Newark Bay females produced few viable eggs, while at the same time Tuckerton females produced large numbers of viable eggs. There was a significant increase in the percent of pre-vitellogenic follicles (43% at Tuckerton, 64% at Newark Bay) and a significantly decreased percent of follicles at the mid-vitellogenic and mature stages (25% at Tuckerton, 3% at Newark Bay) in Newark Bay females ($p<0.05$). Vitellogenin mRNA and protein (egg yolk-protein) was significantly decreased in Newark Bay females (6-fold lower mRNA, 27-fold lower protein, $p<0.05$) while gonadal aromatase (produces 17β-estradiol) was significantly increased (210-fold higher, $p<0.05$). Killifish in the NY-NJ Harbor Estuary are exposed to high amounts of PAHs and aryl hydrocarbon agonists and their reproductive health is impaired. Impaired reproductive health is possibly due to disruption of steroid signaling by aryl hydrocarbon receptor-estrogen receptor crosstalk leading to decreased vitellogenin production.

DEDICATION

I dedicate this work to my family – my Mother Mary Jo, my Father George, my Brother Gregory and my Sister Mary Catherine. I'm ever grateful for their love and support.

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LIST OF ABBREVIATIONS

AHR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
bp	base pairs
cDNA	complimentary deoxyribonucleic acid
CT	threshold cycle
CYP1A	cytochrome p4501A
CYP19A1	ovarian cytochrome 19A1
CYP19A2	brain cytochrome 19A2
DDD	*p,p'*-dichlorodiphenyldichloroethane
DDE	*p,p'*-dichlorodiphenylchloroethylene
DDT	*p,p'*-dichlorodiphenyltrichloroethane
DEPC	diethyl pyrocarbonate
DLC	dioxin like compound
DNA	deoxyribonucleic acid
E2	17-β estradiol
EDC	endocrine disrupting compound
EDTA	ethylene diaminetetraacetic acid
EE_2	ethinylestradiol
EGTA	ethylene glycol tetraacetic acid
EtOH	ethanol
g	grams

HAH	halogenated aromatic hydrocarbon
IBA	Integrated Biomarker Assessment
kDa	kilodalton
mL	milliliter
mM	millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
MS222	tricaine methane sulphonate
MT	metallothionein
N	number of animals in a test group
NCBI	National Center for Biotechnology Information
ng	nanogram
nm	nanometer
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PeCDF	pentachlorodibenzo-*p*-furan
PCDD	polychlorinated dibenzo-*p*-dioxins
PCDF	polychlorinated dibenzo-*p*-furans
pg	picogram
ppb	parts per billion
ppm	parts per million
pptr	parts per trillion
PCDD	polychlorinated dibenzo-*p*-dioxin

PCDF	polychlorinated dibenzo-*p*-furan
PMSF	phenylmethanesulphonylfluoride
PVDF	polyvinylidene fluoride
µL	microliter
qPCR	quantitative polymerase chain reaction
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
SFS	synchronous fluorescent spectroscopy
TCDD	tetrachlorodibenzo-*p*-dioxin
TCDF	tetrachlorodibenzo-*p*-furan
µM	micromolar
µm	micron
UV	ultraviolet
v	volts
VTG	vitellogenin I
x g	times gravity

1.0. GENERAL INTRODUCTION

The general hypothesis of this study was that Newark Bay killifish (*Fundulus heteroclitus*) exposed to complex mixtures in the harbor would exhibit biomarker responses consistent with impaired health when compared to a reference population from Tuckerton, NJ, due to contaminant exposure. The main objectives were to (1) develop a battery of biomarkers for evaluating the general and reproductive health of killifish in the NY-NJ Harbor Estuary, (2) make recommendations as to the suitability of each specific biomarker, (3) determine chemical exposure and effects of contaminants and (3) determine whether endocrine disruption was occurring in fish inhabiting the NY-NJ Harbor Estuary.

1.1. BIOMARKER APPROACH IN THE NY-NJ HARBOR ESTUARY

The New York-New Jersey Harbor Estuary (Figure 1) has a large biotic community living within these waters which are heavily impacted by anthropogenic sources of historical and emerging contaminants (Assaf-Anid, 2003; Litten, 2003) including, but not limited to, dioxins/furans (Muñoz et al., 2006), polycyclic aromatic hydrocarbons (Valle et al., 2007), polychlorinated biphenyls (Panero et al., 2005) and heavy metals (de Cerreño et al., 2002; Boehme and Panero, 2003). Evaluating environmental quality in this ecosystem is essential to assure success of ongoing efforts to monitor and reduce toxic inputs and to re-establish healthy and viable populations of aquatic species. A widely accepted and commonly used method for assessing environmental quality is the Sediment Quality Triad, which includes gathering three lines of evidence consisting of sediment chemical characterization, sediment toxicity (acute

toxicity using amphipods) and benthic community structure (Long and Chapman, 1985; Chapman, 1990; Chapman et al., 1997). This method examines the potential impact of sediment associated toxicants on invertebrate species living in or on the sediment. The Sediment Quality Triad has been previously used to evaluate sediment quality in the NY-NJ Harbor including locations throughout the Passaic (Iannuzzi et al., 2007) and Hackensack Rivers (Sorensen et al., 2007). However, limiting ecological risk assessment studies to these sediment quality parameters is shortsighted and does not fully estimate the extent of contamination and its impact on higher organism health. Bioaccumulation of sediment bound toxicants in wildlife has been proposed to be an additional line of evidence for Sediment Quality Assessments to help facilitate a more comprehensive ecological risk assessment (Chapman and Anderson, 2005). Additional and alternative lines of evidence have also been recommended to supplement the classical approach, including fish histopathology, PAH concentrations in various tissues (i.e. bile), surface water quality, field biomarker studies, *in situ* toxicity assays to determine resistant species and mechanism specific bioassays using environmental samples such as sediments and pore waters (Chapman and Hollert, 2006).

Incorporating biological based endpoints is important to better characterize and evaluate the extent of contaminant exposure and effects in ecosystems that have complex mixtures of contaminants. Biomonitoring is the centerpiece of ecological assessment and is essential for assessing the well-being of any ecosystem (Burger, 2006). Monitoring biological effects is critical for evaluating effectiveness of management decisions and success of restoration efforts within the estuary and changes in contaminant profiles over time. However, there are currently no established or standardized finfish biological based

indicators of ecosystem health. The use of an integrated biomarker approach for assessing fish health can serve as a biological-based metric for estimating an ecosystem's health as it relates to finfish and provide a comprehensive measure of multiple contaminant exposure in commercially and ecologically important species. Estimating adverse impacts to an ecosystem is accomplished ideally by directly measuring the health of sentinel organisms that represent important trophic levels (i.e. small fish, bivalves, aquatic insects). Using a sentinel bioindicator approach may allow for integration of biotic and abiotic factors to better estimate biological impact from multiple chemicals as opposed to chemical analysis of contaminant concentrations in water, sediment or biotic (e.g. fish, shellfish) tissue, which provide a list of detected compounds but no indication of biological effects. These indirect methods of measuring exposure may not accurately represent animal health, whereas full contaminant analysis can be very expensive and do not indicate whether or not there are biological impacts.

Biomarkers are broadly defined as biochemical, physiological or ecological responses that are linked to or correlated to biological effects measured at one or more levels of biological organization (McCarty and Munkittrick, 1996). The World Health Organization defines a biomarker as "any substance, structure or process that can be measured in the body…and influence or predict the incidence or outcome of disease" (WHO, 2001). Bartell (2006) and Burger (2006) have independently made the suggestion that biomarkers be limited to effects at the sub-organism level of biological organization and that bioindicators include structures and processes at the higher levels of organization (e.g. population, community and ecosystem). Biomarkers can be as simple as measuring organ weight as an indicator of altered organ function. They can also be as

specialized as measuring the metabolic activity of a biochemical enzyme performing a specific function (e.g. CYP1A activity). When conducting an Integrated Biomarker Assessment (IBA) it is important to choose endpoints that allow for both a mechanistic understanding of thee contaminant exposure-effect relationship and extrapolation to organism and population-level effects that have an ecologically relevant impact (Figure 2). Many biomarkers that have been well-characterized are specific responses to short term exposure and to specific types of contaminant (e.g. cytochrome p450, metallothionein, vitellogenin). However, the utility of a biomarker needs to be evaluated for a given population and species because the endpoints employed were developed as acute responses to short term exposures of single compounds. Killifish (*Fundulus heteroclitus*) throughout the NY-NJ Harbor Estuary are being chronically exposed to complex mixtures, which have been shown to have decreased sensitivities to compounds such as methylmercury and dioxin (Weis, 1985; Prince and Cooper, 1995a/b). With this in mind, the biomarkers that were selected to be evaluated are listed in Table 1 and were chosen because of their well known and systematic responses to both general and specific classes of contaminants. This project was a direct outcome of previous efforts to develop an Integrated Biomarker Assessment for evaluating the health of finfish living in the chronically contaminated NY-NJ Harbor Estuary (Cooper and Buchanan, 2007).

1.2. MAJOR CONTAMINANTS IN THE NY-NJ HARBOR ESTUARY

The contaminants known to be present and contaminants likely to be present in the future directed the development of this IBA. The NY-NJ Harbor Estuary has a long history of economical and industrial development which has led to contamination and

accumulation of a broad spectrum of anthropogenic pollutant (i.e. heavy metals, dioxins, polycyclic aromatic hydrocarbons, pesticides, pharmaceuticals, brominated flame retardants, etc.). This ecosystem is also at the center of the most densely populated region in the country allowing it to be heavily contaminated by anthropogenic sources. While contamination comes from many point-sources, non-point sources (e.g. atmospheric deposition, sewer outflows) generally contribute a significant amount to the total input in this ecosystem which makes it difficult to regulate. Contamination is widespread and many classes of compounds are often found together in this estuary and as a result biological effects are likely consequence of complex mixtures. The extent of the complex contaminant mixtures in this estuarine system exemplifies the need for an integrated biomarker assay for evaluating fish health. Many biomarkers are not compound specific but are general responses to classes of structurally or functionally similar chemicals. Although there are many inorganic contaminants present in the estuary the primary contaminants that will be discussed include dioxin-like-compounds, polycyclic aromatic hydrocarbons and endocrine disrupting compounds. These have widespread occurrence, are risk drivers for cleanup and can have major impacts on finfish inhabiting these waters.

1.2.1. DIOXIN-LIKE-COMPOUNDS

The NY-NJ Harbor Estuary is heavily contaminated by aryl hydrocarbon receptor (AHR) agonists (e.g. dioxins, furans, polychlorinated biphenyls, etc.). In 1985, the NJ Department of Environmental Protection reported tissue levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) in fish from Newark Bay that exceeded the

50 parts-per-trillion (pptr) level concern set by the Food and Drug Administration (Belton et al., 1985). A major source of dioxins and furans in this system have been attributed to the 80 Lister Avenue chemical plant where polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzo-*p*-furans (PCDFs) were released as industrial byproducts as early as 1948 (Bopp et al., 1991). 2,3,7,8-TCDD and 2,3,7,8-tetrachlorodibenzo-*p*-furan (TCDF) contamination have since remained some of the highest sediment concentrations ever reported. In 1985±1 year, 2,3,7,8-TCDD and 2,3,7,8-TCDF was determined to be 310 and 290 parts-per-trillion (pptr) in surface sediments, and 260pptr and 280pptr in suspended solids, respectively, in Newark Bay (Bopp et al., 1991). This historical data demonstrates that aquatic species have been chronically exposed to high concentrations of DLCs in this habitat for several decades.

There have been many efforts to comprehensively characterize the mass balance of PCDD/Fs in this watershed for management and monitoring purposes (Huntley et al., 1998; Dimou and Pecchioli, 2006; Muñoz et al., 2006). Ongoing contamination of PCDD/Fs in this watershed is due to municipal discharge, wastewater effluents, atmospheric deposition, stormwater runoff, incinerators, waste management burning and structural fires (Huntley et al., 1998). A major report for the pollution prevention and management strategy for dioxins in the NY-NJ Harbor Estuary has concluded that (1) mass balances show higher accumulation rates for PCDD/Fs than they are being removed (based ON 17 dioxins), (2) octachlorodibenzo-*p*-dioxin accounts for ~80% of the PCDD/F input by mass (3) the most toxic congeners (2,3,7,8-TCDD, 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF) are being lost faster than they accumulate and (4) more than 90% of sediment toxicity is accounted for by three congeners, 2,3,7,8-TCDD, 2,3,7,8-TCDF and

2,3,4,7,8-PeCDF (Muñoz et al., 2006). Despite the fact that the most toxic congeners are being depleted, total PCDD/Fs are accumulating and will continue to be an increasing problem in this estuary as this is expected to result in increasingly toxic sediments. Dimou and Pecchioli (2006) also studied the distribution of 17 PCDD/F congeners throughout the estuary as part of the *NJ Toxics Reduction Workplan* and found that (1) 2,3,7,8-TCDD was as high as 210pptr at Newark Bay, exceeded 440pptr in the Passaic River and exceeded 170pptr in the Hackensack River, (2) total PCDD/F sediment concentrations were highest at locations in the Elizabeth River, Rahway River and Raritan River (exceeding 38, 22 and 40ppb, respectively). These current studies have shown that dioxin concentrations in sediments throughout the NY-NJ Harbor Estuary remain a concern to present day and that aquatic organisms are being exposed to contaminated sediments which may be impacting organism and population health. These findings have also shown that PCDD/F contamination is widespread and inputs are ongoing with high concentrations being reported in the upstream rivers.

The toxicity of exposure to PCDD/Fs is well understood and exposure leads to a wide variety of biological responses which are reviewed here. Toxic effects of dioxins and dioxin-like-compounds are generally mediated through the aryl hydrocarbon receptor pathway (Figure 3) which is well conserved amongst vertebrates (Hahn, 1998; Hahn, 2002). Exogenous ligands (halogenated aromatic hydrocarbons, polychlorinated biphenyls) bind to the cytosolic aryl hydrocarbon receptor (AHR), translocate into the nucleus and form a heterodimer with the aryl hydrocarbon nuclear translocator (ARNT) leading to DNA binding (via xenobiotic responsive elements) and altered gene expression (reviewed in Denison and Nagy, 2002; Hahn, 2002). A wide variety of biochemical,

cellular and tissue responses are regulated by the AHR pathway. Acute dioxin exposure has been shown to cause developmental abnormalities, pericardial edema and mortality in several teleost species including zebrafish (*Danio rerio*) and killifish (Prince and Cooper, 1995a; Henry et al., 1997). 2,3,7,8-TCDD exposure to developing killifish also results in induction of cytochrome P4501A, the biochemical endpoint of AHR activation (Prince and Cooper, 1995b) and apoptosis which co-occurs in tissues with altered cytochrome P4501A expression (Toomey et al., 2001). These findings have also been repeated in zebrafish (*Danio rerio*), demonstrating that local circulation failure in the mid-brain is associated with increased apoptosis (Dong et al., 2002). Chronic dioxin exposure and the subsequent AHR activation has been shown to promote hepatocarcinogenesis in rats (Pitot et al., 1980) although the promotion of tumors by AHR agonists has not been thoroughly investigated in fish species. Other studies have shown involvement of AHR with cell cycle signaling, normal homeostasis and development (reviewed in Kawajiri and Fujii-Kuriyama, 2007). Studies with AHR null mice have found differences in expression of Transforming Growth Factor ß1 in fibroblasts which correlated with decreased proliferation, suggesting the AHR pathway plays an important role in cell cycle regulation (Chang et al., 2007). Other studies *in vitro* have shown that 2,3,7,8-TCDD leads to increased expression of the potent mitogen epiregulin (Patel et al., 2006). Consequently, exposure to DLCs is involved in a myriad of both acute and chronic conditions which can severely impact the health of a population of aquatic species. Furthermore, there are an increasing number of studies showing a relationship between DLC exposure and harmful effects on reproductive health through endocrine disruption. 2,3,7,8-TCDD has been shown to exhibit endocrine activity (reviewed in: Hutz, 1999)

and disrupt gonad development in zebrafish (Heiden et al., 2008), the eastern oyster (Wintermyer and Cooper, 2007) and salmon (Bemanian et al., 2004).

1.2.2. POLYCYCLIC AROMATIC HYDROCARBONS

PAHs are another widespread contaminant in the NY-NJ Harbor Estuary with many point and non-point sources (fuel combustion, automobile exhaust, tire wear, leaking petroleum products, creosote-treated wood and industrial activity). A recent report for PAH pollution prevention and management has characterized the fate and transport in the NY-NJ Harbor and estimates the relative PAH discharges in this watershed to be primarily atmospheric (814,000kg/yr) while land release accounts for the second most (11,400kg/yr) and water release accounts for 1,800kg/yr (Valle et al., 2007). This study estimates that the primary sources of all PAHs entering the entire system are creosote treated wood (47%), residential wood combustion (38%), transportation related (15%) and oil spills (<1%). The PAHs released into the aquatic environment are from creosote treated marine pilings (76%) and discarded motor oil (20%) account for the majority of contamination. While the dominant medium of release is atmospheric, gas absorption, dry particle and wet particle deposition to water bodies contributes a significant amount of PAHs to the NY-NJ Harbor Estuary (Gigliotti et al., 2005). However, it is currently unknown whether atmospheric deposition in this ecosystem is greater than volatilization rates, and although likely for low molecular weight PAHs, this is unlikely for high molecular weight PAHs.

The *Contaminant Assessment and Reduction Plan* (New York State Department of Environmental Conservation) has also reported elevated PAH concentrations for water

and sediments throughout the NY-NJ Harbor Estuary (Litten, 2003). Total PAH concentration estimates in ambient water (filtered and particulate phases) in Newark Bay was 1,760ng/L. Other sites in the watershed have much higher total PAH concentrations such as Hudson River (500-10,700ng/L), Passaic River (6,389-15,600ng/L) and Hackensack River (4,000-5,120ng/L). These ambient water samples are much lower than concentrations measured in raw or treated wastewaters. The highest concentrations of PAHs were reported from combined sewer outflows and range in concentrations from Newtown Creek (800,000ng/L total PAHs) to the lowest at storm water overflow in Jamaica Bay (5,000ng/L total PAHs).

The findings reported by Litten (2003) and others demonstrate the magnitude of PAH contamination throughout the NY-NJ Harbor Estuary. Since PAHs are hydrophobic, very little total PAH is found freely available in water and they tend to associate with the hydrophobic compartment of the environment and therefore concentrate in sediments and suspended particles. The abundance and exposure to PAHs poses a significant risk to the health of many aquatic organisms. PAH metabolism and acute toxicity, like DLCs, is generally attributed to AHR binding and activation (reviewed for teleosts in Billiard et al., 2002). Unlike DLCs, PAHs are readily metabolized by cytochrome P4501A by addition of hydroxyl groups, conjugation and elimination. Biotransformation of some of the PAHs results in the formation of mutagenic and carcinogenic metabolites which have been shown to result in tumor formation in high dose or chronic exposure situations in various fish species (reviewed in Eisler, 1987; Baumann and Harshbarger, 1995; Pinkney et al., 2004). Killifish exposed to PAHs by inhabiting creosote contaminated sites have been found to exhibit increased

incidences of hepatic lesions (altered staining focii, hepatocellular adenomas, hepatocellular carcinomas, cholangiocellular proliferation) and an increased incidence of DNA adducts and bile PAH concentrations (Vogelbein et al., 1990). The EPA has classified 7 PAHs as probable human carcinogens (Group B2) with the most widespread and potent PAH being benzo[a]pyrene. In addition, PAHs can bioaccumulate in aquatic organisms with highly variable bioaccumulation factors for fish (12-9,200), crustaceans (200-134,248) and bivalves (8-242) with a tendency to be greater in higher molecular weight PAHs (Eisler, 1987). In addition to being mutagenic and carcinogenic, several PAHs are suspected of behaving as estrogens and anti-estrogens in various *in vitro* and *in vivo* models (Santodonato, 1997). Several PAHs (dibenz[*a*,*h*]anthracene, 6-hydroxy-chrysene, 2,3-benzofluorene, and benzo[a]pyrene) have been shown to interact with the human estrogen receptors and inhibit an estrogen-sensitive reporter in yeast (Tran et al., 1996). Others have shown several PAHs (benzo[a]anthracene, benzo[b]fluorene and benzo[c]phenanthrene) to be weakly agonstic with the estrogen receptor in human MCF-7 breast cancer cells, and although they do not bind strongly enough to displace estradiol from the estrogen receptor they can still compete for binding (Fertruck et al., 2001). Most studies of the endocrine activity of PAHs have been conducted *in vitro* using human estrogen receptors and few studies have investigated the relationship between PAHs and (anti-)estrogen activity in teleost species. Benzo[a]pyrene has been shown to inhibit gonadal aromatase expression in killifish *in vivo* (Patel et al., 2006). Due to the complexity of the hypothalamus-pituitary-gonad axis and likeliness of PAHs to be found in complex mixtures, it will be necessary to study the effects of individual PAHs *in vivo* and the interaction of multiple PAHs on endocrine pathways in finfish.

1.2.3. ENDOCRINE ACTIVE COMPOUNDS

In addition to historically documented contaminants are a class of compounds (tributlytin, surfactants, phthalates, pesticides and synthetic steroids) that are now recognized to have important biological effects, endocrine disruptors (in the reproductive context) that over the past decade have emerged as significant contaminants in rivers and estuaries (Sumpter and Jobling, 1995; Gimeno et al., 1996/1997; Houtman et al., 2004; Denslow and Larkin, 2006). The World Health Organization defines endocrine disruptors as "…exogenous substances or mixtures that alter function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or sub(populations)" (WHO, 2002). Endocrine disrupting compounds are defined by their potential for their reproductive effects on humans and aquatic biota rather than defined as being chemically similar (reviewed in Hutz, 1999).

The central tenet of endocrine disruption in the reproductive system is that contaminants can enter an organism and interfere with the normal processes of the hypothalamus-pituitary-gonad (HPG) axis (Figure 4). Many pollutants are known for their ability to interact directly and indirectly with the estrogen receptor (ER) pathway in agonistic or antagonistic ways (Figure 5), which can lead to disruption of reproductive biochemistry, altered gene expression and ultimately altered gonadal development. Effects can be estrogenic, anti-estrogenic, androgenic or anti-androgenic, leading to feminization and/or demasculinization of males and defeminization and/or masculinization of females. Reproductive effects in female killifish were a focus of this study because efforts to strip spawn female killifish at Newark Bay failed to produce eggs

in 2007 and 2008 (10-20 eggs per female on average) while at the same time killifish at a reference site were reproducing normally (100-200 eggs per female on average).

The HPG axis drives gametogenesis, in the ovaries which consists of highly conserved biological events that can easily be disrupted by exogenous compounds in various organs (Thomas, 2008). Oogenesis generally occurs in 6 steps: (1) germline segregation (formation of primordial germ cells), (2) sexual differentiation of primordial germ cells into oogonia, (3) meiotic transformation of oogonia into oocytes, (4 and 5) growth and maturation of oocytes and (6) expulsion of ovum from follicle (reviewed in Patiño and Sullivan, 2002). The events of teleost oogenesis (folliculogenesis and vitellogenesis) are summarized and shown in Figure 6. Generally, oogenesis in females is initiated by hypothalamus and pituitary production and release of gonadotropins (luteinizing hormone, follicle stimulating hormone) which stimulates the ovaries to begin steroid production (steroidogenesis) and follicle development (folliculogenesis). There are generally 3 stages of follicular growth (pre-vitellogenic, early vitellogenic, mid-vitellogenic growth) which ends with a mature oocyte). Gonadal aromatase (CYP19A1) converts androgen steroids to 17β-estradiol (E2) in the granulosa cells of the developing follicle at the pre/early-vitellogenic stages. Estrogen then circulates to the liver and drives hepatic vitellogenesis through ER-dependent activation of the vitellogenin genes. The production of vitellogenin proteins in the liver are required for growth and maturation of the developing oocyte. Vitellogenesis begins generally in the pre/early-vitellogenic stages and accumulation of vitellogenin pushes the oocyte into the mid-vitellogenic stage. Vitellogenin I and II is synthesized in the liver and circulates to the ovaries where it is cleaved by many cathepsins in the developing oocyte into

approximately a dozen different vitellin proteins during growth and maturation at the mid/late-vitellogenic stages (reviewed in LaFleur et al., 2005). The biological processes of oogenesis are complex and occur in various organ systems that can be disrupted by many types of contaminants.

