CONTAMINANT EFFECTS ON VITELLOGENESIS AND OOGENESIS IN ZEBRAFISH (*DANIO RERIO*), AND KILLIFISH (*FUNDULUS HETEROCLITUS*) FROM THE CHEMICALLY IMPACTED NEWARK BAY, NJ

by

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A Dissertation submitted to the

Graduate School–New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Environmental Sciences

Written under the direction of

Professor Keith R. Cooper

and approved by

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New Brunswick, New Jersey

October, 2011

ABSTRACT OF THE DISSERTATION

CONTAMINANT EFFECTS ON VITELLOGENESIS AND OOGENESIS IN ZEBRAFISH (*DANIO RERIO*), AND KILLIFISH (*FUNDULUS HETEROCLITUS*) FROM THE CHEMICALLY IMPACTED NEWARK BAY, NJ

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Dissertation Director: Professor Keith R. Cooper

Vitellogenins are large glycolipoprotein precursors to yolk–proteins that act as vital biomolecules and growth substrate in developing oocytes of teleosts and are synthesized exclusively in the liver. The studies in this Dissertation tested the general hypothesis that aryl hydrocarbon receptor agonist contaminants in Newark Bay, NJ down–regulate hepatic vitellogenin synthesis, resulting in inhibition of oogenesis and reproductive dysfunction. This hypothesis was examined using a variety of studies with a field population of killifish (*Fundulus heteroclitus*) from the contaminated Newark Bay and relatively clean Tuckerton, NJ, and using lab studies with zebrafish (*Danio rerio*) as a model teleost. In killifish native to Newark Bay, inhibition of oocyte yolk–development resulted in decreased egg production, decreased embryo mass and reduced yolk–volume. The cause of these effects was shown to be the down–regulation of vitellogenesis in the liver. Decreased vitellogenin expression during spawning was demonstrated to be due to deficient levels of circulating 17β–estradiol, and a decreased sensitivity of the vitellogenin pathway to induction (protein and mRNA levels) by physiological doses of

17β–estradiol. In the Newark Bay population, vitellogenin expression was inversely correlated with CYP1A, a biomarker for aryl hydrocarbon receptor 2 (AhR2) activity. I therefore propose that the down–regulation of the vitellogenin pathway is phenotypic of aryl hydrocarbon receptor mediated cross–talk inhibition of the estrogen receptor (ER). The role of AhR2 in mediating AhR–ER cross–talk inhibition of vitellogenin was examined using the zebrafish as a model teleost. The potent AhR agonists 2,3,7,8–tetrachlorodibenzo–*p*–dioxin and 1,2,3,7,8–pentachlorodibenzo–*p*–dioxin were found to inhibit the induction of vitellogenins 1, 2 and 3 by 17α–ethynylestradiol in zebrafish. Transient knock–down of AhR2 levels showed that the AhR2 mediates inhibition of vitellogenesis by 2,3,7,8–tetrachlorodibenzo–*p*–dioxin, demonstrating that AhR2 activation plays a role in AhR–ER cross–talk. Taken together, these studies demonstrated that the contaminant induced down–regulation of vitellogenesis in the liver can cause reproductive dysfunction in the ovary and that the mechanism for these effects is mediated through AhR2–ER signaling pathways.

DEDICATION

I dedicate this work to my family – my Father George, my Mother Mary Jo, my older Brother Gregory and my younger Sister Mary Catherine. My passion for wildlife and the great outdoors comes from my Father who instilled many things in me, including spending time outside and how to fish. My value of an education comes from my Mother, who tirelessly pushed me to pursue an education and taught me how to learn. Considering their influence together, it only made sense to pursue a career in Environmental Toxicology. My Brother and Sister are also an inspiration to me for pursuing their own passions in their own lives because they are successful in their own right. I wouldn't be who I am today without them and I'm ever grateful for their love and support.

ACKNOWLEDGEMENTS

I'd like to acknowledge two people that made my experience as a Graduate Student not only possible and enjoyable, but exciting. Josephine Bonventre: I'm not sure I could have made it through Graduate School without her. She was the rock I leaned on through all the stress and difficulty and was an endless supply of moral support and clarity. Dr. Keith Cooper: I only hope that someday I can equal his skill as a Mentor, Comparative Toxicologist, and Environmental Scientist. He was a fantastic P.I. with an incredibly strong work ethic that went above and beyond everything I expected.