The evidence that endocrine-disrupting chemicals are widespread in the United States due to anthropogenic sources (Kolpin et al., 2002; Barnes et al., 2008; Focazio et al., 2008) and are having effects on invertebrates and fish reproduction is growing (Arcand-Hoy and Benson, 1998; Nash et al., 2004). Contaminants that have been shown to be present in the NY-NJ Harbor Estuaries and exhibit endocrine activity, and are therefore likely impacting fin and shellfish include the following: tributlytin from shipping, 17-*B* estradiol (E_2) and ethinylestradiol (EE_2) from the large metropolitan human populations (Desbrow et al., 1996), nonionic surfactants such as nonylphenol and octylphenol (Jobling et al., 1996; Gronen et al., 1999), phthalate esters (Patyna et al., 1999; Patyna et al., 2005), bisphenol A from secondary water treatment facilities, various pesticides (pyrethroids, DDT/DDE, endosulfan, methoxychlor), various dioxins and AHR agonists (Hutz, 1999; Bemanian et al., 2004; Wintermyer and Cooper, 2007; Heiden et al., 2008) and PAHs (Santodonato, 1996; Navas and Segner, 2000; Fertruck et al., 2001). There is not only concern for aquatic and terrestrial wildlife (Tyler et al., 1998), but also the exposure to humans through drinking water (Kolpin et al., 2002; Zogorski et al., 2006) and consumption of contaminated food (NAS, 2003). Sediment concentrations of the endocrine disrupting pesticides *p,p'*-dichlorodiphenyltrichloroethane (DDT), *p,p'*-dichlorodiphenyldichloroethane (DDD), and *p,p'*-dichlorodiphenylchloroethylene (DDE) have been found at concentrations as high as 473, 429 and 111 parts-per-billion (ppb) for

DDT, DDD and DDE, respectively, in Newark Bay (Gillis et al., 1994). PCBs are an abundant contaminant in this ecosystem, especially the Hudson River, and may be having effects on finfish (Panero et al., 2005).

There is a wide variety of contaminants that could potentially act as an EDC and there is a lack of entirely understanding which compounds act as endocrine disruptors and the mechanism by which they exert endocrine disrupting effects. Therefore, a biomarker approach is an inexpensive and fast approach for assessing whether endocrine disruption is a concern in an ecosystem. Alternatively, it is cost-effective and faster to evaluate total endocrine activity of sediments and pore waters using one of the many bioassays available rather than use analytical techniques to quantify presence of known EDCs (reviewed in Campbell et al., 2006). These bioassays are not compound specific but instead measure the total endocrine activity of environmental samples using biomarker endpoints.

1.3. KILLIFISH – A SENTINEL SPECIES MODEL

Fundulus heteroclitus heteroclitus (Linnaeus, 1766) (common name: killifish) was chosen as the sentinel organism to develop a biomarker assay for several reasons. First, it has been extensively studied in the NY/NJ Harbor Estuary in response to contaminants (Weis et al., 1985; Khan and Weis, 1987; Prince and Cooper, 1995a/b; Arzuaga, 2002; Monosson et al., 2003) Second, it is a major prey species in the food web where it is ecologically important (Abraham, 1985). Third, it is abundant at many contaminated and non-contaminated sites throughout the estuary. In addition, they are omnivores eating detritus and small benthic invertebrates which provides a link to

chronic exposure to the local contamination. They also overwinter in sediments, are non-migratory and have home ranges of less than 100 meters throughout all seasons (Fritz et al., 1975; Lotrich, 1975; Skinner et al., 2005). Killifish are also commonly found along the entire Atlantic Coast from the Southeast Atlantic to Northeast Atlantic region (Abraham, 1985).

Killifish play an important and complex role in the food web as they are both a predator of other smaller aquatic organisms (small crustaceans, grass shrimp, insects, amphipods, etc.) and also an important food source for top predators (migratory birds, white perch, eel, snapper, etc.) in this ecosystem (reviewed in Able, 2007). Contamination in the tissue of killifish is a concern because of potential trophic transfer to predatory fish, birds and crustaceans. It has been shown that feeding PCB contaminated killifish collected from the Hackensack River to young-of-the-year bluefish (*Pomatomus saltatrix*) leads to increased tissue PCB concentration and decreased feeding and swimming activity (Candelmo et al., 2007). The increased use of killifish in toxicological research has resulted in the development of molecular tools allowing researchers to pursue mechanistic exposure-effect relationships. The killifish model has led to the development of morpholinos for targeting specific pathways, knockout strains, transgenic killifish and microarray technologies in killifish (reviewed in Burnett et al., 2007). Even with these advancements, there is a lag in the development of a genomic toolbox for this species compared to some of the small aquarium species, such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), both of which have their genome sequenced compared to killifish which does not (circa 2009).

1.4. RESISTANT POPULATIONS

While biochemical biomarkers (e.g. mRNA, protein and activity) are useful for indicating exposure to contaminant loads, interpretation can be complicated by locally resistant and insensitive populations (Yuan et al., 2001). Several populations of fish have been shown to be resistant to the toxic effects of several classes of chemicals including dioxins (Prince and Cooper, 1995a/b), PAHs (Nacci et al., 1999; Bello et al., 2001; Meyer et al., 2002), PCBs (Elskus et al., 1999; Roy and Wirgin, 1997; Courtenay et al., 1999) and heavy metals (Weis et al., 1985). Resistant phenotypes in a population are a consequence of exposure to chronically contaminated habitats resulting in decreased toxicity to the particular contaminant (or class of contaminants). This is usually due to alteration of biochemical and metabolic pathways leading to an attenuated toxicological response, which may confound the nature of biomarker-based assessments of fish health.

Several fish species and populations have been demonstrated to exhibit altered dose-response relationships to various contaminants such as PAHs, PCBs and dioxins/furans. Prince and Cooper (1995a/b) have previously shown that killifish inhabiting Newark Bay exhibited increased basal activity of cytochrome P4501A activity, and an inability to induce CYP1A activity or pathological lesions in embryos challenged with graded doses of 2,3,7,8-TCDD. A separate study using depurated killifish (80% reduction of total embryo PCB burden) collected from the Newark Bay also reported decreased induction of CYP1A activity and protein levels when challenged with various PCBs indicating that this refractory phenotype is persistent long after they were removed from the contaminated habitat (Elskus et al., 1999). Findings similar to those found in the Newark Bay killifish have been reported in killifish inhabiting New Bedford Harbor,

MA, a site heavily contaminated by PCBs and HAHs (Nacci et al., 1999; Bello et al., 2001). Adult killifish collected from New Bedford Harbor and their F1 generation did not respond to various challenge studies using DLCs and 3-methyl cholanthrene in a dose-dependent manner at the level of mRNA, protein and activity for CYP1A (Nacci et al., 1999; Bello et al., 2001). Resistance to PAHs has also been documented in a population of killifish inhabiting the heavily creosote contaminated Elizabeth River, VA. This population exhibited decreased induction of CYP1A protein and activity in the parental generation and 3 sequential generations when exposed to the prototypical PAHs β-naphthoflavone and 3-methylcholanthrene (Meyer et al., 2002). Although the refractory phenotype was evident for 3 generations of offspring, the second and third generation's ability inhibition was not as pronounced suggesting that the etiology of the refractory phenotype, while long lasting, is not permanent.

Atlantic Tomcod (*Microgadus tomcod*) inhabiting the Hudson River (heavily contaminated by PAHs and PCBs) have also been shown to have altered liver biochemical responses to HAHs (but not to PAHs) demonstrated by decreased CYP1A mRNA inducibility, decreased nuclear protein binding to CYP1A enhancer elements and altered CYP1A kinetic profiles (Roy and Wirgin, 1997; Courtenay et al., 1999). AHR mRNA regulation was similar between populations and was thus ruled out as the etiology of this refractory phenotype, however analysis of the AHR DNA sequence using RFLP genome mapping suggested the refractory phenotype may be caused by variation in the sequence of a critical exon of the AhR promoter known to be involved in AHR/ARNT heterodimer translocation (Roy and Wirgin, 1997). Furthermore, Hudson River tomcod that were treated with graded high doses of AhR agonists did exhibit induced hepatic

CYP1A mRNA levels in the liver (but not in heart, intestine, spleen or kidney) with maximum induction values comparable to fish from the reference site which suggests that the AhR pathway was functional, only much less sensitive (Yuan *et al*., 2006).

In conclusion, many studies have demonstrated the potential of killifish and other teleost species to develop altered biochemical pathways and altered pathologies in populations that have acclimated to contaminants commonly encountered in the environment. While the majority of these types of studies demonstrate acquired resistance to HAHs, PAHs and DLCs, other studies have also shown killifish from heavy metal contaminated locales in the NY-NJ Harbor were also able to develop resistance to mercury (Weis et al., 1981).

Acquired resistance to various contaminants leads to altered biochemical pathways and phenotypes and may potentially complicate biomarker assessments. Commonly used biomarkers (e.g. CYP1A, metallothionein) may be regulated differently in insensitive populations and therefore stresses the importance of validating and developing a unique biomarker toolkit for the specific ecosystem in question.

1.5. BIOMARKER SELECTION

The biomarkers that were selected for evaluation are listed in Table 1. These biomarkers were chosen for their potential to respond to various types of contaminants known to be present in the NY-NJ Harbor Estuary and its tributaries.

Organ to body weight ratios are simple and rapid biomarkers that indicate enlarged or atrophied organs due to chemical exposure, altered biochemistry or histopathologic lesions. For example, killifish exposed to 2,3,7,8-TCDD exhibited

increased liver to body weight ratios concomitant with cytochrome P4501A induction in comparison to untreated fish (Prince and Cooper, 1995b). Hematocrit and red blood cell morphology indicate anemic populations with altered circulating red blood cell volume and/or altered fragility of red blood cells due to chemical or biological stressors. Blood smears of erythrocytes can allow for quantification of genotoxic events such as micronuclei – small extra-nuclear chromosomal fragments caused by DNA damage and disrepair (Schmid, 1975). Micronuclei prevalence indicates exposure to genotoxic contaminants (Bolognesi et al., 2006). Histopathology of the liver and gonads is a classic biomarker that measures prevalence of acute and chronic lesions. Histological assessment of the gonads can indicate decreased reproductive health in male and females by identifying altered gonadal development or presence of ovotestis which indicates feminization of males (Blazer, 2002; Pait and Nelson, 2003).

PAHs are a major contaminant in the NY-NJ Harbor (Valle et al., 2007) and most are readily metabolized, concentrated in bile and excreted. Therefore bile fluorescence was chosen as a biomarker of exposure to PAHs. Scanning fluorescent spectroscopy is a rapid and inexpensive method to qualitatively and semi-quantitatively measure presence of PAHs in fish bile and relative amounts between populations. Fluorescence of PAHs in bile and other tissues (e.g. hemolymph in crustaceans) has been used previously to show exposure of aquatic organisms to PAHs and oil spills (Ariese, 1993; Aas, 2000).

Certain genes and proteins whose expression can be upregulated in response to increasing contamination are sensitive biomarkers that can indicate presence of specific classes of pollutants and therefore can serve as biomarker of exposure and subsequent biochemical changes (reviewed in Tom and Auslander, 2005). This study employed

several biochemical endpoints including hepatic cytochrome P4501A, hepatic metallothionein, hepatic vitellogenin and gonadal aromatase. These biomarkers are mechanism specific for exposure to specific classes of contaminants.

One of the most common biochemical biomarkers is mRNA expression, protein levels and protein activity of cytochrome P4501A. Cytochrome P4501A is involved in phase I metabolism and is induced by a wide variety of aromatic hydrocarbons and can acts as a surrogate biochemical measurement of contaminant exposure to the organism. CYP1A is a membrane bound oxidative enzyme primarily found in endoplasmic reticulum and is responsible for metabolizing drugs and xenobiotics for phase II glucuronidation which facilitates excretion and detoxification. Halogenated aromatic hydrocarbons are known to induce hepatic mRNA expression and the relative induction levels between sites and variation amongst each site gives valuable information regarding exposure (Hahn, 1998b; Yuan et al., 2001).

Metallothionein (MT) is a hepatic protein that sequesters and binds physiological and xenobiotic heavy metals and is induced in response to exposure to certain heavy metals (Tom et al., 2004). It is a low-molecular-weight (6-7kDa) metal binding protein that consists of a multigene family that has a high capacity to bind metallic cations due to its high content of cysteine. The cysteine sulfhydryl groups are capable of binding Zn^{2+}, Cd^{2+}, Hg^{2+}, methyl-Hg^{+}, Cu^{2+}, Ag^{+} and other cations. Metallothionein is a non-specific biomarker of metal exposure because it can be upregulated by and sequester a variety of heavy metals. Xenobiotic heavy metals that bind to MT are subsequently excreted from the organism.

Vitellogenin I (VTG) and gonadal aromatase are tightly regulated genes involved in reproductive pathway that can be disrupted by exposure to a wide variety of endocrine disrupting compounds. Vitellogenin is a large egg yolk protein precursor produced in the liver of female oviparous fish and shuttled to the gonads where it is cleaved into smaller egg yolk proteins that serve as nutrients for developing follicles (LaFleur et al., 2005). Normally, male fish do not express vitellogenin, which has been shown to be induced in male killifish that were exposed to estrogenic compounds (Pait and Nelson, 2003). Alternatively, expression and protein levels of this reproductive endpoint can also be used to show anti-estrogenic effects in female fish populations. Another commonly used and important reproductive endpoint is aromatase, a cytochrome P450, which are tightly regulated enzymes involved in the conversion of androgens to estrogen. Two isoforms of aromatase are found in the ovaries (CYP19A1) and brain (CYP19A2) of killifish (Greytak et al., 2005). CYP19A2 is predominantly expressed in the brain where it is generally thought to be involved in early development, neuronal development, survival and sexual behavior whereas CYP19A1 is expressed predominantly in the ovary and is generally accepted to be involved in sexual differentiation and oocyte growth. Altered regulation of vitellogenin and/or aromatase (expression and protein) can indicate exposure to endocrine disrupting compounds. Ultimately, exposure to endocrine disrupting compounds can alter development and maturation of the gonads which is a higher level effects compromising the ability of the population to reproduce.

1.6. RESEARCH OBJECTIVES

As previously stated, the purpose of this study was to evaluate a suite of biomarkers in a population of killifish (*Fundulus heteroclitus*) inhabiting the heavily contaminated Newark Bay to (1) develop a biomarker battery that can be used to evaluate fish health in the NY-NJ Harbor Estuary, (2) collect a wide range of observations using biomarkers appropriate for the contaminant history in this ecosystem for comparison with future studies and policy changes implemented in the harbor, (3) recommend improvements for our biomarker assay to advance the application and (4) collect preliminary data on altered gonad development from the NY-NJ Harbor. The primary hypothesis tested was that Newark Bay fish would exhibit significantly altered biomarker responses from the reference population consistent with exposure to pollutants. Findings were also anticipated to determine which class of compounds killifish are being exposed to and determine whether there are biological effects which are therefore impacting fish health.

2.0. MATERIALS AND METHODS

A summary of all biomarkers and endpoints measured are listed in Table 1. Each biomarker and endpoint is discussed in the Introduction.

2.1. ANIMAL PROTOCOLS

All methods for collection, handling and sampling of tissues from the fish were approved by the Rutgers University Animal Rights Committee in accordance with AALAC accreditation and NIH guidelines (Protocol #03-014 and 04-013).

2.2. SITE SELECTION AND ANIMAL COLLECTION

Atlantic Killifish (*Fundulus heteroclitus*) were collected from two estuaries in New Jersey using baited minnow traps. Specifically, 25 fish of breeding size and age were collected from Newark Bay (Richard Rutkowski Park, Bayonne, 40° 41’ 17.0”N and 74° 06’ 42.0”W) and the reference site in Tuckerton (Little Sheepshead Creek, 39° 31’ 17.0”N and 74° 19’ 10.0”W). These specific locations were chosen for their access to wetland habitat where killifish may be caught. Collections were carried out June 27/July 2 for Tuckerton and July 1, 2007 for Newark Bay to coincide with the full moon (July 30, 2007) which is the peak of the killifish's lunar-dependant spawning cycle. Fish were transported immediately back to the laboratory to reduce stress in aerated containers with water from the collection site.

The collection site within Newark Bay is formerly known as the “North 40 Park” and was renamed the Richard Rutkowski Park. Restoration of this wetland was completed by May 2006 and dedicated as a wetland preserve by the City of Bayonne.

The city invested $500,000 to restore the 32 upland acres and 10 wetland acres by widening and deepening the small channels innervating the wetland that were already present to increase tidal flow into the wetland. This wetland was restored to provide additional habitat for aquatic species, including migratory fish and shellfish that will use this habitat for breeding and nursery purposes.

The reference site at Tuckerton is part of the Great Bay Wildlife Management Area (approximately 6,000 acres), which lies on the Northeast shore of Great Bay. Also sharing the Great Bay on the Southwestern shore is the Edwin B. Forsythe National Wildlife Refuge. Tuckerton was chosen as the reference site because these wetlands are relatively pristine and undeveloped. Killifish from this site have served as a reference population for several decades.

2.3. NECROPSY PROTOCOL

Fish were necropsied immediately upon arrival at the laboratory to ensure quality and accuracy of the more sensitive biochemical biomarkers. Each fish was euthanized in a small container of water with an overdose of MS222 and given a unique accession number for that animal. When the animal lost the righting ability it was removed, weighed and measured for total length (snout to tail) and snout to peduncle length. The animal was then examined for external abnormalities and parasites of the skin, gills and fins. The caudal portion of the fish just anterior to the caudal fin was severed and blood was collected into a heparinized micro-capillary tube. A drop of blood was placed on a glass slide and a blood smear was made. An incision was then made along the ventral peritoneal area from the anus to pericardial cavity. The endometrial lining of the

peritoneal cavity was observed for any hemorrhagic areas or other visible lesions. At the same time any grossly visible abnormalities on the liver, spleen, GI tract, and gonadal tissues were observed. The liver was then removed, weighed and divided into two portions, one for histopathology and the second for biochemical analyses (apex of left lobe). The liver was stored in RNA*later*® (Ambion®, Austin, TX), snap frozen and maintained at -80°C until processed for quantitative polymerase chain reaction (qPCR) and immunoblotting. Bile was then collected from the gall bladder when possible, snap frozen, and stored at -80°C for determination of polycyclic aromatic hydrocarbons. The spleen and gonads were then removed and weighed. Gonadal tissue was also divided into two portions, one for histopathology and the second for biochemical endpoints (~100mg). The carcass and organs were saved for histopathology were then fixed in 10% buffered formalin for 48 hours before being stored indefinitely in 70% ethanol. The liver to body weight ratio, gonad to body weight ratio and spleen to body weight ratio were also calculated.

2.4. HEMATOLOGY

Blood samples were collected in heparinized micro-capillary tubes during necropsy and immediately spun on a hematocrit centrifuge for ten minutes. Following centrifugation the percentage of packed cell volume was determined. Any hemolysis in the plasma layer was noted as this can indicate fragility of the red blood cells or improper handling of the blood. Blood smears were stained with a Giemsa Stain (Richard-Allan Scientific, Kalamazoo, MI) to evaluate the presence of micronuclei in the cytoplasm of erythrocytes by counting the prevalence in 1000 red blood cells from each individual in

multiple fields (at least four in different areas). In addition, the staining characteristics of the red blood cells were also noted if there were alterations in the nuclear membrane crenulations. White blood cell differentials were done by counting 100 white blood cells in the blood smear of each individual and calculating the percent of each type of white blood cell.

2.5. HISTOPATHOLOGY

Liver and gonadal tissues were embedded in paraffin and cut into six-micron sections and stained with Hematoxylin and Eosin. The tissues were examined by light microscopy for lesions and other abnormalities. All tissue slides were evaluated without knowing the location history of the animal. All female gonads were evaluated for developmental stages by counting at least 150 developing follicles from each individual and determining the percent of each stage. Follicles were counted as either pre-vitellogenic, early vitellogenic, mid-vitellogenic or mature. Pre-vitellogenic follicles were those as having a cortical alveoli, early vitellogenic follices were those having the formation of yolk globules with cortical alveoli intermixed, mid-vitellogenic follicles were those with a visibly high yolk globule content and cortical alveoli pushed to the periphery of the follicle and mature eggs were those with any amount of fused hyaline yolk material (Figure 6). Each stage of the follicle is roughly two times the size of the previous stage.

2.6. BILE PAH DETERMINATION

Synchronous Fluorescence Spectrophotomety (SFS) methods were used (modified from Ariese et al., 1993; Aas et al., 2000) to quantify naphthalene, pyrene and benzo[a]pyrene as surrogate measures of PAH exposure. Upon removal from the freezer prior to fluorescence the bile was diluted 1:1500 in 50% ethanol and vortexed. A 3mL sample was then placed in a Cary Eclipse Fluorescent Spectrophotometer and the total fluorescence was measured using an excitation/emission wavelength offset of 44nm, an excitation/emission slit width of 5nm/5nm and maximum detector voltage (800v). The spectrum between 250nm-450nm was obtained for quantification of peaks for naphthalene (290nm), pyrene (346nm) and benzo[a]pyrene (380nm). Intensities of these peaks were used to semi-quantify concentrations of these compounds.

Standard curves of β-naphthol (Sigma-Aldrich, St. Louis, MO), 1-hydroxypyrene (Sigma-Aldrich, Milwaukee, WI) and 3-hydroxybenzo[a]pyrene (Midwest Research Institute, Kansas City, MO) were generated using detectable concentration ranges of 0.1-10μM, 1-50nM and 1-100nM, respectively. Concentration estimates were converted and recorded as mass of standard equivalent per microliter of bile. Threshold values were set at 5ng/μL for naphthalene, 0.5ng/μL for pyrene and 50pg/μL for benzo[a]pyrene. Any sample below these set detection limits were recorded as threshold values for all analyses. A standard sample was also scanned daily to ensure spectrophotometer variation from day to day was not an issue.

2.7. RNA AND PROTEIN ISOLATION

Total RNA was extracted as described by Chomczynski & Sacci (1987). Approximately 100mg of each frozen liver and gonadal tissue was directly homogenized in 1mL TRIZOL® Reagent in 1.5mL microcentrifuge tubes (RNase, DNase, pyrogen and DNA free). Homogenates were left to sit at room temperature for 5 minutes and then 0.2mL of chloroform was added, vortexed and incubated at room temperature for 3 minutes. Samples were then centrifuged at 14,000 x g for 15 minutes at 4°C. The aqueous phase (top layer) was transferred to new 1.5mL microcentrifuge tubes and precipitated with 0.5mL of isopropyl alcohol. Samples were incubated for 10 minutes at room temperature and then centrifuged at 12,000 x g for 10 minutes at 4°C. Supernatant was discarded and the pellets were washed and suspended with 0.1mL of cold 70% ethanol. The samples were then centrifuged at 7,500 x g for 5 minutes at 4°C. The supernatant was discarded and RNA pellets were left to air dry before being resuspended with an appropriate volume of 0.1% diethyl pyrocarbonate (DEPC) treated water. Each sample was incubated at 60°C for 10 minutes to fully dissolve the RNA before concentrations of each sample were determined on a UV spectrophotometer (Beckman, Germany). Representative RNA samples were analyzed with gel electrophoresis to verify mRNA integrity. Integrity was determined by loading 6μg of total RNA on a 1% denaturing agarose gel cast in 1X MOPS containing 1% (v/v) formaldehyde to be electrophoresed and stained with ethidium bromide. The gel was visualized under UV illumination to assess quality of the 28S and 18S ribosomal RNA bands.

Cytosolic protein and microsomal protein were isolated through differential centrifugation. Approximately 100mg of each frozen liver was homogenized in a

microcentrifuge tube on ice with 150µL RIPA buffer composed of 50mM Tris-HCl pH 7.4, 150mM NaCl, 0.5M EDTA, 0.5M EGTA, 1% Igepal, 0.1% SDS, 10mM $NaMoO_4$, 1mM Na_3VO_4, 40mM NaF, 1mM PMSF(EtOH) and Sigma P8340 Protease Inhibitor cocktail (100µL in 10mL final volume). Tissues were incubated on ice for 10minutes to allow complete lysis of the tissue with intermittent inversion. The homogenate was then centrifuged at 10,000 x g for 10minutes at 4°C to pellet cellular debris and nuclear complexes. The resulting supernatant was then removed, placed into an ultramicrocentrifuge tube and centrifuged 108,000 x g for 90minutes at 4°C in a Beckman Optima™ TLX Ultracentrifuge. Upon completion of the spin the lipid layer formed on the surface was removed if present. The supernatant (cytosolic proteins) were saved for vitellogenin immunoblotting and the microsomal pellet resuspended in 100µL of RIPA buffer by homogenizing on ice with vortexing if necessary.

2.8. MESSENGER RNA ANALYSIS

A portion of each RNA sample (10µg) was treated with DNA-free (Ambion®, Austin, TX) to remove any residual genomic DNA. Reverse transcription was then performed on 1µg of DNase treated RNA to create cDNA template necessary for qPCR using iScript™ cDNA Synthesis Kit (BIORAD, Hercules, CA).

Genes targeted for mRNA quantification with qPCR included β-actin, cytochrome P4501A (CYP1A), metallothionein (MT), vitellogenin I (VTG) and the gonadal aromatase cytochrome P45019A1 (CYP19A1) (Table 2). PCR products for each primer pair were confirmed by verifying the correct anticipated amplicon size with electrophoresis on a 1.4% agarose gel made with 0.5X tris-borate EDTA (TBE). Melt

curves were also used to confirm no production of secondary products for all genes and in all samples. qPCR was carried out using 100ng total cDNA template using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) for amplification and Bio-Rad iCycler iQ real-time PCR Detection System (Bio-Rad, Hercules, CA) for fluorescent detection. Each gene was run in triplicates to use average threshold values for quantification.

Standard curves were generated by using serial dilutions (0.1ng template-0.00001ng template) of each gene. Threshold cycle (CT) values were used for quantification. For each group of samples amplified, β-actin was included for each sample to standardize all samples to the median β-actin CT value of the group. This method normalizes all genes to β-*actin*, a reference housekeeping gene that is purported to remain constant throughout a population. Normalized CT values were used to quantify each template and were expressed as nanograms of mRNA per microliter of template.

Threshold concentrations for VTG and CYP19A1 were set at detection limits of $1.5x10^{-7}$ ng/µL and $2.0x10^{-8}$ ng/µL, respectively. These values were threshold for the linear range of the instrument. Data below these values were set at the detection limit.

2.9. PROTEIN ANALYSIS

Protein samples were quantified by the Modified Lowry Protein Assay (Pierce Biotechnology) using a bovine serum albumin standard immediately before immunoblotting. For CYP1A analysis 10µg of microsomal protein for each sample was reduced with 1.5µL of 500mM dithiothreitol for 10 minutes at 80°C. Samples for CYP1A analysis were separated on a Novex 4-12% Bis-Tris SDS polyacrylamide gel (Invitrogen) for 60 minutes using 150volts. For vitellogenin analysis 75µg of cytosolic

protein was reduced with 1.5µL of 500mM dithiothreitol for 5 minutes at 55°C. Samples for vitellogenin analysis were separated on a Novex 3-8% Tris-Acetate SDS polyacrylamide gel (Invitrogen) for 60minutes using 150volts. The resulting gels were then transferred to an Immobilon PVDF membrane for 3.0 hours using 150 milliamperes (constant) in 1X transfer buffer (25mM Tris-Base, 192mM Glycine, 20% methanol). Membranes were stained with ponceau and photographed to demonstrate equal loading of samples in each lane. After staining they were blocked for 60 minutes in 5% non-fat milk dissolved in 1X TBST (200mM NaCl, 50mM Tris, 0.1% Tween-20). CYP1A membranes were then incubated at 4°C for approximately 20 hours in 5% non-fat milk in 1X TBST with 1.0µg/mL CYP1A (scup) monoclonal antibody 1-12-3 (gift of Dr. John J. Stegeman, Woods Hole, MA). Vitellogenin membranes were incubated at 4°C for approximately 20 hours in 5% non-fat milk in 1X TBST with vitellogenin (striped-bass) monoclonal antibody ND-1C8 diluted 1:500 (Cayman Chemical). Membranes were washed 3 times for 10 minutes in TBST (350mM NaCl, 50mM Tris, 0.1% Tween-20) and then incubated at room temperature in 1:5000 (CYP1A) or 1:2000 (vitellogenin) ECL anti-mouse IgG Horseradish peroxidase-linked whole antibody (NA931, GE Healthcare) with 5% non-fat milk in 1X TBST. Membranes were washed 2 times for 10 minutes in TBST (350mM NaCl, 50mM Tris, 0.1% Tween-20) and once for 10 minutes in water. The Amershasm ECL Advance Western Blotting Detection Kit (RPN2135, GE Healthcare) was used to detect immunoreactivity.

Bands were quantified by densitometry with ImageJ (Abramoff et al., 2004) and converted to relative intensity to a standard sample. For CYP1A analysis an individual from Newark Bay was selected and run on all gels for the normalization of all samples to

the CYP1A signal of a single sample. For vitellogenin, two samples of purified rainbow trout vitellogenin (Vtg-51, Biosense Laboratories, Bergen, Norway) was run on each gel (1:5 and 1:10 dilution of stock). All samples were normalized to the 1:10 stock sample vitellogenin signal. Traditionally protein analyses of killifish has been normalized to total protein mass loaded rather than an internal standard (such as β-actin or GAPDH). β-actin was not chosen for normalization for several reasons. First, there is no known reactive antibody for β-actin in killifish. Secondly β-actin is not expected to be isolated and present in hepatic microsomes. Thirdly, histopathology of both Tuckerton and Newark Bay livers indicates a high prevalence of necrosis which can alter β-actin levels.

2.10. STATISTICAL ANALYSES

All data was separated by gender and statistical tests were performed using SigmaStat™ version 1.01. A p-value< 0.05 is regarded as significantly different from control. Unpaired t-tests were used to compare two different treatments. When normality failed, a Student-Neuman-Keuhls test was used. When equal variance failed a Mann-Whitney Rank Sum test was used. Discrete data such as that obtained from histopathology were run using Chi Square Analysis or Fisher's Exact Test.

3.0. RESULTS

Refer to Table 1 for information regarding the purpose and endpoint of each biomarker. Table 3 contains the summary data for all biomarker parameters measured and the number of animals analyzed for each biomarker and group.

3.1. GROSS AND EXTERNAL OBSERVATIONS

In all fish examined there were no major ulcerations, fin erosions or major external lesions. Some fish had livers with petechial hemorrhages, a mottled appearance and/or pale coloration (10-15% of fish at both sites). However there were no significant differences between populations and therefore no correlation for exposure. The majority of female fish from Newark Bay were initially described as having small or undeveloped gonads with few to no mature eggs. Efforts to strip spawn female killifish in the field at Newark Bay failed to procure eggs at the time of collection, and several other attempts during the breeding seasons of summers 2007 and 2008 when at the same time Tuckerton females were producing large numbers of viable eggs. Young of the year were however observed in the field at both sites although in seemingly greater numbers at Tuckerton.

3.2. MORPHOMETRICS AND CONDITION INDICES

There were no significant differences between sites when comparing to average snout to peduncle length, average snout to tail length, average body weight, average liver weight, average length to body weight ratio, average liver to body weight ratio and spleen to body weight ratio for either gender (Table 3). However, both genders at Newark Bay exhibited significantly decreased average gonad weight and average gonad to body

weight ratio in comparison to Tuckerton (Figure 7). Spleen weight of males at Newark Bay (9.4±4.1mg) was significantly decreased compared to Tuckerton males (16.1±4.5mg).

Females at Newark Bay had higher average snout to peduncle lengths, average snout to tail lengths and average liver weights than males while there were no gender differences at Tuckerton (Table 3). Females had significantly higher liver to body weights than males at both sites.

3.3. HEMATOLOGY

There were no differences for hematocrit between populations for either gender (Table 3). There were no differences between sites for presence of red blood cells with crenulated nuclei (Figure 8). Females at Newark Bay had a significant 3-fold lower prevalence of micronuclei in red blood cells (Figure 8). Photomicrographs of micronuclei and crenulated nuclei are shown in Figure 9. Although not significant, there was a trend that Newark Bay males had a decreased prevalence of micronuclei and crenulated nuclei. Newark Bay males had approximately an 11-fold average lower prevalence of micronuclei ($p=0.06$) and approximately 3-fold lower average prevalence of crenulated nuclei ($p=0.07$).

White blood cell differentials were evaluated qualitatively by looking for major shifts in cell types in the differentials (Figure 10). Photomicrographs of several white blood cells are shown in Figure 11. Overall, Newark Bay appeared to have a higher percent of eosinophils although this is not statistically proven. Males from both populations generally had fewer eosinophils, fewer lymphocytes and more thrombocytes

than females. There was a high variation in the types of white blood cells which some individuals having 100% eosinophils while some individuals having a very low percent of eosinophils and presence of several other types of leukocytes. Anecdotally, there was a large difference in the time needed to count 100 lymphocytes per animal between Tuckerton and Newark Bay fish, with Tuckerton taking longer time than Newark Bay animals.

3.4. HISTOPATHOLOGY

There were no major significant differences in the liver histopathological findings between sites and genders (Table 4). However, there was a high prevalence of necrosis, macrophage aggregates and lymphocyte infiltrate in both hepatic and pancreatic tissue of both populations and genders, indicating a high degree of damage in both populations (Figure 12). All individuals examined had at least one or all of these pathologies in the pancreas (Table 5). Although not statistically significant, both males and females at Tuckerton had a 25% prevalence of parasitism compared to Newark Bay males and females (both 0%). This was not significant probably due to the small sample size, but qualitatively it demonstrates that parasitism is present in the Tuckerton population although not seen in the Newark Bay killifish.

Histopathological evaluations of the testis showed a high prevalence of thickened walls, macrophage aggregates and empty follicles at both sites (Figure 13). Photomicrographs of these commonly observed lesions in the testis are shown (Figures 14). Newark Bay tended to have a higher prevalence of these lesions however this was not significant (Table 6). All animals evaluated from Tuckerton exhibited normal

spermatogenesis evident by a wide margin of follicles containing spermatocytes at the periphery of the testis. In contrast, all males from Newark Bay had a markedly reduced margin of follicles containing spermatocytes (Figure 14).

Ovaries of Newark Bay fish were significantly less developed than Tuckerton fish of the same size class. Representative photomicrographs for Tuckerton and Newark Bay ovaries are shown (Figures 15 and 16). Newark Bay had both an increased percentage of follicles at the pre-vitellogenic stage (43% at Tuckerton, 64% at Newark Bay) and a decreased percentage of follicles at the mid-vitellogenic (22% at Tuckerton, 17% at Newark Bay) and mature stages (25% at Tuckerton, 3% at Newark Bay) indicating altered follicle development (Figure 17).

3.5. BILE PAH CONCENTRATIONS

For all compounds measured, Newark Bay females consistently had significantly higher bile concentrations than Tuckerton females (Figure 18). Newark Bay males had only significantly higher concentrations of pyrene (5.11±2.74ng/µl) than Tuckerton males(1.07±0.87ng/µl), although there was a trend for naphthalene ($p=0.06$) and benzo[a]pyrene ($p=0.07$) showing higher concentrations in Newark Bay males. There were no significant gender differences for any PAH at either site. Table 7 shows the fold difference between concentrations at Newark Bay over those at Tuckerton for all PAHs. Newark Bay consistently had higher concentrations for all compounds measured and for both genders, although not significant for naphthalene and benzo[a]pyrene between male populations.

3.6. MESSENGER RNA AND PROTEIN EXPRESSION

Hepatic CYP1A mRNA expression was significantly induced in both genders of Newark Bay compared to expression levels at Tuckerton (Figure 19). Males from Newark Bay had approximately a 7-fold higher mRNA expression while females from Newark Bay had approximately a 3-fold higher mRNA expression (using median values). The distribution plot shows the large variation in expression seen in field populations, both sites having a range of over 10 fold (Figure 19). There were no gender differences in CYP1A expression at either site. Immunoblot analysis of microsomal CYP1A protein showed similar trends. The median relative CYP1A protein signal to the standard for Newark Bay males was approximately 5-fold higher than Tuckerton males and 8-fold higher in Newark Bay females in comparison to Tuckerton females. (Figures 20 and 21). Females at Tuckerton had significantly lower CYP1A protein than males, however, there were no gender differences at Newark Bay.

Metallothionein expression yielded no significant differences between genders or sites (Figure 22). This indicates no exposure of Newark Bay killifish to heavy metals known to induce metallothionein expression (cadmium, chromium, mercury).

Hepatic vitellogenin I mRNA expression was significantly higher in females than males for both sites (Figure 23). There was also a significant difference between female populations. Expression of vitellogenin mRNA in Newark Bay females was approximately 6 times lower than that of Tuckerton females (using median values). The distribution plot below also shows how tightly regulated vitellogenin is in the Tuckerton female population in comparison to Newark Bay females whose expression spans over 3-orders of magnitude. The immunoblot analyses of hepatic cytosolic vitellogenin levels

agree with the mRNA expression results. Males at both sites do not express vitellogenin at detectable levels. Newark Bay females have significantly decreased levels of vitellogenin protein, 27-fold lower than Tuckerton females (Figures 24 and 25).

Both genders at Newark Bay had significantly different gonadal aromatase mRNA expression than Tuckerton (Figure 26). Aromatase mRNA expression in Newark Bay Females was approximately 210-fold higher (using median values) than Tuckerton females. Expression in Newark Bay males was approximately 3-fold lower than Tuckerton males. Females at Newark Bay were also significantly higher than male although there was no significant different between genders at Tuckerton.

4.0. DISCUSSION

In summary, findings indicated that killifish from Newark Bay are being exposed to increased levels of PAHs and exhibit higher constitutive AHR pathway activity than reference fish indicating chronic exposure in this population. Newark Bay fish also exhibit endocrine disruption in males and females, demonstrated by decreased gonad to body weight ratio, altered gonad development and altered biochemistry (vitellogenin and aromatase). Overall, the two populations had similar disease states of the liver, pancreatic and gonadal tissues tissues which was not expected.

4.1. DEMOGRAPHICS AND GENERAL FISH HEALTH

Biomarker assessments must meet the condition that population samples are of the same age and size demographic so that data from each sampling population are comparable. Tuckerton and Newark Bay killifish collected were no different with respect to the basic morphometric measurements body length, body weight, length to body weight ratio, liver weight and liver to body weight (Table 3). This is especially important when assessing reproductive health so that differences between populations are due to differences in environmental quality rather than reproductive age. In order to ensure valid biomarker comparisons for assessing reproductive health, killifish were collected from both sites at the peak of the full moon, when killifish are at the peak of their reproductive cycle.

4.2. CONTAMINANT EFFECTS AND PAH EXPOSURE

Newark Bay fish exhibited significantly higher levels of hepatic CYP1A mRNA expression and protein than Tuckerton fish (Figures 19 and 20). These findings have been previously demonstrated in this population (Prince and Cooper, 1995a/b) indicating the chronic exposure of killifish to AHR agonists. Females in Newark Bay were highly variable with several individuals forming small sub-populations having similar CYP1A mRNA expression (3 of 13 animals, or 23%) and protein levels (4 of 13 animals, or 31%) to reference values. These small sub-populations of non-responders are significantly different from the remaining females at Newark Bay (distribution plots of Figures 19 and 20). This is demonstrated in Figure 21, where sample 12 is a female having no immunoreactivity for CYP1A. The individuals in the Newark Bay population with levels roughly equal to Tuckerton (low levels) may represent non-responders, individuals that are more resistant to AHR-mediated toxicity than the rest of the population that exhibit high levels of CYP1A. However, because of the number of individuals in this sub-population (3-4 animals), bile data is not available for all of these animals (only 1-2 have bile PAH data). Therefore this study was not able to ascertain whether these animals have lower concentrations of PAHs.

Results from the bile PAH analysis are reflective of exposure to the extensive PAH contamination known to be present throughout this ecosystem. All compounds measured (pyrene, naphthalene, benzo[a]pyrene) were 4-9 fold higher in Newark Bay fish (Figure 18), and although significant for all 3 compounds in females, only pyrene was significantly higher in Newark Bay males compared to Tuckerton males (Table 7). Pyrene in particular was found at significantly higher concentrations in both genders of

Newark Bay which is commonly reported in bile PAH studies. These results suggest killifish are being exposed to carcinogenic PAHs.

Due to the high levels of PAHs in the bile, an increased prevalence of micronuclei and crenulated nuclei was expected to be found in the red blood smears of Newark Bay fish. Micronuclei are the result of genotoxic events, and an increased prevalence of micronuclei has been correlated with exposure to a wide variety of contaminants including PAHs (Al-Sabti and Metcalfe, 1995; Aas et al., 2000). However, both micronuclei and crenulated nuclei tended to be lower in Newark Bay fish, but only micronuclei in Newark Bay females was significantly lower than in Tuckerton females (Figure 8). This may be due to development of a resistant phenotype to genotoxic compounds through genetic selection in Newark Bay fish as a survival adaptation from living in a chronically contaminated habitat. This may be possible by increased elimination of contaminants or by altered phase I and phase II metabolism leading to decreased production or accumulation of genotoxic metabolites. The Newark Bay killifish have been previously demonstrated to have altered response of CYP1A phase-I metabolism to agonist exposure (Prince and Cooper 1995a/b). Based on these results, it is recommended that alternative genotoxicity biomarkers be evaluated in future studies. PCR-based techniques have been developed for detecting and quantifying DNA damage in killifish (Jung et al., 2008). This method has the advantage of being more sensitive, as well as faster than blood smear-based assays, and relatively inexpensive. In addition, this method can quantify tissue specific DNA damage (e.g. liver, gonads, kidney, etc.).

Newark Bay fish did not exhibit a significantly increased prevalence of parasites, macrophage centers, necrosis, vacuolization in hepatocytes, hemosiderin/lipofuschin

deposits, basophilic/eosinophilic foci, hyaline deposits, lymphocyte infiltrate or granulomas, compared to Tuckerton fish. Newark Bay fish did not exhibit any neoplastic or pre-neoplastic lesions indicative of exposure to carcinogenic contaminants (Tables 4 and 5). These findings demonstrate that killifish inhabiting the Newark Bay are not exhibiting an increased prevalence of liver lesions, including neoplastic or pre-neoplastic lesions. These findings are similar to those of the micronuclei biomarker, and also support the hypothesis that Newark Bay killifish may have adapted to living in this contaminated habitat by having developed altered metabolism of carcinogenic compounds to decrease liver lesions. Both populations exhibited a high degree of necrotic damage to both hepatic and pancreatic tissues overall with several fish being highly parasitic and almost all had an abundance of macrophage centers (Figure 12). Newark Bay fish had a more active immune response (as measured by time-to-count in the white blood cell differentials). Prevalence of macrophage centers and parasites in the pancreas and liver were statistically similar (Tables 4 and 5), probably due to similar metazoan parasites being present in bird populations at both locations. Although not significant, Newark Bay males and females exhibited a 0% prevalence of hepatic parasites compared to Tuckerton (25% in males and females). This was not significant probably due to the small sample sizes. Alternatively, Tuckerton may have a larger bird community (Great Bay Wildlife Management Area and Edwin B. Forsythe National Wildlife Refuge). Overall, Newark Bay fish had similar disease states of liver and pancreatic tissue, which was unexpected for fish living within sediments containing carcinogenic PAHs and dioxins at Newark Bay. This may be due to a decreased sensitivity to contaminants in the Newark Bay population as a survival adaptation.

However, because of the small sample size of this study the prevalence of lesions with low occurrence is difficult to estimate.

4.3. REPRODUCTIVE HEALTH OF FEMALES

Field observations in the summers of 2007 and 2008 demonstrated that female killifish at Newark Bay were unable to be strip spawned because they produced fewer eggs compared to female killifish at Tuckerton. Gonad to body weight ratio was decreased in Newark Bay (0.041±0.023) compared to Tuckerton (0.089±0.033). This was an early indicator of impaired reproductive health in females at Newark Bay. Histological analysis of the female gonads confirmed field observations. Qualitatively, Newark Bay females exhibited markedly different gonads with follicles that did not appear to develop to maturity (Figures 15 and 16). Quantitative analysis of the 4 follicular stages of vitellogenic growth demonstrated that Newark Bay females exhibited significantly impaired gonad development. Newark Bay females had significantly decreased percentages of eggs at the pre-vitellogenin, mid-vitellogenin and mature stages of follicular development (Figure 3). Few Newark Bay female follicles developed to maturity (3%) compared to Tuckerton females (25%). Conversely, Newark Bay females had an increased percentage of follicles at the pre-vitellogenic stage (64%) compared to Tuckerton females (43%). Follicle formation was occurring normally in Newark Bay females (as demonstrated by the presence of pre-vitellogenic follicles) which indicates that early folliculogenesis was normal. However this suggests that the ovaries were forming follicles which are not then stimulated to initiate vitellogenic growth. Histology results demonstrate that the Newark Bay female ovaries were arrested in steps 4 and 5 of

teleost oogenesis, the growth and maturation of oocytes (events outlined in Figure 6). At this stage pre-vitellogenic follicles were not being stimulated to develop into early or mid-vitellogenic follicles. Vitellogenin mRNA expression and protein levels support these findings (Figures 23 and 24). Decreased vitellogenin production in Newark Bay females may be the reason for the inhibition of oocyte development (6-fold lower hepatic VTG mRNA and 27-fold lower hepatic VTG protein). Interestingly, aromatase expression in the Newark Bay females was significantly higher than in the reference population by 210-fold (Figure 26). These findings suggest that estrogen synthesis was active in the ovaries of the Newark Bay fish (specifically, in the granulosa cells of the follicle) at the aromatase mRNA level due to being stimulated by gonadotropins (from pituitary). However, this is not leading to a subsequent activation of vitellogenesis in the liver. Because of this lack of vitellogenesis activation, the follicles in the gonads do not progress beyond the pre-vitellogenic stage, leading to few mature eggs in Newark Bay females. Based on histopathological and biochemical biomarkers it is concluded that the endocrine disruption exhibited in Newark Bay killifish is anti-estrogenic.

4.4. AHR-ER CROSSTALK

This study has demonstrated a correlation between exposure to AHR agonists and impaired reproductive health and proposes a cross-talk mechanism between the two pathways that explains the decreased vitellogenin production and altered ovary development (Figure 27). Other studies have demonstrated a correlation between exposure to AHR agonists (such as dioxins and PAHs) and decreased reproductive health in finfish (Navas and Segner, 2000; Bemanian et al., 2004; King Heiden et al, 2006;

Mortenson and Arukwe, 2007). Other studies in higher vertebrates have demonstrated direct crosstalk between the AHR pathway and ER pathways. AHR activation is now suspected of directly down-regulating the ER pathway in several ways. The non-genomic mechanism of crosstalk involves the AHR complex binding directly to the ER in the nucleus which leads to ubiquitination and proteasomic degradation of the AHR-ER complex (Wormke et al., 2004). The genomic mechanism of crosstalk involves AHR binding to DNA regions such as inhibitory xenobiotic response elements (iXREs) which lies both upstream of the gene and downstream of estrogen response elements (EREs), thereby interfering with ER-dependent gene translation (Safe et al., 2000). Similar mechanisms may underlie the decreased vitellogenin production seen in Newark Bay females. Newark Bay killifish exhibit significantly higher CYP1A expression and protein levels which is a biomarker for exposure to AHR agonists. Bile fluorescence also shows exposure of Newark Bay killifish to PAHs, known inducers of the AHR pathway. This data suggests that exposure to a complex mixture of AHR agonists (PAHs and dioxins) may be down-regulating hepatic vitellogenesis, which is ER-dependent.

Alternatively, the phenotype seen in the Newark Bay females may be due to decreased aromatase activity. Exposure to benzo[a]pyrene has been demonstrated to inhibit gonadal aromatase activity in killifish (Patel et al., 2006). While results suggest that aromatase expression is up (210-fold in Newark Bay females), estrogen production (17β-estradiol) may be decreased. However, this study does support this possibility and does not have data regarding gonadal aromatase activity, circulating sex steroids or estrogen receptor levels, which is necessary to determine at which point vitellogenesis and sex steroid signaling is disrupted.

Ultimately, due to the nature of complex mixtures of contaminants in Newark Bay, the anti-estrogenic endocrine disruption may be the cumulative result of many mechanisms of action. However, due to the increasing body of knowledge of AHR-ER crosstalk, and the correlation between high AHR activity and low vitellogenin production in this study, crosstalk between AHR contaminants with the ER is the proposed mechanism of vitellogenesis disruption in Newark Bay females (Figure 27).