Members of the Cooper/White Lab:

The Cooper/White lab team was a fantastic group because we all worked on widely different projects. Dr. Lori White was a fantastic secondary P.I. and was instrumental helping me to think outside the box and more mechanistic. Caren Villano, Kyle Murphy and Tiffany Kung: They were all fantastic in helping me out and were invaluable resources for me to access for getting an opinion on one of my ideas or giving me sound technical advice. I'd also like to thank Marisol Gutierrez, Jedd Hillegass, Kristin Pangallo, Kristen Bircsak, Aaron Lulla and David Clemson.

Committee Members:

This Dissertation was made possible by my committee members, who came together to help me get this project off the ground when it was in its infancy as a Master's Thesis and helped it mature. Their constructive criticism was greatly appreciated.

Others:

Dr. Carolyn Bentivegna of Seton Hall University: She was instrumental to me for helping find my hidden passion for science during my Undergraduate career. Her passion for Environmental Sciences excited and inspired me.

Dr. Gavin Swiatek: He was an invaluable resource to me for his vast expertise in biochemical methods and techniques.

Janessa Maguire and Peter Anderson: These two were fantastic and regularly went above and beyond what I ever needed or asked from you.

Dr. William Belden: In the last year of my Graduate school he inspired me with his knowledge of epigenetics and was a fantastic source for troubleshooting molecular techniques.

Craig Harvey: I appreciate all his help with my 17β–estradiol analyses.

Stacy Bromberger, Evan Reed, Matt Schuster, Mike Perlson, and Chris Caprino: Through all the difficult times, they were there and that's what got me through the stress.

Financial Support:

My research was carried out at the New Jersey Agricultural Experiment Station (NJAES) with funding from NJAES (*01201)* through Cooperative State Research, Education, and Extension Services, The Environmental and Occupational Health Sciences Institute (ES05022), the New Jersey Water Resources Research Institute (2009NJ198B), New Jersey Department of Environmental Protection Agency, Division of Science, Research and Technology (SR09–019) and The National Oceanic and Atmospheric Administration (432742).

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Chapter 6: General discussion

ABBREVIATIONS

AhR	aryl hydrocarbon receptor
AhR2–MO	aryl hydrocarbon receptor 2 morpholino
AHRR	aryl hydrocarbon receptor repressor
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
bp	base pairs
cDNA	complimentary deoxyribonucleic acid
CHG	choriogenin
cm	centimeter
C–MO	control morpholino
CT	threshold cycle
CYP19A1	ovarian cytochrome P450 19A1
CYP19A2	brain cytochrome P450 19A2
CYP1A	cytochrome P450 1A
DD	dibenzo–*p*–dioxin
DNA	deoxyribonucleic acid
dpf	days post fertilization
E2	17β–estradiol
E2–I2	Exon 2–Intron 2
EDC	endocrine disrupting compounds
EDTA	ethylene diaminetetraacetic acid
EE2	17α–ethynylestradiol

ABBREVIATIONS (continued)

EGTA	ethylene glycol tetraacetic acid
ER	estrogen receptor
ERE	estrogen response element
EtOH	ethanol
FSH	follicle stimulating hormone
g	gram
GABA	gamma–aminobutyric acid
GtH	gonadotropin hormone
GtRH	gonadotropin releasing hormone
hpf	hours post fertilization
HPGL	hypothalamus–pituitary–gonad–liver axis
HSP90	heat shock protein 90
iXRE	inhibitory xenobiotic response element
kDa	kilodalton
LH	leutinizing hormone
Mg	milligram
mL	milliliter
mM	millimolar
MO	morpholino
MOPS	3–(N–morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
MS222	tricaine methane sulphonate

ABBREVIATIONS (continued)

N	number of samples/animals in group
NADPH	nicotinamine adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
ng	nanogram
NIC	Non–injected control
nL	nanoliter
nm	nanometer
nM	nanomolar
P450scc	Cytochrome P450 cholesterol side chain cleavage enzyme (CYP11A)
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo–*p*–dioxin
PCDF	polychlorinated dibenzofuran
PCR	polymerase chain reaction
PE	pericardial edema
PeCDF	pentachlorodibenzofuran
pg	pictogram
pmol	picomole
PMSF	phenylmethanesulphonylfluoride
ppb	parts per billion
ppm	parts per million
pptr	parts per trillion

ABBREVIATIONS (continued)

PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
S9	supernatant 9000 fraction
SDS	sodium dodecyl sulfate
SFS	synchronous fluorescent spectroscopy
StAR	steroidogenic acute regulatory protein
TBST	tris–buffered saline with tween–20
TCDD	tetrachlorodibenzo–*p*–dioxin
TCDF	tetrachlorodibenzofuran
µL	microliter
µM	micromolar
µm	micron
UV	ultraviolet
v	volts
VTG	vitellogenin
× g	times gravity
YP	yolk–protein

CHAPTER 1

General Introduction

Anthropogenic chemicals found in aquatic systems can alter normal reproductive biochemistry and disrupt hormone signaling in aquatic species. Endocrine disruption can potentially lead to decreased reproductive success, which ultimately poses a risk to the population and community sustainability. The general purpose of the work presented in this Dissertation was to investigate how contaminants within the heavily polluted Newark Bay, NJ (Fig. 1.1), disrupt the reproductive physiology of oviparous fish. This work is a continuation of the research carried out for my Masters of Science degree. In my Masters work I demonstrated that a population of killifish living within Newark Bay exhibited signs of endocrine disruption indicative of potential impacts on their reproductive success (Chapter 2). Females had decreased expression of vitellogenin, the liver derived egg–yolk protein precursor which is required for oocyte growth and development in all oviparous species. This biochemical impact correlated with altered morphology of the ovary, which showed that Newark Bay females have an inhibition of follicular development at vitellogenin dependent stages of oocyte growth. Based on these observations, studies were designed to further characterize contaminant impacts on oogenesis and explore the relationship between exposure to aryl hydrocarbon receptor agonists (e.g., dioxin like compounds) and the alteration of vitellogenin gene–regulation. Because of the complexity of the hypothalamus–pituitary–gonad–liver (HPGL) axis, I decided to focus primarily on the liver–gonad interaction with an emphasis on hepatic vitellogenesis.

Within Chapter 3, the hypothesis tested was that the inhibition of oocyte development in Newark Bay killifish is due to (1) deficient levels of circulating 17β–estradiol, and (2) a decreased sensitivity of the vitellogenin pathway to physiological doses of 17β–estradiol. Within Chapter 4, the hypothesis tested was that the Newark Bay killifish have a decreased inducibility (mRNA expression) of estrogen responsive genes (vitellogenin, choriogenin, ER α) due to elevated cytochrome P450 metabolism (clearance) of 17β–estradiol. In Chapter 5, I tested the hypothesis that activation of the aryl hydrocarbon receptor 2 (AhR2) by 2,3,7,8–tetrachlorodibenzo–*p*–dioxin (2,3,7,8–TCDD) mediates the inhibition of vitellogenesis. Overall, these studies were designed to link contaminant impacts on the gene regulation of vitellogenin to effects on the reproductive success and sustainability of a population.

1.1. Killifish (Fundulus heteroclitus) and zebrafish (Danio rerio) as model teleosts for reproductive toxicology

Atlantic killifish (Linnaeus: *Fundulus heteroclitus heteroclitus*) was chosen as the primary teleost model for all field–studies for several reasons. First, this model has an extensive history of being used to study contaminant effects in Newark Bay and elsewhere in the greater NY–NJ Harbor Estuary (Weis et al., 1981; Khan and Weis, 1987; Prince and Cooper, 1995a,b; Arzuaga and Elskus, 2002; Monosson et al., 2003). Second, it is commonly used because of its widespread abundance in contaminated and non–contaminated sites. Killifish 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). Therefore, contaminant effects can be attributed to local exposures. Third, killifish play

an important role in the food web of this ecosystem as both a predator and a prey species. For example, studies by Candelmo et al. (2010) demonstrated that bluefish (*Pomatomus saltatrix*) fed contaminated killifish from the Hudson River bioaccumulated PCBs. The killifish is one of the most commonly used small model estuarine/marine species used in ecotoxicology because of these traits. Due to this, there have been major advances in the molecular and biochemical tools available for the killifish, which increases their utility for mechanistic based studies (Burnett et al., 2007). However, the killifish genome is not yet available (circa 2011).

The zebrafish has become one of the major laboratory teleost models and there are considerably more molecular tools available for pursuing mechanistic studies in the zebrafish than in killifish (Hill et al., 2005). For example, the genome of the zebrafish is currently annotated and available and there is a broader body of literature for toxicological studies using the zebrafish. Therefore, the zebrafish was employed as a laboratory model to study contaminant effects on the estrogen receptor regulation of vitellogenin. Zebrafish studies were based on the findings from the field–based studies of the Newark Bay killifish. The conserved nature of the reproductive physiology in fish allows for findings obtained in the zebrafish to be applied to the killifish with appropriate caveats.