4.5. REPRODUCTIVE HEALTH OF MALES

The gonad to body weight ratio was significantly decreased in Newark Bay males (0.023±0.005) compared to Tuckerton males (0.035±0.007). Histology of the male gonads did not demonstrate significant differences in pathologies (thickened walls, macrophage centers, empty follicles, hemosiderin) between sites suggesting that Newark Bay and Tuckerton males have similar gonad disease states (Table 6). Newark Bay males did however tend to have a slightly higher prevalence for all lesions. Sample sizes for males were small (8 at Tuckerton, 10 at Newark Bay) which increases uncertainty regarding the prevalence of these lesions. Gonad development however was significantly different between sites. All Newark Bay males exhibited a decreased margin of peripheral follicles at the primary spermatocyte stage. Tuckerton normally exhibited a wide margin of follicles at the primary spermatocyte with spermatozoa and spermatid follicles at the center of the tissue. Follicles containing spermatids were present in Newark Bay males, however there was an increased prevalence of empty follicles (although not significant at $p<0.05$). These findings indicate that males at Newark Bay have a decreased reproductive capacity due to impaired gonad development. In addition,

males at Newark Bay did not exhibit vitellogenin production, suggesting no exposure to estrogenic contaminants.

5.0. CONCLUSIONS

The hypothesis tested was that Newark Bay killifish, which are chronically exposed to multiple contaminants, would have altered biomarker responses when compared to a reference killifish population and that this could result in impacts at the population or higher organization levels within the ecosystem (Figure 2). The major findings of altered biomarkers and the biological ramifications in Newark Bay killifish are summarized in Table 8. Burger (2006) discussed how biomarkers that measure organism health at the sub-organismal level ultimately have ramifications on higher levels of biological organization, such as population and community structure. Many of the altered endpoints in Table 8 ultimately reduce the overall health of the individual fish by affecting parameters essential for survival and reproduction, such as, nutritional state, metabolism, fecundity, lifespan, growth, and behavior. This compromises the population by affecting overall growth rate, survival rate, demographics, biomass and genetic diversity. It is also likely that the biological effects observed in the Newark Bay killifish population occur in other fish within the NY-NJ Harbor, and other urban harbors with similar anthropogenic inputs. Impacted health of killifish is important considering *Fundulus heteroclitus* is much more resistant to contaminant effects than other aquatic organisms, which suggest that species that are less tolerant may have difficulty in surviving.

This study demonstrated that killifish inhabiting the Newark Bay had significantly higher levels of PAHs in their bile (Figure 18 and Table 7) than bile collected from the reference site fish. The increased bile levels of PAHs were not surprising since high concentrations of polycyclic aromatic hydrocarbons have been previously shown to be

widespread throughout the Newark Bay system (Litten, 2003; Valle et al., 2007). Chronic exposure to complex mixtures of PAHs and dioxins has led to high levels of hepatic CYP1A expression and protein (Figures 19 and 20) in Newark Bay killifish. Constitutive up-regulated CYP1A expression may be energetically costly, leading to decreased organism health, behavior, and ultimately survival.

Reproductive health of Newark Bay killifish was severely impacted as demonstrated by significantly decreased gonad weight and gonad to body weight ratios in both sexes (Table 3), altered morphology of the testis, altered gonad development in females (Figure 17), altered aromatase expression in both sexes (Figure 26) and decreased vitellogenin production (Figures 23 and 24) in females. The results from this study demonstrated the presence of endocrine disruption effects and impaired reproductive health in both males and females at Newark Bay.

Results of reproductive biomarkers in females suggest that aromatase estrogen synthesis is active at the mRNA level, but is unable to stimulate down-stream events like vitellogenin production, which the developing early-vitellogenic oocytes are dependent for development. Up-regulation of AHR has been previously demonstrated to cross-talk with the ER by binding to the ER complex (leading to degradation) or by binding downstream of the ERE thereby inhibiting ER activation of ER-dependent genes (i.e. vitellogenin). It could be hypothesized from the above data that PAH exposure (or general AHR activity) may be altering the ER-signaling pathway of Newark Bay killifish in an antagonistic way by interfering with ER activation of the vitellogenin gene and ultimately inhibiting gonad development of vitellogenin-dependent follicles (Figure 27).

As previously discussed, the anti-estrogenic effect exhibited in females may be due to AHR disruption of ER signaling, which may preclude vitellogenin from being a valid biomarker for exposure to estrogenic compounds in Newark Bay males. Therefore, this study does not rule out the possibility for exposure of killifish to estrogenic compounds, as measured by vitellogenin production in males, and therefore requires further investigation. In addition to these molecular findings, Newark Bay males exhibited altered testis development and decreased gonad weights which suggested that males have a reduced ability to produce spermatozoa.

The reproductive health of killifish is concluded to have been impacted by exposure to endocrine active contaminants in the NY-NJ Harbor. This study demonstrated that both males and females have a significantly decreased capacity to successfully reproduce. This study also suggests that PAH exposure (more likely, general AHR activity) may be regulating the ER-signaling pathway of Newark Bay killifish in an antagonistic way by interfering with ER activation of the vitellogenin gene (Figure 27).

Chronic contamination by complex mixtures of organic compounds in the NY-NJ Harbor has led to an acquired resistance in Newark Bay killifish (Prince and Cooper 1995a/b). This may explain why Newark Bay killifish in this study had similar disease states of the liver and pancreas as the reference population. Altered metabolism of carcinogens and genotoxicants known to be present in this harbor may be an acquired adaptive response to decrease the prevalence of toxicant associate pathologies in this population (demonstrated by liver histology and red blood cell micronuclei). This study also demonstrated the variability in biomarker responses (i.e. vitellogenin expression and

protein levels) in a wild population and how there can be responders and non-responders in a population of killifish inhabiting a contaminated system. In addition to histopathology findings, approximately 20% of killifish from Newark Bay exhibited CYP1A expression and protein levels similar to Tuckerton fish (Figures 19 and 20). Approximately 20-30% of the female killifish from Newark Bay in this study also exhibited vitellogenin levels similar to the reference population (Figure 23 and 24). These sub-populations are considered to be relatively normal and represent non-responders – individuals that are exposed to contaminants but are resistant to the effects and function at a relatively normal capacity because of acquired resistance and/or variability in the population's hardiness. In addition, the average percent of follicles reaching maturity in the Newark Bay female ovaries was 3%, several individuals had as high as 10% mature follicles (compared to Tuckerton females which had 25% mature follicles). This also explains how a population such as Newark Bay can sustain itself despite the population's significantly impaired reproductive capacity. This is supported by field observations in 2007 and 2008 that noted the presence of young-of-the-year killifish, regardless of the inability to strip spawn adults in the field. It is assumed that a small percentage of the population is performing all reproductive duties to carry the population to the next generation, which may have led to genetic bottlenecking.

If the mechanism of endocrine disruption proposed in Figure 27 is a plausible explanation of impaired reproductive health in killifish at Newark Bay, then these findings emphasize the need for sediment remediation prior to repopulation efforts in the NY-NJ Harbor. However, further studies are required to confirm this proposed mechanism of AHR-mediated estrogen receptor disruption. The collection site (Richard

Rutkowski Park, see Materials and Methods) is a small restored wetland within Newark Bay. Despite habitat restoration efforts, toxicants in the harbor continue to impact this population of fish. This stresses the importance of sediment remediation and toxicant reduction in the harbor to increase the probability of success of aquatic species repopulation efforts. While habitat restoration and conservation is an important first step in restoring an ever-shrinking urban ecosystem, sediment remediation is necessary to improve environmental quality to the point where finfish and shellfish are able to repopulate without contaminant related reproductive health impacts.

Integrated Biomarker Assessments (IBAs) are essential for long-term monitoring of both finfish health and the effectiveness of remediation efforts in the NY-NJ Harbor Estuary, as it pertains to contaminant impacts. This study has laid the groundwork for development of a standardized IBA for the NY-NJ Harbor Estuary by establishing baseline data on many common biomarkers used for assessing fish health. This study also exemplifies the need for an integrated biomarker approach to comprehensively measure fish population health. No single biomarker is useful alone in habitats with complex mixtures and many possible biological outcomes. One challenge with the development and application of an IBA in the NY-NJ Harbor Estuary is the ever-changing contaminant profile and the many possible resulting biological impacts. However, many toxicants present are legacy contaminants (i.e. dioxins/furans, PCBs, heavy metals) or are contaminants likely to be an ongoing problem in this heavily urbanized/industrial area (i.e. PAHs). This study will serve as a basis for an IBA for biological impacts of types of compounds in finfish of the NY-NJ Harbor Estuary and will complement Sediment Quality Triad assessments. However, another challenge

facing the development of a standardized IBA for the NY-NJ Harbor Estuary will be emerging contaminants and the addition of appropriate biomarkers for future contaminants of concern, which have biological effects that require alternative biomarkers in order to study their biological impact.

6.0. FUTURE STUDIES

The presence of an anti-estrogenic phenotype in Newark Bay females is a novel finding for this population and is hypothesized to be due to chronic exposure to AHR agonist contaminants and subsequent ER degradation by AHR crosstalk. This is an area requiring further study to understand reproductive impacts by AHR agonists as well as how reproductive pathways differ in chronically exposed populations. Alternatively, complex mixtures of endocrine disrupting compounds may also be the cause of the decreased reproductive health in killifish at Newark Bay. Large numbers of endocrine active compounds may be coming through sewage treatment plants in the NY-NJ Harbor Estuary which are contributing endocrine active contaminants to the system and are ultimately affecting finfish reproductive health.

Challenge studies of killifish to 17ß-estradiol will allow for the determination of estrogen receptor pathway functionality in the Newark Bay population (both males and females). This approach can further characterize how the hypothalamus-pituitary-gonadal axis is altered in a chronically contaminated population. The assay will also provide a method for studying the cause and effect relationship between contaminant exposure (AHR agonists) and the disruption of folliculogenesis and vitellogenesis. Co-treatments of 17ß-estradiol and AHR agonists can be used to determine inhibition potential of select compounds (PAHS and dioxins) known to be present in the NY-NJ Harbor.

Figure 1. Satellite image of the NY-NJ Harbor Estuary and its tributaries. The sampling site at Newark Bay, NJ, is circled. The reference site at Tuckerton, NJ, is not shown.

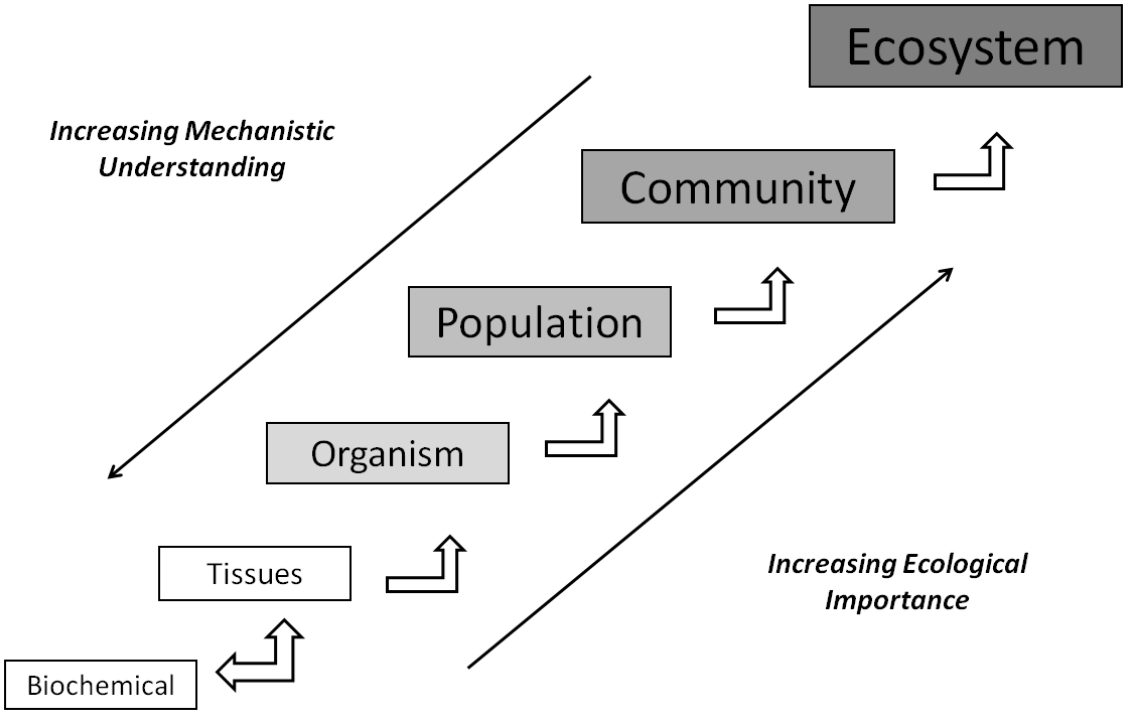


Figure 2. Biomarker relationship to hierarchy of biological structure, ecological importance and mechanistic understanding.

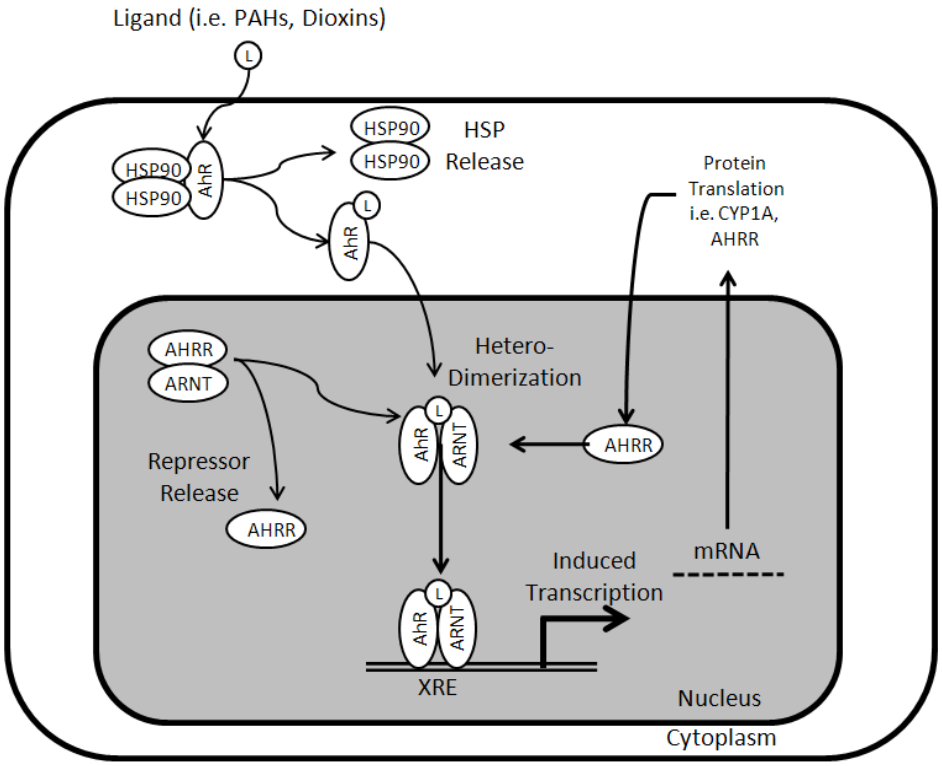


Figure 3. Schematic of the aryl hydrocarbon receptor pathway and ligand activation.

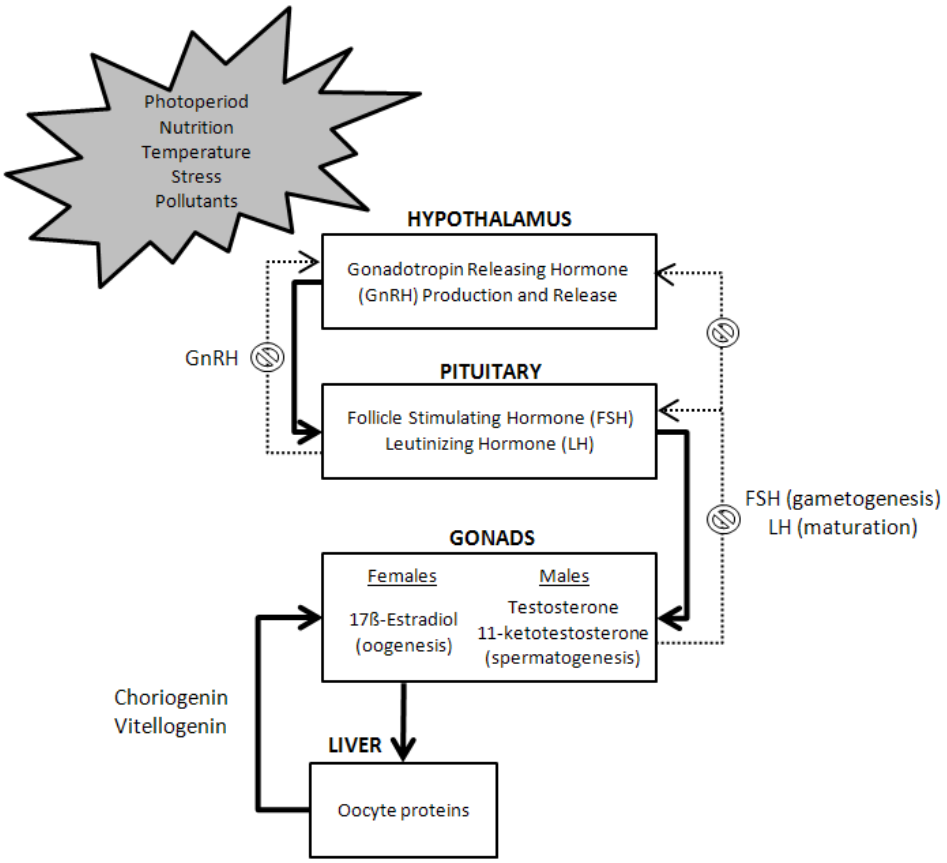


Figure 4. The hypothalamus-pituitary-gonad (HPG) axis for teleosts.

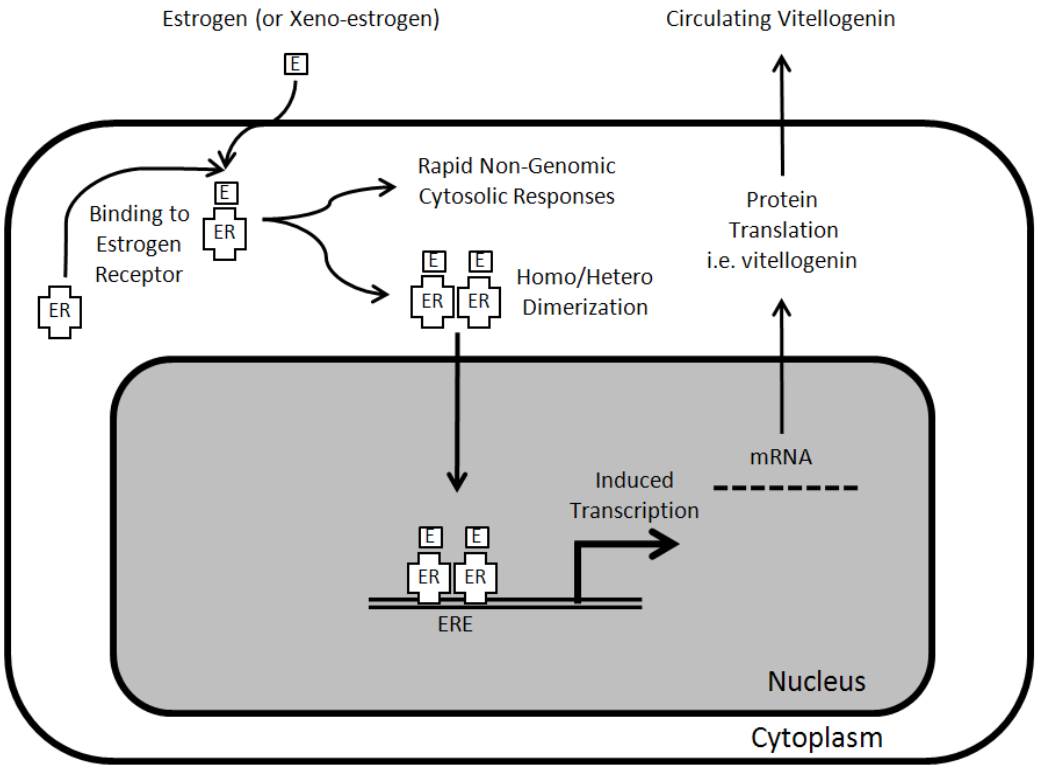


Figure 5. Schematic of the estrogen receptor pathway.

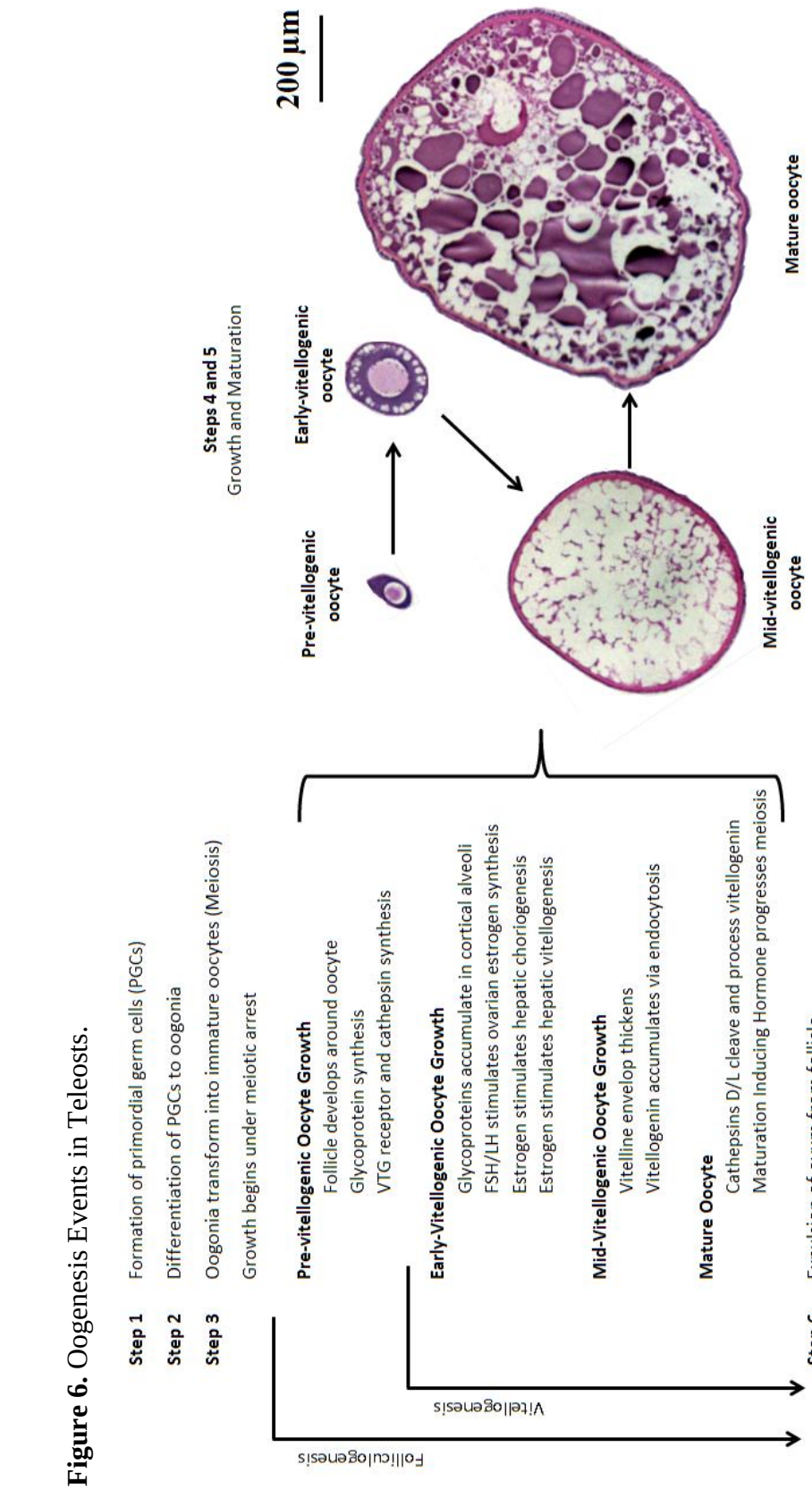


Figure 6. Oogenesis Events in Teleosts.

Sizes of follicles are relative. Magnification is 40X.

Table 1. Table of all biomarkers evaluated.

Endpoint	Biomarkers	Purpose
Gross Examinations	Prevalence of external and grossly visible lesions	Examine fish for external lesions involving skin, fins, gills or eyes and color and shape of internal organs
Morphometrics	Organ to body weight ratios (liver, spleen, gonads), length to body weight	Determine whether impacted fish exhibit altered liver to body weight ratios as an indicator of disease
Hematology	Packed cell density, micronuclei in red blood cells, red blood cell morphology, white blood cell differentials	Determine presence of micronuclei and blood cell morphology correlating with contaminants. Determine whether white blood cell differentials are altered in contaminated population
Histopathology	Prevalence of lesions (gonads, liver)	Evaluate liver cellular structure at light microscopic level for abnormal pathological lesions indicating disease or stress
Bile PAH Loads	Fluorescence of aromatic hydrocarbons (naphthalene, pyrene, benzo[a]pyrene)	Determine fluorescent activity to semi-quantitatively estimate PAH levels
mRNA Expression	Hepatic Cytochrome P4501A	Evaluate expression of key genes
	Hepatic Metallothionein	
	Hepatic Vitellogenin I	
	Gonadal Aromatase	
Protein Quantification	Hepatic Cytochrome P4501A Hepatic Vitellogenin	Evaluate levels of endogenous proteins

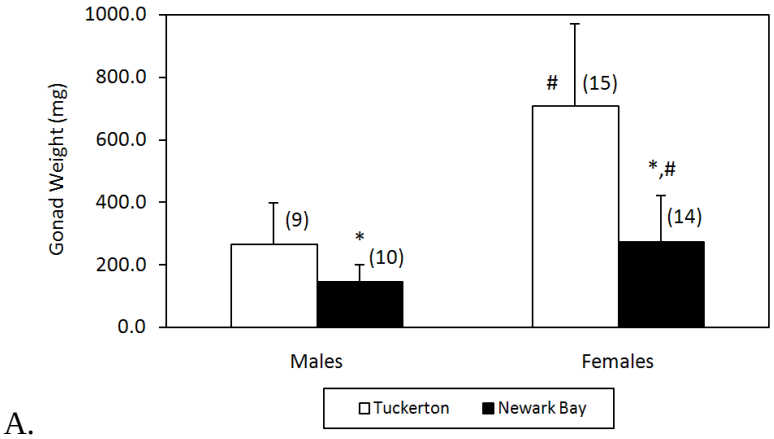
Table 2. Primers used for qPCR.

Gene	NCBI ID#	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product Size (bp)	Annealing Temp
β-actin	AY735154	GCTCTGTGCAGAACAACCACACAT	TAACGCCTCCTTCATCGTTCCAGT	136	59.7
CYP1A	AF026800	TGTTGCCAATGTGATCTGTG	CGGATGTTGTCCTTGTCAAA	258	53
MT	AB426465	ATGGATCCCTGCGATTGC	GCACACGCAGCCAGAGG	147	57.3
VTG I	U07055	AGGATTCGTCCGAACAACAC	TTTCAGACGGCACTCAGATG	416	55
CYP19A1	AY428665	ACGAGAAAGAGCTGCTGCTGAAGA	TGATGTCCAGCTTATCTGCCTGCT	198	60.1

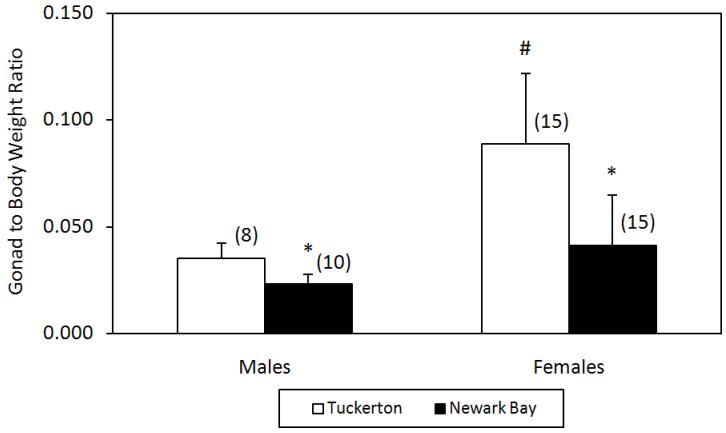
Table 3. Summary statistics for all biomarkers.

	Tuckerton Males	Newark Bay Males	Tuckerton Females	Newark Bay Females
Snout to Peduncle Lengths (cm)	7.00±0.79 (9)	6.80±0.41 (10)	7.18±1.16 (16)	7.34±0.54 (15) #
Snout to Tail Lengths (cm)	8.08±0.88 (9)	7.84±0.51 (10)	8.15±1.31 (16)	8.36±0.60 (15) #
Body Weight (g)	7.92±2.96 (9)	6.38±1.78 (10)	8.30±4.26 (15)	7.14±1.31 (14)
Liver Weight (mg)	155.5±77.0 (9)	149.7±68.2 (9)	230.3±174.1 (15)	222.9±51.1 (14) #
Spleen Weight (mg)	16.1±4.5 (7)	9.4±4.1 (8) *	17.1±17.4 (14)	8.0±2.2 (15)
Gonad Weight (mg)	266.9±131.7 (9)	147.0±52.8 (10) *	708.0±262.5 (15)	274.6±147.7 (14) *
Length to Body Weight	1.11±0.29 (9)	1.3±0.29 (10)	1.07±0.32 (16)	1.18±0.15 (14)
Liver to Body Weight	0.018±0.003 (8)	0.023±0.007 (9)	0.026±0.008 (16) #	0.030±0.004 (14) #
Spleen to Body Weight	0.0025±0.0007 (8)	0.0014±0.0004 (8) *	0.0015±0.0008 (14) #	0.0011±0.0003 (15)
Gonad to Body Weight	0.035±0.007 (8)	0.023±0.005 (10) *	0.089±0.033 (15) #	0.041±0.023 (15) *
Hematocrit (%)	47.3±11.9 (7)	42.8±5.8 (9)	46.2±10.2 (8)	38.1±4.7 (11)
# Micronuclei/1000 Red Blood Cells	3.5±3.99 (7)	0.33±0.50 (9)	1.60±1.40 (16)	0.49±0.58 (11) *
# Crenulated Nuclei/1000 Red Blood Cells	18.5±17.7 (7)	6.7±4.4 (9)	7.4±6.3 (15)	4.2±2.9 (11)
Bile Naphthalene (ng/µL)	189.8±143.2 (4)	604.6±370.2 (8)	199.0±108.9 (10)	504.4±210.7 (11) *
Bile Pyrene (ng/µL)	1.07±0.87 (4)	5.11±2.74 (8) *	0.93±0.51 (11)	4.18±1.77 (11) *
Bile Benzo[a]pyrene (pg/µL)	65.0±29.9 (4)	406.6±323.4 (8)	66.0±28.4 (10)	174.8±135.8 (10) *
Hepatic CYP1A mRNA (ng/µL)	3.3E-04±3.3E-04 (7)	1.6E-03±9.5E-04 (10) *	4.0E-04±3.5E-04 (16)	9.5E-04±6.9E-04 (13) *
Hepatic Metallothionein mRNA (ng/µL)	3.7E-04±1.5E-04 (6)	2.7E-04±1.6E-04 (9)	5.8E-04±2.5E-04 (14)	6.2E-04±5.8E-04 (13)
Hepatic Vitellogenin mRNA (ng/µL)	2.3E-04±4.6E-04 (9)	1.1E-05±1.5E-05 (9)	1.9E-01±5.7E-02 (15) #	6.5E-02±9.4E-02 (13) *,#
Gonadal Aromatase mRNA (ng/µL)	2.1E-07±1.6E-07 (9)	8.2E-08±6.5E-08 (10) *	4.3E-06±9.7E-06 (12)	2.6E-05±1.7E-05 (14) *,#
Hepatic CYP1A Protein Relative Signal	0.16±0.08 (7)	0.60±0.10 (6) *	0.04±0.01 (14) #	0.42±0.35 (13) *
Hepatic Vitellogenin Protein Relative Signal	0.00±0.00 (8)	0.00±0.00 (9)	1.24±1.03 (11) #	0.42±0.71 (13) *,#

Data are reported as mean ± standard deviation. *Significantly different from Tuckerton for the respective gender ($p<0.05$). #Indicates significant difference between genders of the respective site ($p<0.05$). Values in parenthesis are number of animals examined for the given endpoint.



A.



B.

Figure 7. Morphometric data for (A) gonad weight and (B) gonad to body weight ratio. Data are reported as mean ± standard deviation. *Significantly different from Tuckerton for respective gender ($p<0.05$). #Indicates significant difference between genders of the respective site ($p<0.05$). Values in parenthesis are N values for each group.

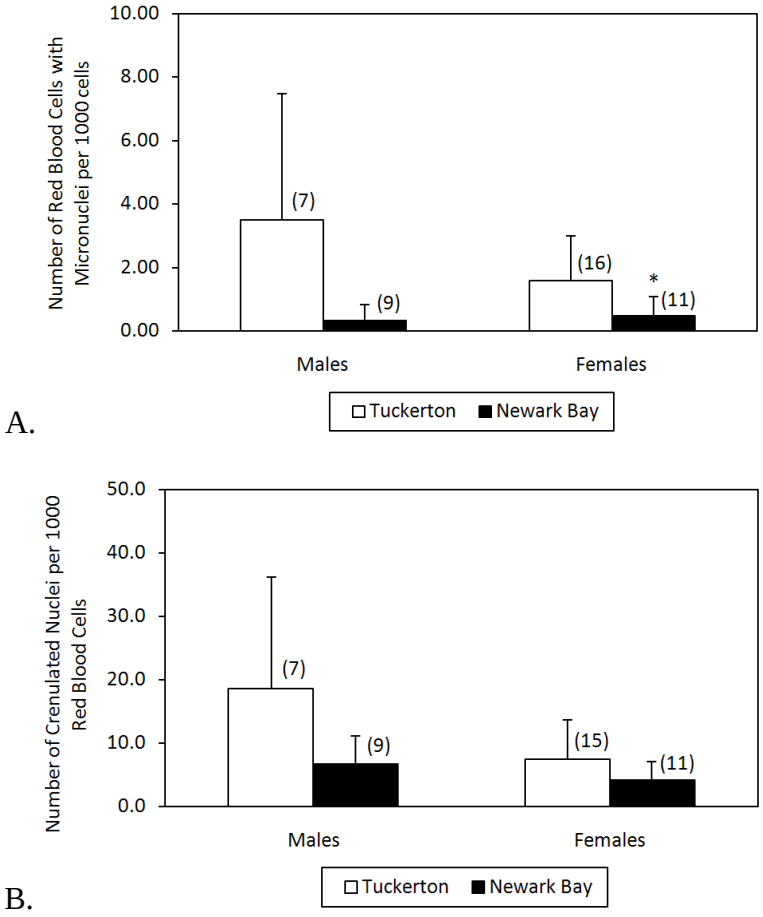


Figure 8. Red blood cell analyses for (A) prevalence of micronuclei in red blood cells and (B) prevalence of crenulated nuclei in red blood cells. Data are reported as mean ± standard deviation. *Significantly different from Tuckerton for respective gender ($p<0.05$). #Indicates significant difference between genders of the respective site ($p<0.05$). Values in parenthesis are N values for each group.

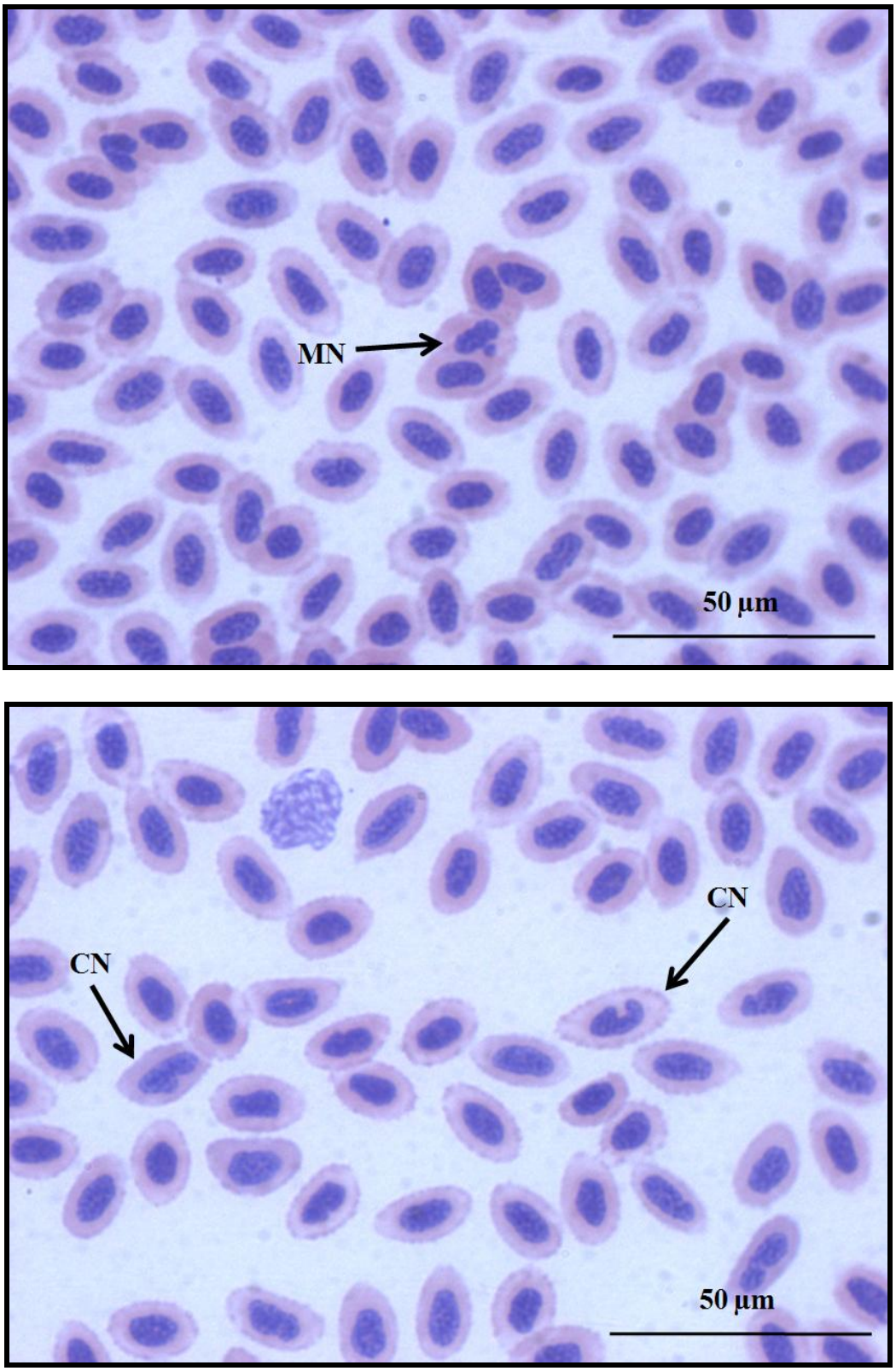


Figure 9. Photomicrographs of micronuclei (MN) and crenulated nuclei (CN) in red blood cells. The magnification is 1000X.

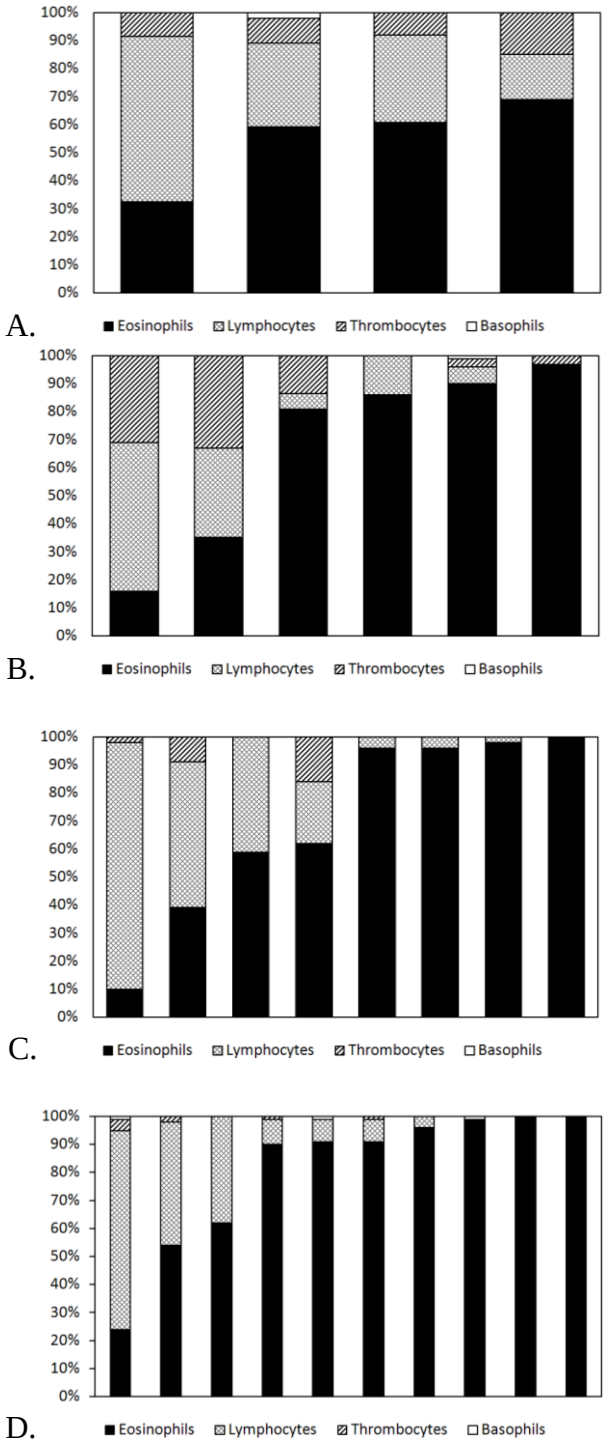


Figure 10. White blood cell differentials for (A) Tuckerton males, (B) Newark Bay males, (C) Tuckerton females and (D) Newark Bay females. Each bar represents a different individual and are lined up in increasing order of percent eosinophils.

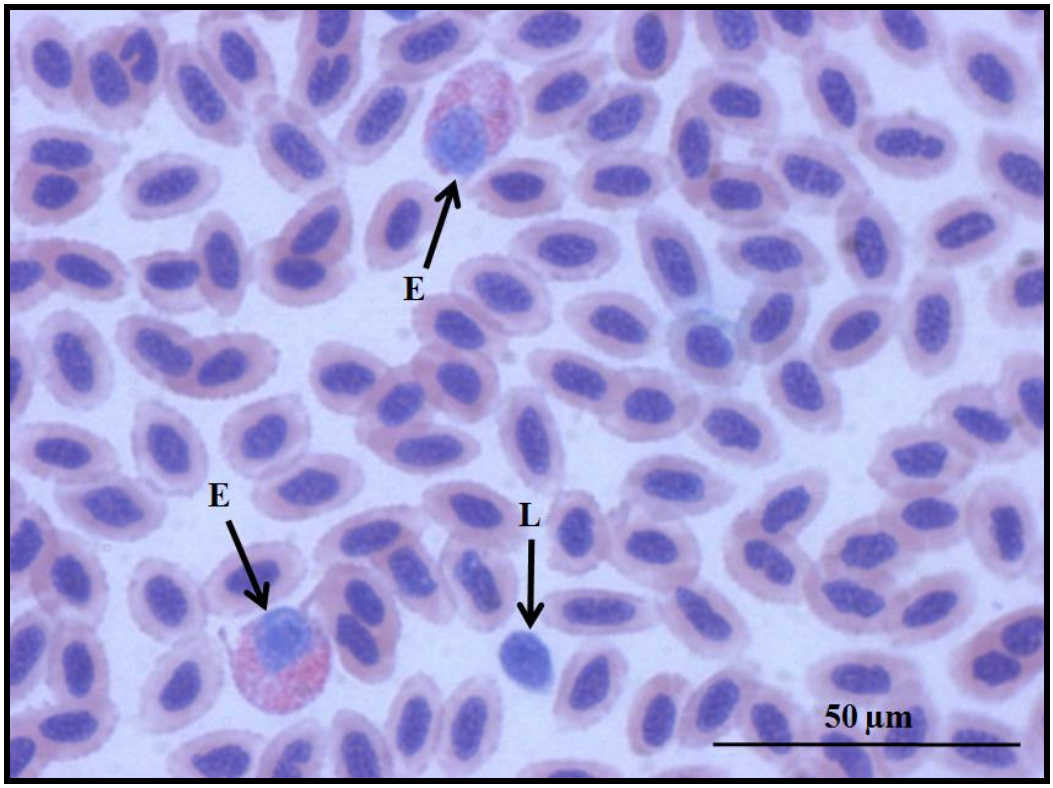


Figure 11. Photomicrographs of eosinophils (E) and lymphocyte (L) in blood smears. Magnification is 1000X.

Table 4. Prevalence of lesions in the liver

	Parasites	Macrophage Centers	Necrosis	Vacuolization	Hemosiderin/Lipofuschin	Basophilic Staining	Hyaline Vacuoles	Lymphocte Infiltrate	Granuloma
Tuckerton Males	25.0	50.0	62.5	12.5	25.0	37.5	12.5	37.5	12.5
Newark Bay Males	0.0	50.0	30.0	0.0	20.0	20.0	0.0	40.0	0.0
Tuckerton Females	25.0	37.5	81.3	37.5	0.0	62.5	25.0	25.0	6.3
Newark Bay Females	0.0	33.3	80.0	20.0	20.0	33.3	0.0	26.7	6.7

Numbers shown are percent of animals that exhibited the given lesion. N=8 for Tuckerton Males, N=10 for Newark Bay Males, N=16 for Tuckerton Females, N=15 for Newark Bay Females.

Table 5. Prevalence of lesions in the pancreas

	Parasites	Macrophage Centers	Necrosis	Hemosiderin/Lipofuschin
Tuckerton Males	12.5	100.0	75.0	50.0
Newark Bay Males	0.0	62.5	56.3	18.8
Tuckerton Females	40.0	130.0	80.0	110.0
Newark Bay Females	0.0	60.0	53.3	26.7

Numbers shown are percent of animals that exhibited the given lesion. N=8 for Tuckerton Males, N=10 for Newark Bay Males, N=16 for Tuckerton Females, N=15 for Newark Bay Females.

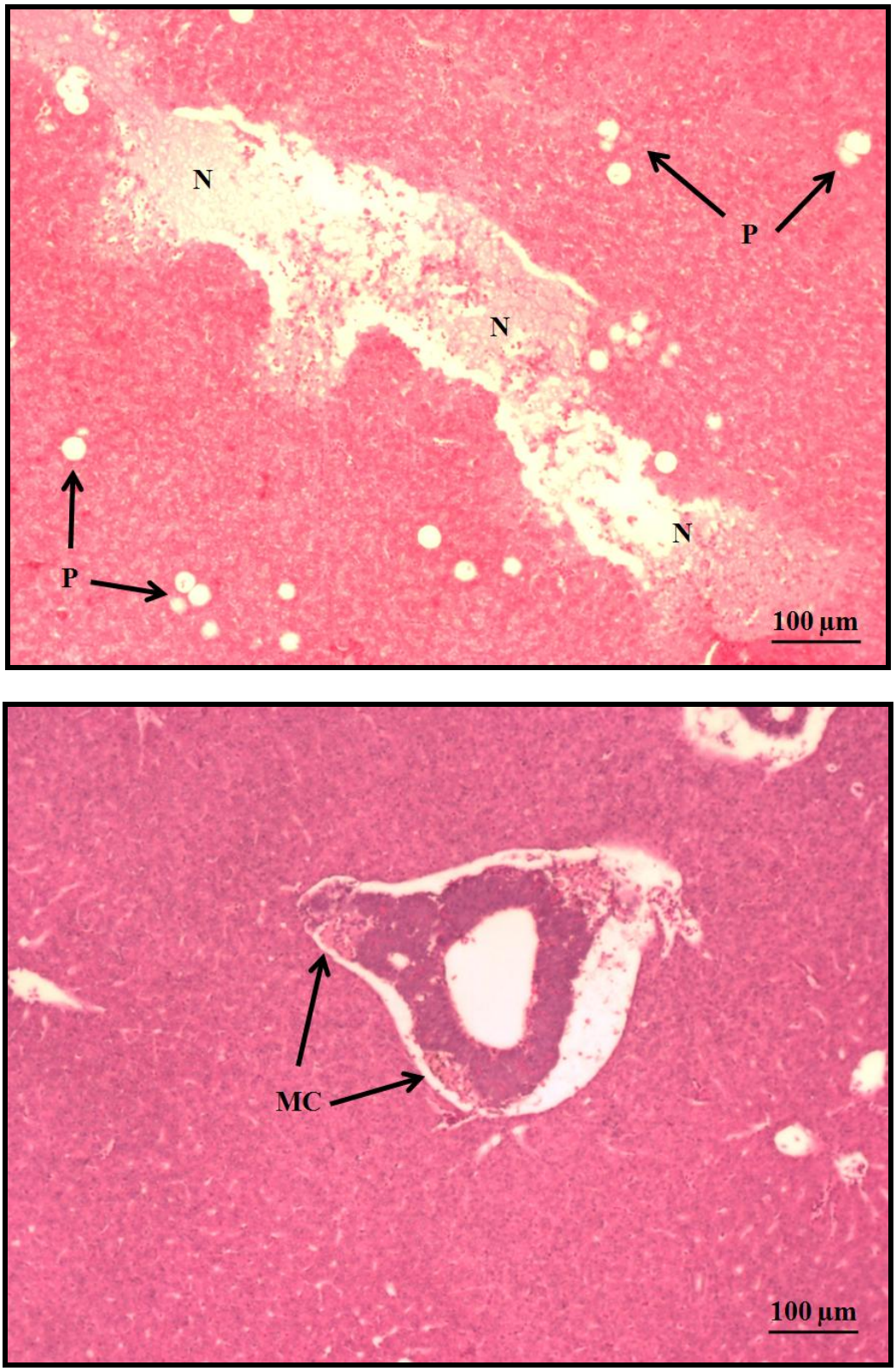


Figure 12. Commonly observed lesions in liver and pancreatic tissue. Necrosis (N), parasites (P) and pancreatic macrophage centers (MC). Magnification is 100X.

Table 6. Prevalence of lesions in the testis

	Thickened Walls	Macrophage Centers	Empty Follicles	Hemosiderin
Tuckerton	6	4	2	1
Newark Bay	10	9	8	1

Numbers presented show how many animals for each group exhibit the given lesion. N=8 for Tuckerton, N=10 for Newark Bay.

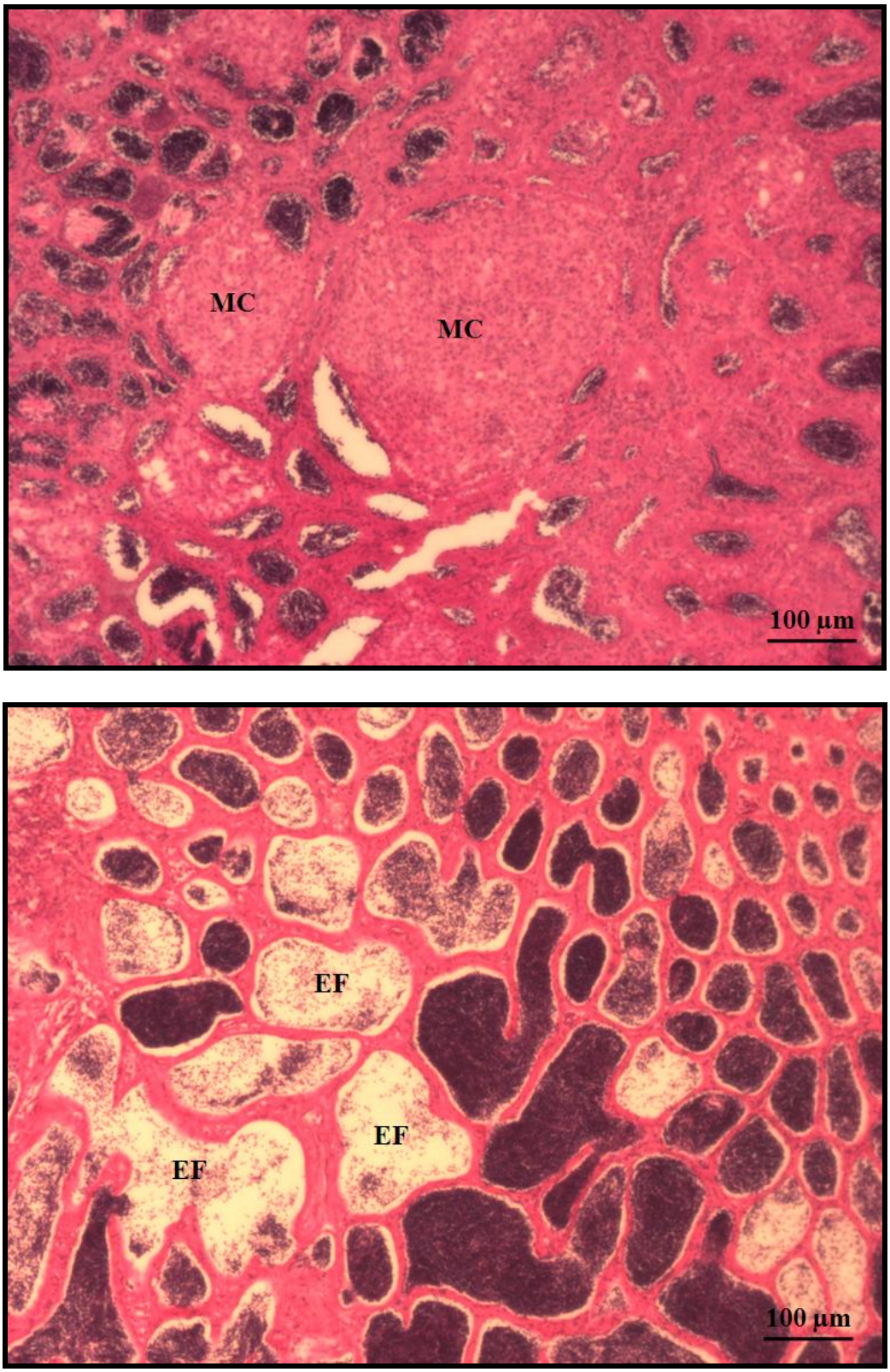
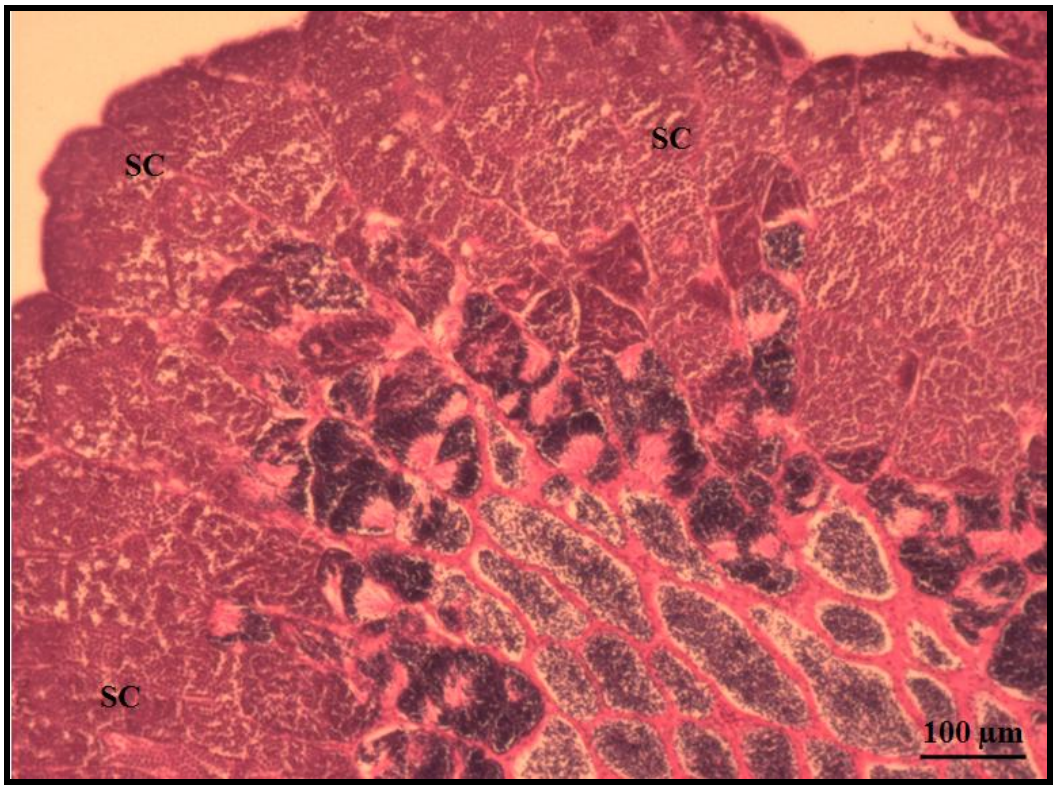


Figure 13. Photomicrographs of commonly observed testis lesions. Macrophage centers (MC) and empty follicles (EF). Magnification is 100X.

A.



B.

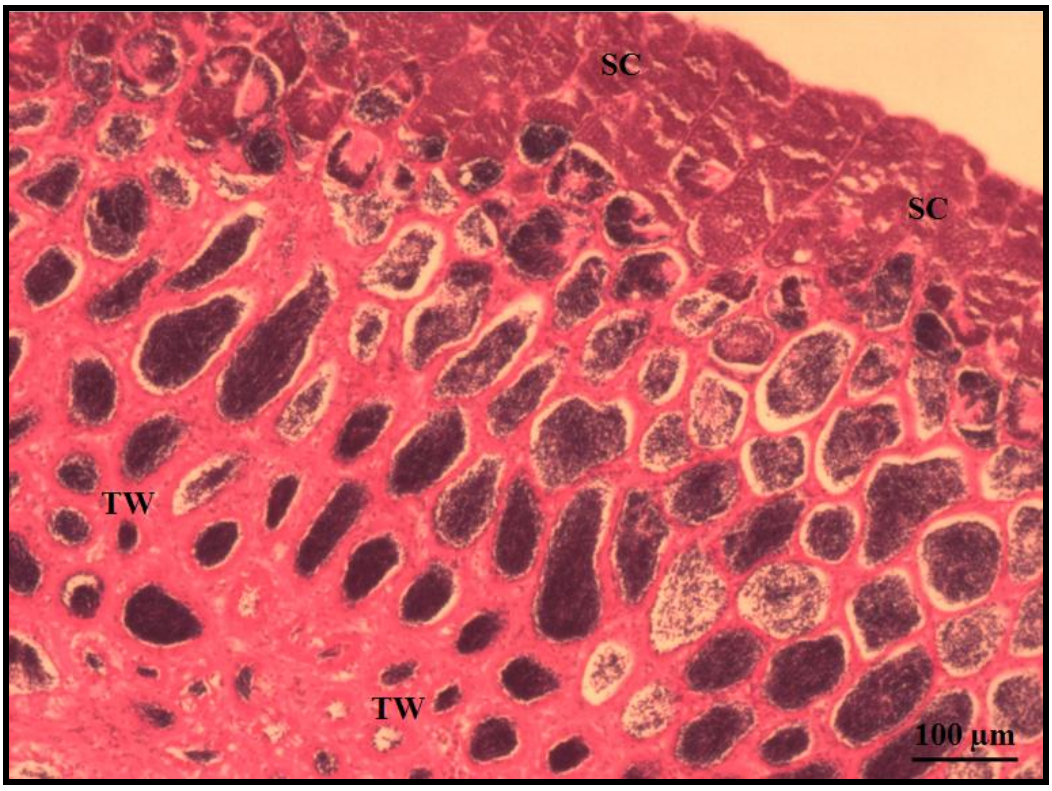


Figure 14. Photomicrographs of testis morphology in (A) Tuckerton and (B) Newark Bay males. Spermatocytes (SC) and thickened walls (TW). Magnification is 100X.

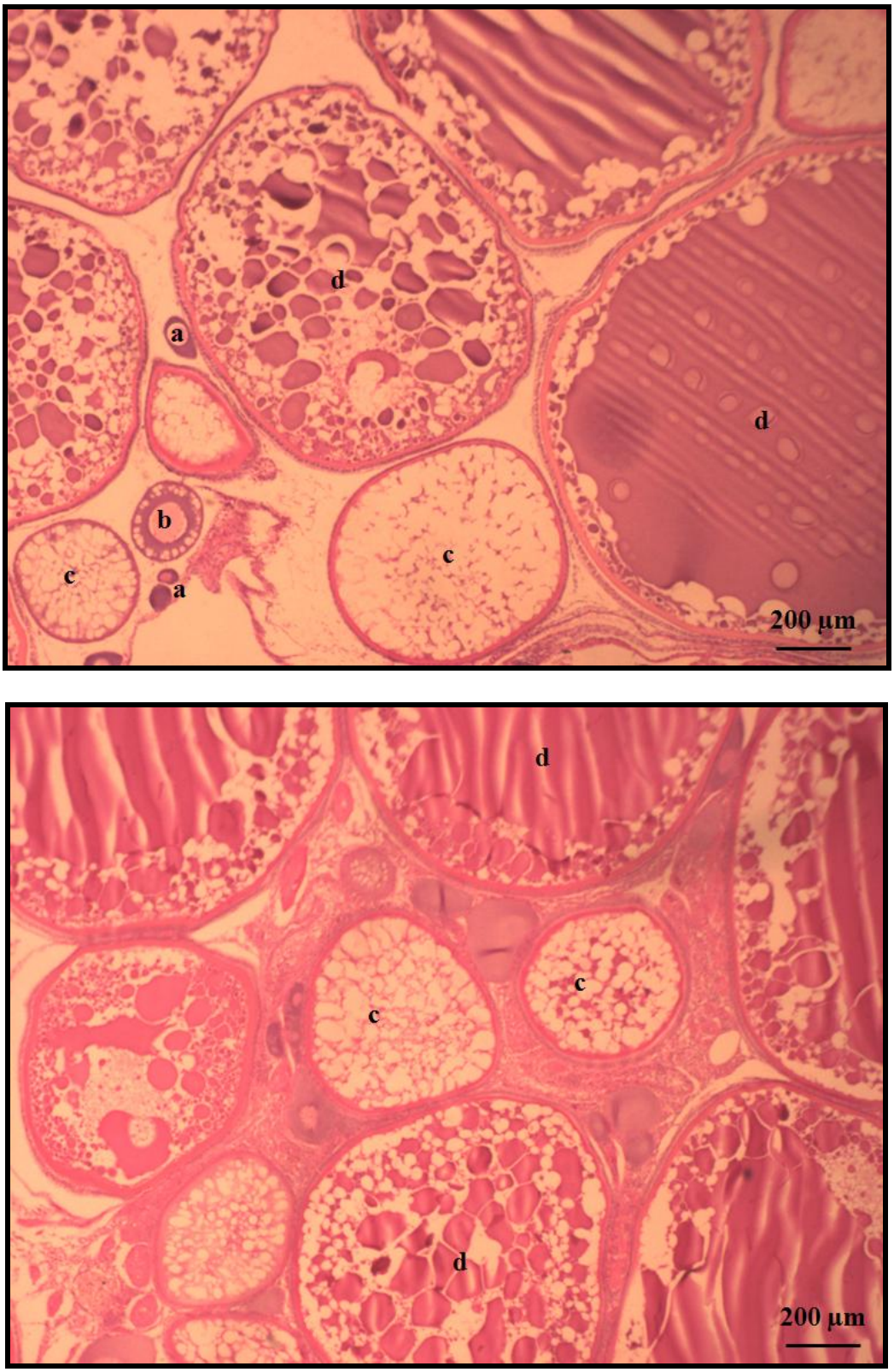


Figure 15. Photomicrographs of Tuckerton ovaries. Pre-vitellogenin (a), early-vitellogenic (b), mid-vitellogenic (c) and mature follicles (d). Magnification is 40X.

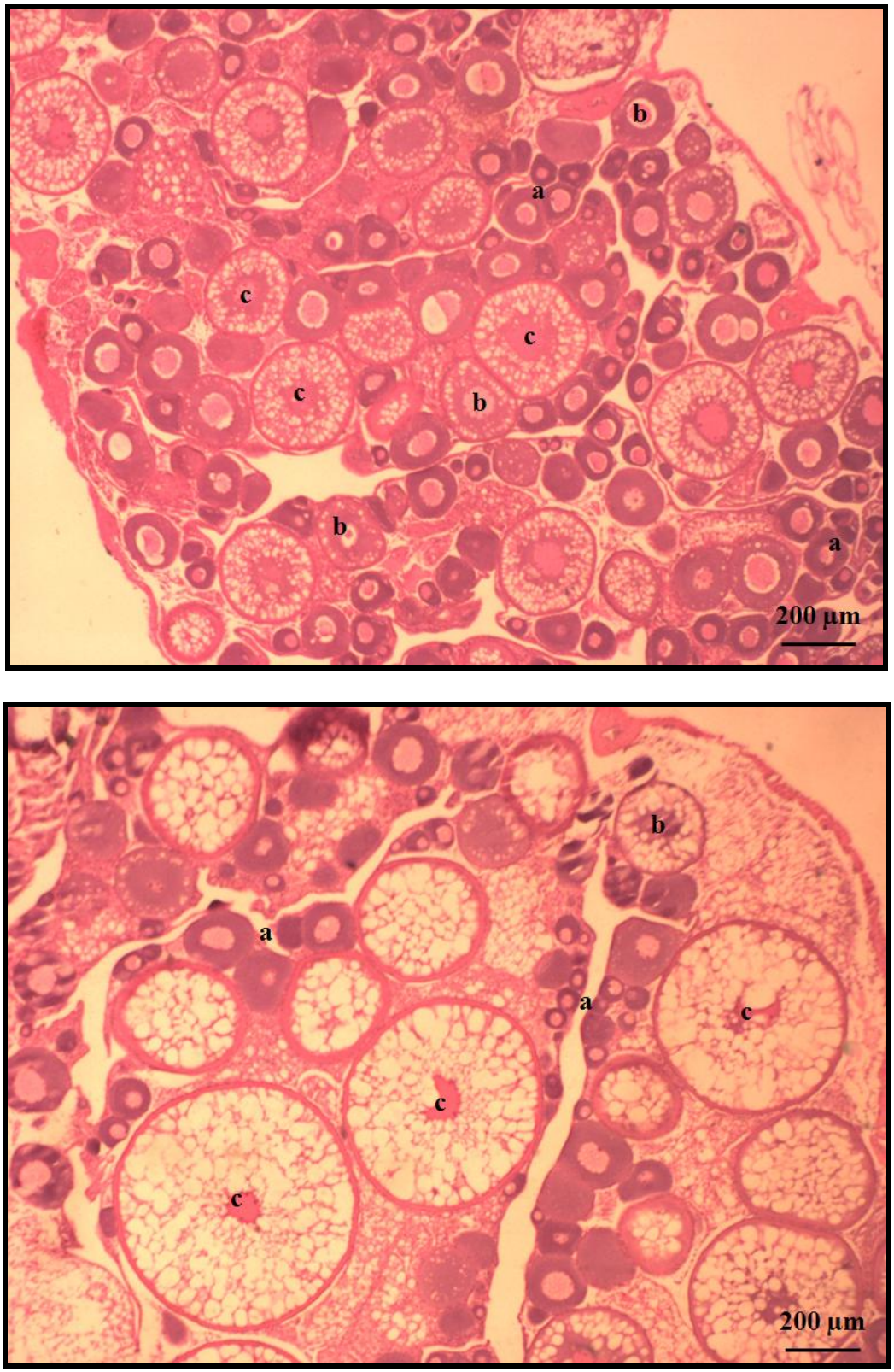


Figure 16. Photomicrographs of Newark Bay gonads. Pre-vitellogenin (a), early-vitellogenic (b) and mid-vitellogenic (c) follicles. Magnification is 40X.

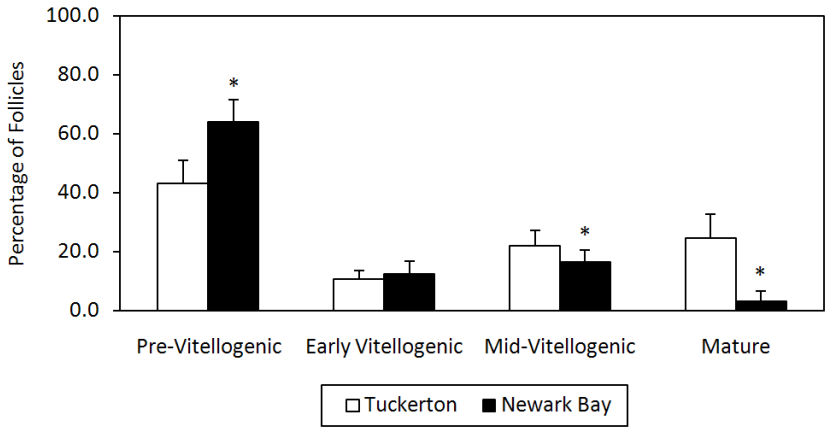


Figure 17. Ovarian follicle developmental stages. Data are reported as mean ± standard deviation. *Significantly different from Tuckerton at $p<0.05$. N=13 for Tuckerton and N=13 for Newark Bay.

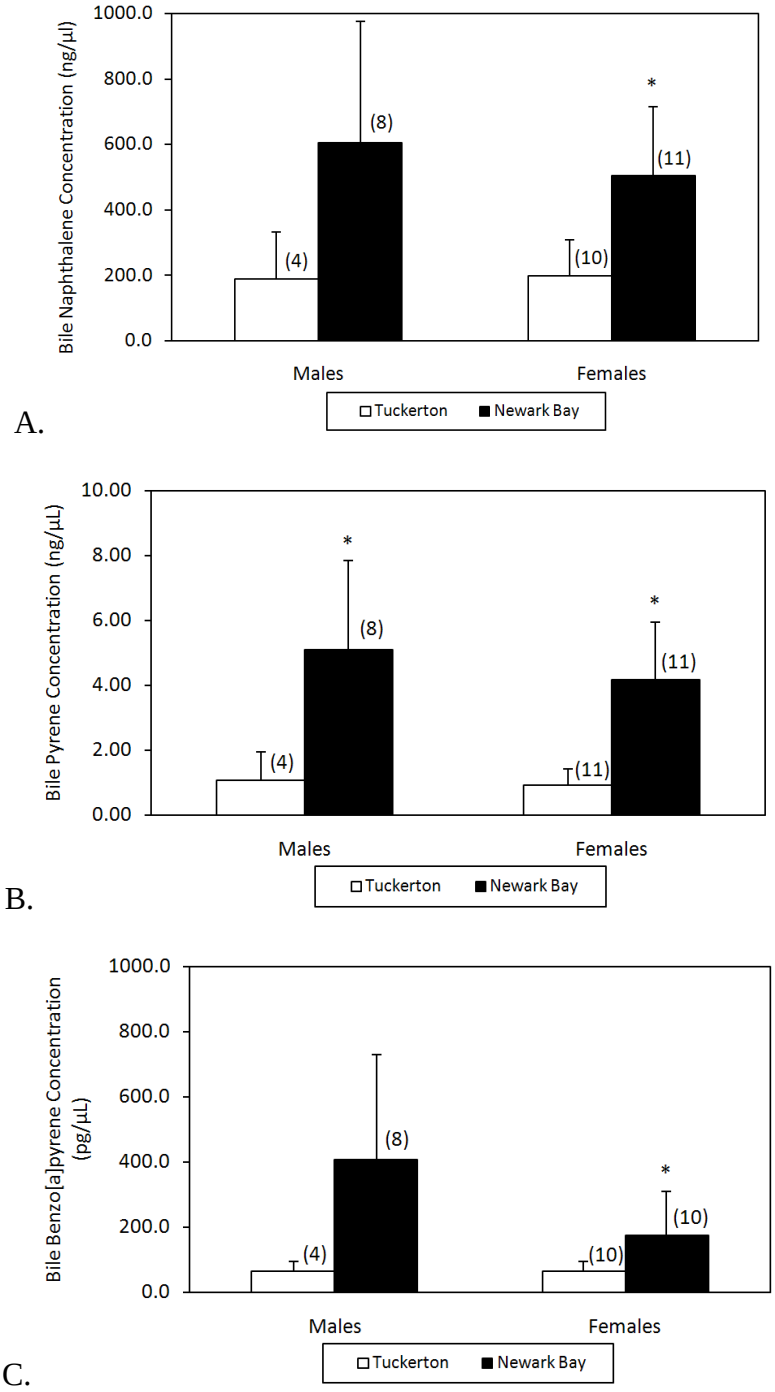


Figure 18. Bile PAH concentrations for (A) naphthalene, (B) pyrene and (C) benzo[a]pyrene. Data are reported as mean ± standard deviation. *Significantly different from Tuckerton for respective gender ($p<0.05$). #Indicates significant difference between genders of the respective site ($p<0.05$). Values in parenthesis are N values for each group.

Table 7. Fold increase of Newark Bay's bile PAH concentrations over Tuckerton

	Males	Females
Naphthalene	4.2	2.6*
Pyrene	7.2*	5.9*
Benzo[a]pyrene	9.3	3.5*

Fold values are calculated using median values. *Newark Bay significantly higher than Tuckerton ($p<0.05$).

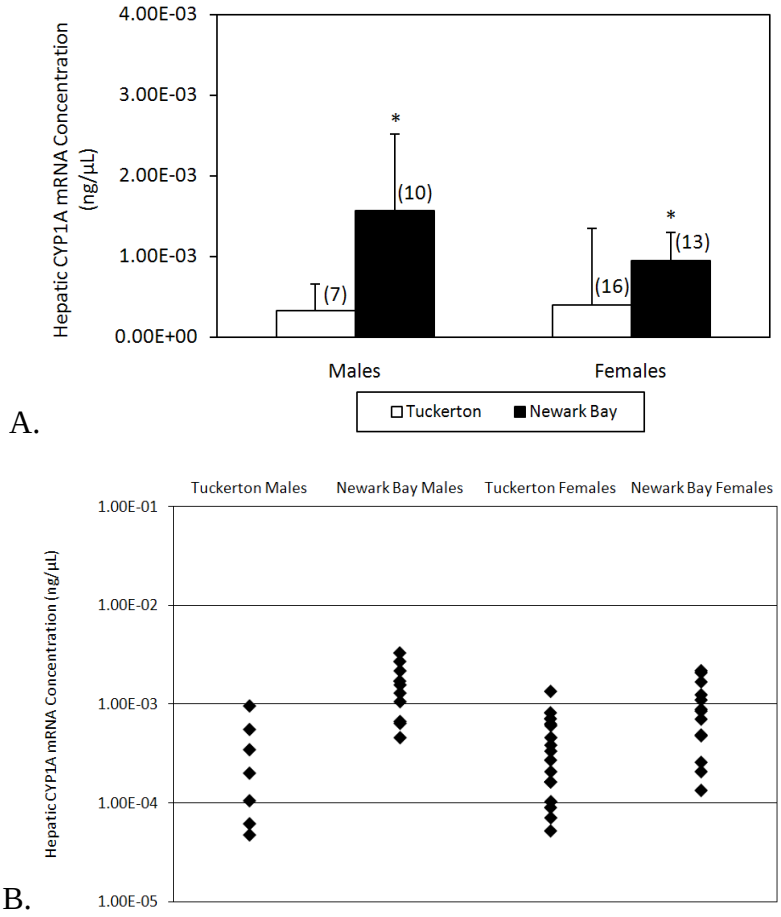


Figure 19. (A) Hepatic CYP1A mRNA expression (data are reported as mean ± standard deviation) and (B) distribution plot of CYP1A values for all individual animals. *Significantly different from Tuckerton for respective gender ($p<0.05$). #Indicates significant difference between genders of the respective site ($p<0.05$). Values in parenthesis are N values for each group.

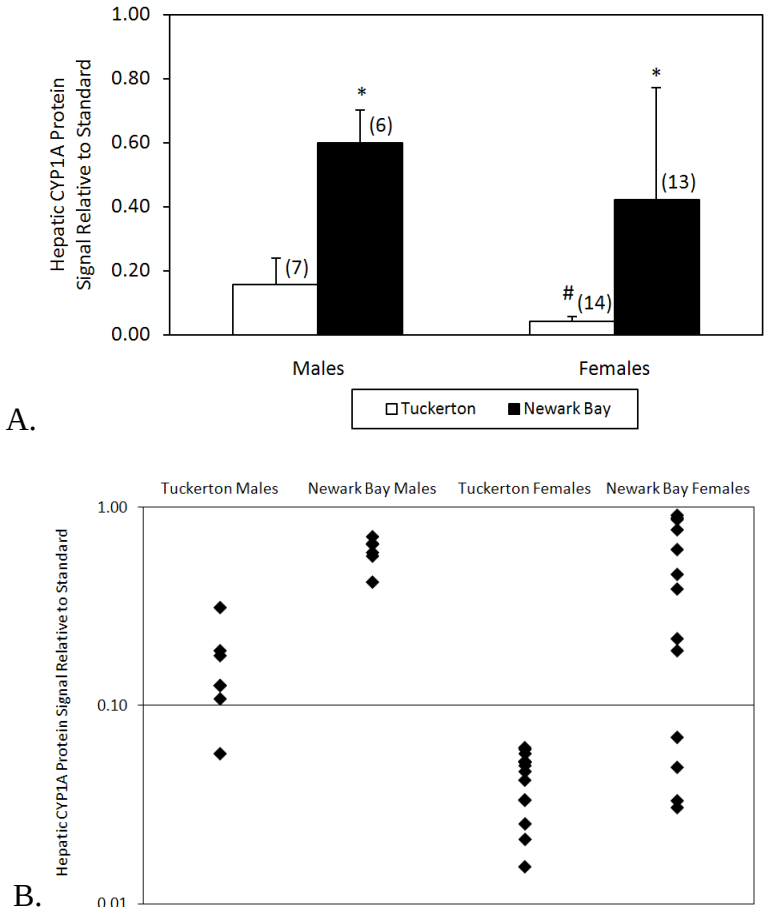


Figure 20. (A) Hepatic microsomal CYP1A protein (data are reported as mean ± standard deviation) and (B) distribution plot of individual animal CYP1A protein values. *Significantly different from Tuckerton for respective gender ($p<0.05$). #Indicates significant difference between genders of the respective site ($p<0.05$). Values in parenthesis are N values for each group.

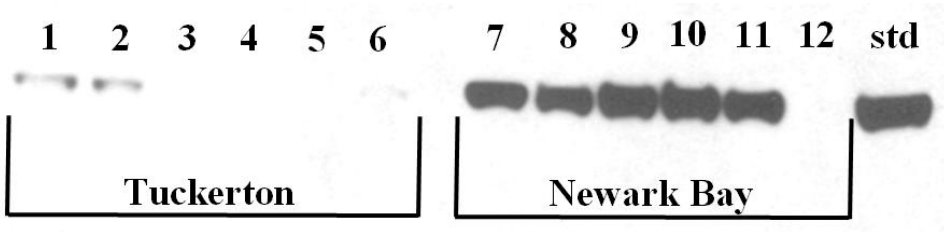
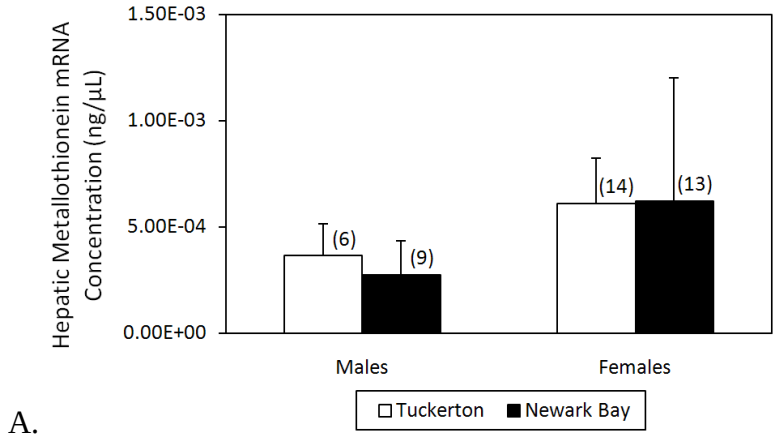


Figure 21. Representative western blot for microsomal CYP1A in the liver. Lanes 1-2 were Tuckerton males, lanes 3-6 were Tuckerton Females, lanes 7-8 were Newark Bay males and lanes 9-12 were Newark Bay females. A standard sample (std) (a Newark Bay sample chosen to be run on every gel) was loaded for calculated relative intensity to a single sample. Bands were approximately 55kDa.



A.

B.

Figure 22. (A) Hepatic metallothionein mRNA expression (data are reported as mean ± standard deviation) and (B) distribution plot of metallothionein values for all individual animals. *Significantly different from Tuckerton for respective gender ($p<0.05$). #Indicates significant difference between genders of the respective site ($p<0.05$). Values in parenthesis are N values for each group.

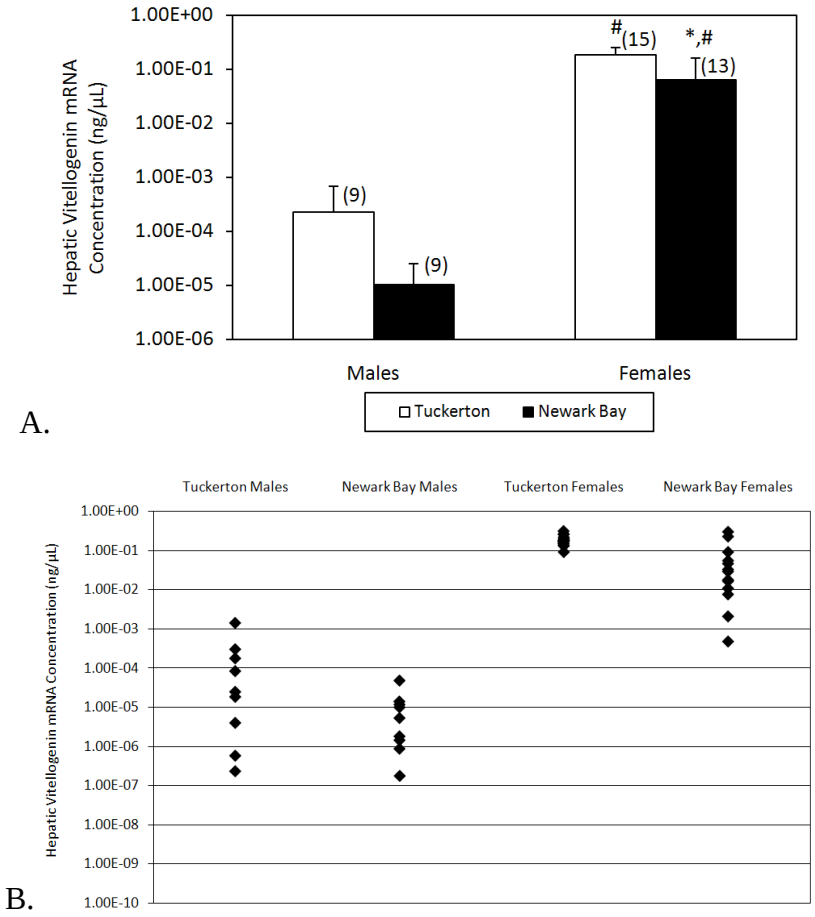


Figure 23. (A) Hepatic vitellogenin mRNA expression (data are reported as mean ± standard deviation) and (B) distribution plot of vitellogenin mRNA values for all individual animals. *Significantly different from Tuckerton for respective gender ($p<0.05$). #Indicates significant difference between genders of the respective site ($p<0.05$). Values in parenthesis are N values for each group.

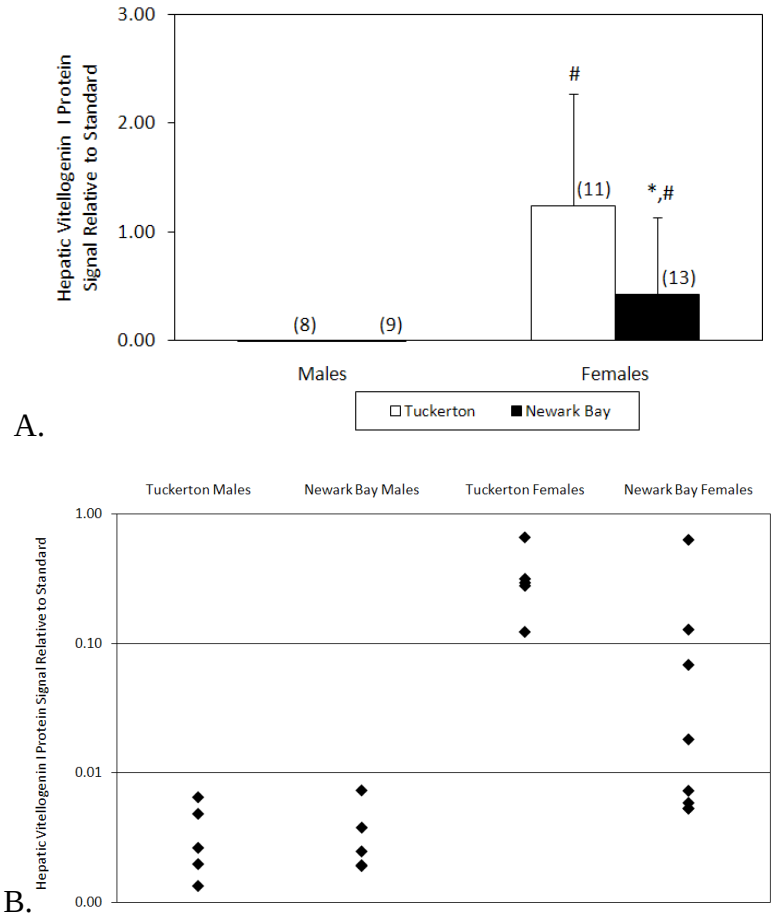


Figure 24. (A) Hepatic vitellogenin protein levels and (B) distribution plot of vitellogenin protein values for all individual animals. *Significantly different from Tuckerton for respective gender ($p<0.05$). #Indicates significant difference between genders of the respective site ($p<0.05$). Values in parenthesis are N values for each group.

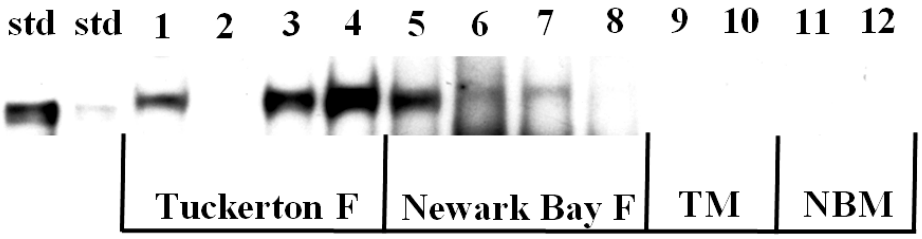


Figure 25. Representative western blot for cytosolic vitellogenin in the liver. Samples 1-4 were Tuckerton females, samples 5-8 were Newark Bay females, samples 9 and 10 were Tuckerton males, samples 11 and 12 were Newark Bay males. Sample 2 was excluded from analysis for not having enough protein loaded (determined by ponceau staining). Standard samples (std) were loaded for calculated relative intensity. Bands were approximately 255kDa.

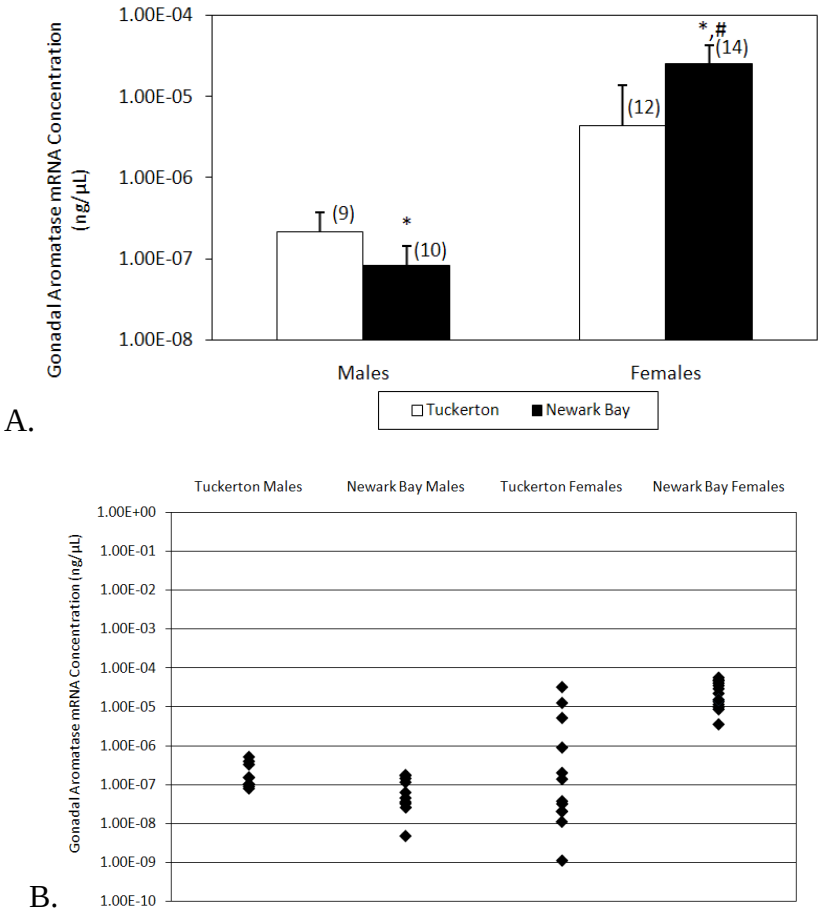


Figure 26. (A) Gonadal aromatase expression (data are reported as mean ± standard deviation) and (B) distribution plot of gonadal aromatase values for individual animals. *Significantly different from Tuckerton for respective gender ($p<0.05$). #Indicates significant difference between genders of the respective site ($p<0.05$). Values in parenthesis are N values for each group.

Table 8. Summary of Alterations in Biomarker Measurements.

Biomarker Endpoint	Newark Bay Males	Newark Bay Females	Importance
Snout to Peduncle Lengths (cm)	-	-	
Snout to Tail Lengths (cm)	-	-	
Body Weight (g)	-	-	
Hepatic Metallothionein mRNA (ng/μL)	-	-	
Spleen Weight (mg)	↓	-	Immune Response
Spleen to Body Weight	↓	-	Immune Response
White Blood Cell Differentials	*	*	Hyperactive Immune System
Hematocrit (%)	-	-	
# Micronuclei/1000 Red Blood Cells	-	↓	Potentially Due to Acquired Resistance to Genotoxicants
# Crenulated Nuclei/1000 Red Blood Cells	-	-	
Liver Weight (mg)	-	-	
Liver to Body Weight	-	-	
Length to Body Weight	-	-	
Bile Naphthalene (ng/μL)	↑ (4-fold, p=0.06)	↑ (3-fold)	
Bile Pyrene (ng/μL)	↑ (7-fold)	↑ (6-fold)	Exposure to Carcinogenic PAHs
Bile Benzo[a]pyrene (pg/μL)	↑ (9-fold, p=0.07)	↑ (4-fold)	
Histopathology of Liver	-	-	Potentially Due to Acquired Resistance to Carcinogens and Hepatotoxicants
Histopathology of Pancreas	-	-	Potentially Due to Acquired Resistance to Carcinogens and Hepatotoxicants
Hepatic CYP1A mRNA (ng/μL)	↑ (7-fold)	↑ (3-fold)	Exposure to AHR Agonists, Constituitive Induction is Energetically Costly
Hepatic CYP1A Protein Relative Signal	↑ (5-fold)	↑ (8-fold)	Exposure to AHR Agonists, Constituitive Induction is Energetically Costly
Gonad Development/Morphology	*	*	Endocrine Disruption of Gonad Development in Both Sexes
Gonad Weight (mg)	↓	↓	Impaired Gonad Growth
Gonad to Body Weight	↓	↓	Impaired Gonad Growth
Gonadal Aromatase mRNA (ng/μL)	↑ (3-fold)	↑ (210-fold)	Active 17β-Estradiol Synthesis
Hepatic Vitellogenin mRNA (ng/μL)	-	↓ (6-fold)	Inhibition/Disruption of Vitellogenesis
Hepatic Vitellogenin Protein Relative Signal	-	↓ (27-fold)	Inhibition/Disruption of Vitellogenesis

Endpoints are shown as being significantly increased (↑), decreased (↓), or no change (-) in Newark Bay fish with respect to Tuckerton fish. Significance is $p<0.05$ unless otherwise noted. *Indicates difference in the subset of measurements for that biomarker. Significant differences between genders at each site are not shown here.

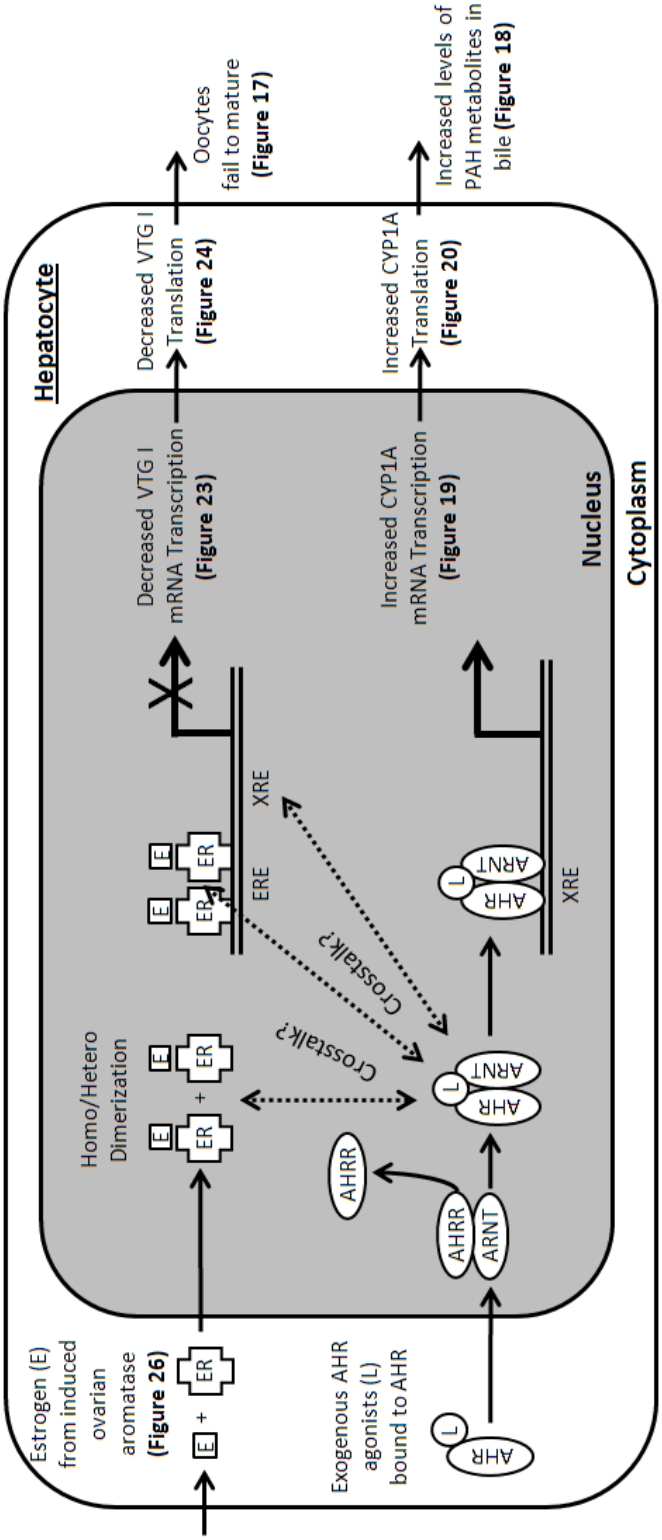


Figure 27. Proposed model of vitellogenesis disruption by crosstalk with the arylhydrocarbon receptor.

REFERENCES

Aas, E., Baussant, T., Balk, L., Liewenborg, B., Andersen, O.K., 2000. PAH metabolites in bile, cytochrome p4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic Cod. Aquat. Toxicol. 51, 241-258.

Able, K.W., Hagan, S.M., Kovitvongsa, K., Brown, S.A., Lamonaca, J.C., 2007. Piscivory by the mummichog (*Fundulus heteroclitus*): evidence from the laboratory and salt marshes. J. Exp. Mar. Biol. Ecol. 345, 26-37.

Abramoff, M.D., Magelhaes, P.J., Ram, S.J., 2004. Image processing with ImageJ. Biophotonics International. 11(7), 36-42.

Al-Sabti, K., Metcalfe, C.D., 1995. Fish micronuclei for assessing genotoxicity in water. Mutat. Res. 343, 121-135.

Arcand-Hoy L.D., Benson, W.H., 1998. Fish reproduction: An ecologically relevant indicator of endocrine disruption. Environ. Toxicol. Chem. 17, 49-57.

Arzuaga, X., Elskus, A., 2002. Evidence for resistance to benzo[a]pyrene and 3,4,3',4'-tetrachlorobiphenyl in a chronically polluted *Fundulus heteroclitus* population. Marine Environmental Research. 54(3-5), 247-251.

Ariese, F., Kok, S.J., Verkaik, M., Gooijer, C., Velthorst, N.H., Hofstaat, J.W., 1993. Synchronous fluorescence spectrometry of fish bile: a rapid screening method for the biomonitoring of PAH exposure. Aquat. Toxicol. 26, 273-286.

Assaf-Anid, N., 2003. PAHs, suspended solids, PBDEs, endocrine active substances, and lead: contaminants of concern to the New York/New Jersey Harbor. New York Academy of Sciences. New York, NY. Available Online: http://www.nyas.org

Barnes, K.K., Kolpin, D.W., Furlong, E.T., Zaugg, S.D., Meyer, M.T., Barber, L.B., 2008. A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States – I) Grounwater. Sci. Total Environ. 402(2-3), 192-200.

Bartell, S., 2006. Biomarkers, bioindicators and ecological risk assessment - a brief review and evaluation. Environmental Bioindicators. 1, 60-73.

Baumann, P.C., Harshbarger, J.C., 1995. Decline in liver neoplasms in wild brown bullhead catfish after coking plant closes and environmental PAHs plummet. Environ. Health Perspect. 103(2), 168-170.

Bello, S.M., Franks, D.G., Stegeman, J.J., Hahn, M.E., 2001. Acquired resistance to Ah receptor agonists in a population of Atlantic Killifish (*Fundulus heteroclitus*)

inhabiting a marine superfund site: *in vivo* and *in vitro* studies on the inducibility of xenobiotic metabolizing enzymes. Toxicol. Sci. 60, 77-91.

Belton, T.J., Hazen, R., Ruppel, B.E., Lockwood, K., Mueller, R., Stevenson, E., Post, J.J., 1985. A study of dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) contamination in select finfish, crustaceans, and sediments of New Jersey waterways final report. New Jersey Department of Environmental Protection and Energy, Trenton, NJ.

Bemanian, V., Male, R., Goksøyr, A., 2004. The aryl hydrocarbon receptor-mediated disruption of vitellogenin synthesis in the fish liver: cross-talk between AHR- and ERα-signaling pathways. Comparative Hepatology. 3(2).

Billiard, S.M., Hahn, M.E., Franks, D.G., Peterson, R.E., Bols, N.C., Hodson, P.V., 2002. Binding of polycyclic aromatic hydrocarbons (PAHs) to teleost aryl hydrocarbon receptors (AHRs). Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. 133, 55-68.

Blazer, V.S., 2004. Histopathological assessment of gonadal tissue in wild fishes. Fish Physiol. Biochem. 26(1), 85-101.

Boehme, S., Panero, M., 2003. Pollution prevention and management strategies for cadmium in the New York/New Jersey Harbor. New York Academy of Sciences. New York, NY. Available Online: http://www.nyas.org

Bolognesi, C., Perrone, E., Roggieri, P., Pampanin, D.M., Andrea, S., 2006. Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions. Aquat. Toxicol. 78, 93-98.

Bopp, R.F., Gross, M.L., Tong, H., Simpsons, H.J., Monson, S.J., Deck, B.L., Moser, F.C., 1991. A major incident of dioxin contamination in sediments of New Jersey estuaries. Environ. Sci. Technol. 25, 951-956.

Burger, J., 2006. Bioindicators: types, development and use in ecological assessment and research. Environ. Bioindicators. 1, 22-39.

Burnett, K.G., Bain, L.J., Baldwin, W.S., Callard, G.V., Cohen, S., Di Giulio, R.T., Evans, D.H., Gómez-Chiarri, Hahn, M.E., Hoover, C.A., Karchner, S.I., Katoh, F., Maclatchy, D.L., Marshall, W.S. Meyer, J.N., Nacci, D.E., Oleksiak, M.F., Rees, B.B., Singer, T.D., Stegeman, J.J., Towle, D.W., Van Veld, P.A., Vogelbein, W.K., Whitehead, A., Winn, R.N., Crawford, D.L., 2007. Fundulus as the premier teleost model in environmental biology: opportunities for new insights using genomics. Comp. Biochem. Physiol. Part D: Genomics Proteomics. 2(4), 257-286.

Campbell, C.G., Borglin, S.E., Green, F.B., Grayson, A., Wozei, E., Stringfellow, W.T., 2006. Biologically directed environmental monitoring, fate, and transport of

estrogenic endocrine disrupting compounds in water: a review. Chemosphere. 65, 1265-1280.

Candelmo, A.C., Deshpande, A., Weis, J.S., 2007. Behavior and condition responses of young-of-the-year bluefish (*Pomatomus saltatrix*) to contaminants accumulated via experimental trophic transfer. Hudson-Delaware Chapter of the Society of Environmental Toxicology and Chemistry. 23rd Annual Meeting. Stockton, New Jersey. Available online: http://www.hdcsetac.org

Chang, X., Fan, Y., Karyala, S., Schwemberger, S., Tomlinson, C.R., Sartor, M.A., Puga, A., 2007. Ligand-independent regulation of transforming growth factor ß1 expression and cell cycle progression by the aryl hydrocarbon receptor. Molecular and Cellular Biology. 27(17), 6127-6139.

Chapman, P.M., Hollert, H., 2006. Should the sediment quality triad become a tetrad, a pentad, or possibly even a hexad? J. Soils and Sediments. 6(1), 4-8.

Chapman, P.M., Anderson, J., 2005. A decision-making framework for sediment contamination. Integrated Environmental Assessment and Management. 1(3), 163-173.

Chapman, P.M., 1990. The sediment quality triad approach to determining pollution-induced degradation. The Science of the Total Environment. 97/98, 815-825.

Chapman, P.M., Anderson, B., Carr, S., Engles, V., Green, R., Hameedi, J., Harmon, M., Haverland, P., Hyland, J., Ingersoll, C., Long, E., Rodgers, J., Jr., Salazar, M., Sibley, P.K., Smith, P.J., Swartz, R.C., Thompson, B., Windom, H., 1997. General Guidelines for using the sediment quality triad. Marine Pollution Bulletin. 34(6), 368-372.

Chomczynski P., Mackey K., 1995. Modification of the TRIZOL reagent procedure for isolation of RNA from polysaccharide and proteoglycan-rich sources. Biotechniques. 19(6), 942-945.

Cooper, K., Buchanan, G.A., 2007. Integrated biomarkers for assessing the exposure and effects of endocrine disruptors and other contaminants on marine/estuarine fish. New Jersey Department of Environmental Protection: Division of Science, Technology And Research. Trenton, New Jersey. Available online: http://www.state.nj.us/dep/

Courtenay, S.C., Grunwald, C.M., Kreamer, G.L., Fairchild, W.L., Arsenault, J.T., Ikonomou, M., Wirgin, I., 1999. A comparison of the dose and time response of CYP1A1 mRNA induction in chemically treated Atlantic Tomcod from two populations. Aquat Toxicol. 47, 43-69.

de Cerreño, A.L.C., Panero, M., Boehme, S., 2002. Pollution prevention and management strategies for mercury in the New York/New Jersey Harbor. New York Academy of Sciences. New York, NY. Available online: http://www.nyas.org

Denison, M.S., Nagy, S.R., 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu. Rev. Pharmacol. Toxicol. 43, 309-34.

Denslow, N.D., Larkin, P., 2006. Utilizing molecular techniques for bioindicator research. Environmental Bioindicators. 1, 40-59.

Thomas, P., 2008. The Endocrine System, in: Di Giulio, R.T., Hinton, D.E. (Eds.), The Toxicology of Fishes. Taylor & Francis Group, Fl, pp. 457-488.

Dimou, K.N., Peccioli., J.A., 2006. The New Jersey toxics reduction workplan for NY-NJ Harbor: distribution of PCDD/Fs in ambient waters. Water, Air, and Soil Pollution: Focus. 6, 5-16.

Dong, W., Teraoka, H., Tsujimoto, Y., Stegeman, J.J., Hiraga, T., 2004. Role of aryl hydrocarbon receptor in mesencephalic circulation failure and apoptosis in zebrafish embryos exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicol. Sci. 77, 109-116.

Eisler, R., 1987. Polycyclic aromatic hydrocarbon hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Fish and Wildlife Service. Laurel, MD. Available online: http://www.pwrc.usgs.gov/

Elskus, A.A., Monosson, E., McElroy, A.E., Stegeman, J.J., Woltering, D.S., 1999. Altered CYP1A expression in *Fundulus heteroclitus* adults and larvae: a sign of pollutant resistance? Aquat. Toxicol. 45, 99-113.

Fertruck, K.C., Kumar, S., Sikka, H.C., Matthews, J.B., Zacharewski, T.R., 2001. Interaction of PAH-related compounds with the α And ß isoforms of the estrogen receptor. Toxicology Letters. 121, 167-177.

Focazio, M.J., Kolpin, D.W., Barnes, K.K., Furlong, E.T. Meyer, M.T., Zaugg, S.D., Barber, L.B., Thurman, M.E., 2008. A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States – II) Untreated drinking water sources. Sci. Total Environ. 402(2-3), 201-216.

Fritz, E.S., Meredith, W.H., Lotrich, V.A., 1975. Fall and winter movements and activity level of the mummichog, *Fundulus heteroclitus*, in a tidal creek. Chesapeake Science. 16, 211-214.

Gigliotti, C.L., Totten, L.A., Offenberg, J.H., Dachs, J., Reinfelder, J.R., Nelson, E.D., Glenn, Eisenreich, S.J., 2005. Atmospheric concentrations and deposition of

polycyclic aromatic hydrocarbons to the Mid-Atlantic east coast region. Environ. Sci. Technol. 39, 5550-5559.

Gillis, C.A., Bonnevie, N.L., Su, S.H., Ducey, J.G., Huntley, S.L., Wenning, R.J., 1995. DDT, DDD, and DDE contamination of sediment in the Newark Bay estuary, New Jersey. Archives of Environmental Contamination and Toxicology. 28(1), 85-92.

Gimeno, S., Komen, H., Venerbosch, P.W., Boomer, T., 1997. Disruption of sexual differentiation in genetic male common carp (*Cyprinus carpio*) exposed to alkylphenol during different life stages. Enviro. Sci. Technol. 31, 2884-90.

Gimeno, S. Gerritsen, A., Bowmer, T, Komen, H., 1996. Feminization of male carp. Nature. 384, 221-222.

Greytak, S.R., Champlin, D., Callard, G.V., 2005. Isolation and characterization of two cytochrome p450 aromatase forms in killifish (*Fundulus heteroclitus*): differential expression in fish from polluted and unpolluted environments. Aquat. Toxicol. 71, 371-389.

Hahn, M.E., 1998. The aryl hydrocarbon receptor: A comparison perspective. Comparative Biochemistry and Physiology Part C. 121, 23-53.

Hahn, M.E., 2002. Aryl hydrocarbon receptors: diversity and evolution. Chemico-Biological Interactions. 141, 131-160.

Heiden, T.C.K., Struble, C.A., Rise, M.L., Hessner, M.J., Hutz, R.J., Carvan III, M.J., 2008. Molecular targets of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) within the zebrafish ovary: insights into TCDD-induced endocrine disruption and reproductive toxicity. Reproductive Toxicology. 25, 47-57.

Heiden,T.K., Carvan III, M.J., Hutz, R.J., 2006. Inhibition of follicular development, vitellogenesis, and serum 17ß-estradiol concentrations in zebrafish following chronic, sublethal dietary exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Chemosphere. 90(2), 490-499.

Henry, T.R., Spitsbergen, J.M., Hornung, M.W., Abnet, C.C., Peterson, R.E., Early life stage toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in zebrafish (*Danio rerio*). Toxicology and Applied Pharmacology. 142, 56-68.

Houtman, C.J., Cenijn, P.H., Hamers, T., Lamoree, M.H., Legler, J., Murk, A.J., Brouwer, A., 2004. Toxicological profiling of sediments using in vitro bioassays, with emphasis on endocrine disruption. Environ. Toxicol. Chem. 23, 32-40.

Huntley, S. L., Carlson-Lynch, H., Johnson, G.W., Paustenbach, D. J., Finley, B.L., 1998. Identification of historical PCDD/F sources in Newark Bay estuary subsurface

sediments using polytopic vector analysis and radioisotope dating techniques. Chemosphere. 36(6), 1167-1185.

Hutz, R.J., 1999. Reproductive endocrine disruption by environmental xenobiotics that modulate the estrogen-signaling pathway, particularly tetrachlorodibenzo-*p*-dioxin (TCDD). J. Reprod. Dev. 45(1), 1-12.

Iannuzzi, T.J., Armstrong, T.N., Long, E.R., Iannuzzi, J., Ludwig, D.F., 2008. Sediment quality triad assessment of an industrialized estuary of the Northeastern USA. Environ. Monit. Assess. 139, 257-275.

Jobling, S., Sheahan, D., Osborne, J.A., Matthiessen, P., Sumpter, J.P., 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environ. Toxicol. Chem. 15, 194-202.

Jung, D., Cho, Y., Meyer, J.N., Di Giulio, R.T., 2008. The long amplicon quantitative PCR for DNA damage assay as a sensitive method of assessing DNA damage in the environmental model, Atlantic Killifish (*Fundulus heteroclitus*). Comparative Biochemistry and Physiology, Part C.

Kawajiri, K., Fujii-Kuriyama, Y., 2007. Cytochrome P450 gene regulation and physiological functions mediated by the aryl hydrocarbon receptor. Archives of Biochemistry and Biophysics. 464, 207-212.

Khan, A.T., Weis, J.S., 1987. Effects of methylmercury on sperm and egg viability of two populations of killifish (Fundulus heteroclitus). Archives of Environmental Contamination and Toxicology. 16(4), 499-505.

King Heiden, T., Carvan, M.J., III., Hutz, R.J., 2006. Inhibition of follicular development, vitellogenesis, and serum 17β-estradiol concentreations in zebrafish following chronic, sublethal dietary exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicol. Sci. 90(2), 490-499.

Kolpin, D.W., Furlon, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, Hormones and Other Organic Wastewater Contaminants In U.S Streams, 1999-2000: A National Reconnaissance. Environ. Sci. Technol. 36, 1202-1211.

Lafleur, G.J., Jr., Raldúa, D., Fabra, M., Carnevali, O., Denslow, N., Wallace, R.A., Cerdá, J., 2005. Derivation of major yolk proteins from parental vitellogenins and alternative processing during oocyte maturation in *Fundulus heteroclitus*. Biology of Reproduction 73, 815-824.

Litten, S., 2003. New York/New Jersey Harbor Contaminant Assessment and Reduction Project (CARP). New York State Department of Environmental Conservation. Albany, New York. Available online: http://www.hudsonriver.org/

Long, E.R., Chapman, P.M., 1985. A sediment quality triad: measures of sediment contamination, toxicity and infaunal community composition in Puget Sound. Marine Pollution Bulletin. 16, 405-415.

Lotrich, V.A., 1975. Summer home range and movements of *Fundulus heteroclitus* (pisces: cyprinodontidae) in a tidal creek. Ecology. 56, 191-198.

Hahn, M.E., 1998. The aryl hydrocarbon receptor: a comparative perspective. Comparative Biochemistry and Physiology Part C. 121, 23-53.

McCarty, L.S., Munkittrick, K.R., 1996. Environmental biomarkers in aquatic toxicology. fiction, fantasy or functional? Hum. Ecol. Risk Assess. 2, 268-274.

Meyer, J.N., Nacci, D.E., Di Giulio, R.T., 2002. Cytochrome P4501A (CYP1A) in killifish (*Fundulus heteroclitus*): heritability of altered expression and relationship to survival in contaminated sediments. Toxicol. Sci. 68, 69-81.

Monosson, E., Ashley, J.T.F., McElroy, A.E., Woltering, D., Elskus, A.A., 2003. PCB congener distributions in muscle, liver and gonad of *Fundulus heteroclitus* from the lower Hudson River Estuary and Newark Bay. Chemosphere. 52(4), 777-787.

Mortenson, A.S., Arukwe, A., 2007. Interactions between estrogen- and Ah-Receptor signaling pathways in primary culture of salmon hepatocytes exposed to nonylphenol and 3,3',4,4'-tetrachlorobiphenyl (congener 77).Comparative Hepatology. 6(2).

Muñoz, G.R., Panero, M.A., Powers, C.W., 2006. Pollution prevention and management strategies for dioxins in the New York/New Jersey Harbor. New York Academy of Sciences. New York, NY. Available online: http://www.nyas.org

Nacci, D., Coiro, L., Champlin, D., Jayaraman, S., McKinney, R., Gleason, T.R., Munns, W.R., Jr., Specker, J.L., Cooper, K.R., 1999. Adaptations of wild populations of the estuarine fish *Fundulus heteroclitus* to persistent environmental contaminants. Marine Biology. 134, 9-17.

Nash, J.P., Kime, D.E., Van der Ven, L.T., Wester, P.W., Brion, F., Stahlschmidt-Allner, P., Tyler, C.R., 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. Envir. Health Persp. 112,1725-33.

National Academy of Science (NAS), 2003. Implications of dioxin in the food supply.

Navas, J.M., Segner, H., 2000. Antiestrogenicity of ß-naphthoflavone and PAHs in cultured rainbow trout hepatocytes: evidence for a role of the arylhydrocarbon receptor. Aquat. Toxicol. 51, 79-92.

Ohtake, F., Baba, A., Fujii-Kuriyama, Y., Kato, S., 2008. Intrinsic AhR function underlies cross-talk of dioxins with sex hormone signalings. Biochemical and Biophysical Research Communications. 370, 541-546.

Pait, A.S., Nelson, J.O., 2003. Vitellogenesis in male *Fundulus heteroclitus* (killifish) induced by selected estrogenic compounds. Aquat. Toxicol. 64(3), 331-342.

Panero, M., Boehme, S. Muñoz, G., 2005. Pollution prevention and management strategies for polychlorinated biphenyls in the New York/New Jersey Harbor. New York Academy of Sciences. New York, NY. Available online: http://www.nyas.org

Patel, M.R., Scheffler, B.E., Wang, L., Willett, K.L., 2006. Effects Of benzo(a)pyrene exposure on killifish (*Fundulus heteroclitus*) aromatase activities and mRNA. Aquat. Toxicol. 77(3), 267-278.

Patel, R.D., Kim, D.J., Peters, J.M., Perdew, G.H., 2006. The aryl hydrocarbon receptor directly regulates expression of the potent mitogen epiregulin. Toxicol. Sci. 89(1), 75-82.

Patiño, R., Sullivan, C.V., 2002. Ovarian follicle growth, maturation, and ovulation in teleost fish. Fish Physiology and Biochemistry. 26, 57-70.

Patyna, P.J., Davi, R.A., Parkerton, T.F., Brown, R.P., Cooper, K.R., 1999. A proposed multigeneration protocol for Japanese Medaka (*Oryzias latipes*) to evaluate effects on endocrine disruptors. Sci. Total Environ. 233, 211-220.

Patyna, P.J., Brown, R.A., Davi, R.A., Letinski, D.J., Thomas, P.E., Cooper, K.R., Parkerton, T.F., 2005. Hazard evaluation of diisononyl and diiodecyl phthalate in a Japanese Medaka multigeneration assay. Ecotoxicology and Environmental Safety. 65(1), 36-47.

Pinkney, A.E., Harshbarger, J.C., May, E.B., Reichert, W.L., 2004. Tumor prevalence and biomarkers of exposure and response in Brown Bullhead (*Ameiurus nebulosus*) from the Anacostia River, Washington, DC and Tuckahoe River, Maryland, USA. Environmental Toxicology and Chemistry. 23(3), 638-647.

Pitot, H.C., Goldsworthy, T., Campbell, H.A., Poland, A., 1980. Quantitative evaluation of the promotion by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin of hepatocarcinogenesis from diethylnitrosamine. Cancer Research. 40, 3616-3620.

Prince, R., Cooper, K.R., 1995a. Comparison of the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on chemically impacted and non-impacted subpopulations of *Fundulus heteroclitus*. I: TCDD toxicity. Envir. Toxicol. and Chem. 14, 579-587.

Prince, R., Cooper, K.R., 1995b. Comparison of the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on chemically impacted and non-impacted subpopulations of *Fundulus heteroclitus*. II: metabolic considerations. Envir. Toxicol. and Chem. 14, 589-596.

Roy, N.K., Wirgin, I., 1997. Characterization of the aromatic hydrocarbon receptor gene and its expression in Atlantic Tomcod. Archives of Biochemistry and Biophysics. 344(2), 373-386.

Safe, S., Wormke, M., Samudio, I., 2000. Mechanisms of inhibitory aryl hydrocarbon receptor-estrogen receptor crosstalk in human breast cancer cells. Journal of Mammary Gland Biology and Neoplasia. 5(3), 295-306.

Santodonato, J., 1997. Review of the estrogenic and anti-estrogenic activity of polycyclic aromatic hydrocarbons: relationship to carcinogenicity. Chemosphere, 34(4), 835-848.

Schmid, W., 1975. The Micronuclei Test. Mutat. Res. 31(1), 9-15.

Skinner, M.A., Courtenay, S.C., Parker, W.R., Curry, R.A., 2005. Site fidelity of mummichogs (*Fundulus heteroclitus*) in an Atlantic Canadian estuary. Water Qual. Res J. Can. 40, 288-298.

Sorensen, M.T., Conder, J.M., Fuchsman, P.C., Martello, L.B., Wenning, R.J., 2007. Using a sediment quality triad approach to evaluate benthic toxicity in the Lower Hackensack River, New Jersey. Arch. Environ. Contam. Toxicol. 5, 36-49.

Tom, M., Auslander, M., 2005. Transcript and protein environmental biomarkers in fish - a review. Chemosphere. 59, 155-162.

Tom, M., Chen, N., Segev, M., Herut, B., Rinkevich, B., 2004. Quantifying fish metallothionein transcript by real time PCR for its utilization as an environmental biomarker. Marine Pollution Bulletin. 48(7-8), 705-710.

Toomey, B.H., Bello, S., Hahn, M.E., Cantrel, S., Wright, P., Tillitt, D.E., Di Giulio, R.T., 2001. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induces apoptotic cell death and cytochrome p4501A expression in developing *Fundulus heteroclitus* embryos. Aquat. Toxicol. 53, 127-138.

Tran, D.T., Ide, C.F., McLachlan, J.A., Arnold, S.F., 1996. The anti-estrogenic activity of selected polynuclear aromatic hydrocarbons in yeast expressing human estrogen receptor. Biochemical and Biophysical Research Communications. 229, 102-108.

Tyler, C.R., Jobling, S., Sumpter, J.P., 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit. Rev. Toxicol. 28, 319-361.

Valle, S., Panera, M.A., Shor, L., Powers, C.W., 2007. Pollution prevention and management strategies for polycyclic aromatic hydrocarbons in the New York/New Jersey Harbor. New York Academy of Sciences. New York, NY. Available online: http://www.nyas.org

Vogelbein, W.K., Fournie, J.W., Van Veld, P.A., Huggett, R.J., 1990. Hepatic neoplasms in the mummichog *Fundulus heteroclitus* from a creosote-contaminated site. Cancer Research. 50, 5978-5986.

Weis, J.S., Weis, P., Heber, M., Vaidya, S., 1981. Methylmercury tolerance of killifish (*Fundulus heteroclitus*) embryos from a polluted vs nonpolluted environment. Mar. Biol. 65, 283-287.

Wintermyer, M.L., Cooper, K.R., 2007. The development of an aquatic bivalve model: evaluating the toxic effects on gametogenesis following 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) exposure in the Eastern Oyster (*Crassostrea virginica*). Aquat. Toxicol. 81, 10-26.

World Health Organization (WHO), 2001. Biomarkers in risk assessment: validity and validation. Environmental Health Criteria 222. Geneva, Switzerland.

World Health Organization (WHO), 2002. Global Assessment of the State-of-the-Science of Endocrine Disruptors. Available online: http://www.who.int

Wormke, M., Stoner, M., Saville, B., Walker, K., Abdelrahim, M., Burghardt, R., Safe, S., 2003. The aryl hydrocarbon receptor mediates degradation of estrogen receptor α through activation of proteasomes. Molecular and Cellular Biology. 23(6), 1843-1855.

Yuan, Z., Wirgin, M., Courtenay, S., Ikonomou, M., Wirgin, I., 2001. Is hepatic cytochrome p4501A1 expression predictive of hepatic burdens of dioxins, furans, and PCBs in Atlantic Tomcod from the Hudson River estuary? Aquat. Toxicol. 54, 217-230.

Yuan, Z., Courtenay, S., Wirgin, I., 2006. Comparison of hepatic and extra hepatic induction of cytochrome p4501A by graded doses of aryl hydrocarbon receptor agonists in Atlantic Tomcod from two populations. Aquat. Toxicol. 76, 306-320.

Zogorski, J.S., Carter, J.M., Ivahnenko, T., Lapham, W.W., Moran, M.J., Rowe, B.L., Squillace, P.J., Toccalino, P.L., 2006. Volatile organic compounds in the nation's ground water and drinking-water supply wells. U.S. Geological Survey. Reston, VA. Available online: http://pubs.er.usgs.gov/