1.2. Reproductive dysfunction of the hypothalamus–pituitary–gonad–liver in oviparous teleosts

Reproductive dysfunction in aquatic species can result from anthropogenic chemicals that disrupt the tightly controlled events in the hypothalamus–pituitary–gonad–

liver (HPGL) axis which regulates reproduction (Arcand–Hoy and Benson, 1998; Rempel and Schlenk, 2008). Reproductive physiology and the HPGL axis in oviparous (egg–bearing) fish species is considered highly conserved, although reproductive strategies vary widely from species to species (e.g. clutch size, egg size, asynchronous/synchronous oocyte development, spawning season). Disruption of the HPGL at any level of regulation is thought to have down–stream effects that may manifest in tissues other than where the chemical disruption occurred (Fig. 1.2). Steroidogenesis and vitellogenesis regulation are two conserved processes critical to oocyte development in all oviparous teleosts, and are discussed below (see reviews by Tyler and Sumpter, 1996; Patiño and Sullivan, 2002; Thomas 2008).

Environmental cues are the major determining factors for the activation of the HPGL and subsequent onset of gonadal recrudescence in adult fish (Vlaming, 1972). Many fish, although active throughout much of the year, may only spawn during a brief period lasting anywhere from a few weeks to several months. The major environmental factors influencing the HPGL are seasonal changes in water temperature, photoperiod and nutritional state (food availability). Fish are sensitive to changes in the season due to sensory receptors in the pre–optic region of the hypothalamus, which is the region of the brain responsible for thermoregulation in higher vertebrates. The initial hormonal and neuroendocrine triggers responsible for stimulating the activation of the hypothalamus are not well understood, but likely involve changes in neurotransmitter, steroid and peptide hormone levels. After gonadal recrudescence and during gametogenesis, neurotransmitters have been shown to regulate pituitary gonadotropin hormone (GtH) synthesis and release through effects on the hypothalamus (Trudeau et al., 2000). The

main hormones produced by the pituitary are gonadotropin hormones I and II (GtH I and GtH II), which are respectively homologous to follicle stimulating hormone (FSH) and luteinizing hormone (LH) in higher vertebrates. Synthesis of GtH I and II is induced by the production and release of gonadotropin releasing hormone I and II (GtRH I and II) in the hypothalamus, respectively. Dopamine production and release by dopaminergic neurons in the hypothalamus has been shown to inhibit the production and release of GtRH II. Gamma–aminobutyric acid (GABA), a dopamine receptor antagonist, has been shown to antagonize the inhibitory effects of dopamine on GtRH by decreasing dopamine turnover and stimulating release of GtRH II, leading to increased GtH II levels (Kah et al., 1992; Trudeau et al., 1993; Trudeau et al., 2000). GtH I has been implicated in stimulating gametogenesis, increasing the uptake of vitellogenin in developing oocytes, and recruitment of a new population of primary oocytes. GtH I and GtH II have both been shown to stimulate steroidogenesis in ovarian follicles, although they are differentially regulated throughout the oocyte developmental process (Slater et al., 1994; Prat et al., 1996). GtH I levels are highest during oocyte stages of vitellogenin–dependent growth, while GtH II expression is low, or not detectable. During follicle maturation, GtH II expression is highest due to its role in stimulating maturation. Various contaminants such as PCBs, cadmium and phenanthrene have been shown to alter gene expression of enzymes involved in gonadotropin release and neurotransmitter and estrogen synthesis (Martyniuk et al., 2009). Effects on the neuroendocrine control of neurotransmitter and gonadotropin synthesis and release are believed to have down–stream effects on steroidogenesis and oogenesis in the ovary.

Primordial germ cells in the ovary differentiate into oogonia which form nests, and upon hormonal stimulation they will undergo meiosis and transform into immature oocytes (Fig. 1.3). The growth and development of the oocyte generally goes through a period of pre–vitellogenic growth, and 3 stages of vitellogenin–dependent growth, which accounts for the majority of growth in size of the oocyte (Fig. 1.3). During the pre–vitellogenic stage, the immature oocyte develops supporting layers of steroid secreting cells (thecal and granulosa cells) and epithelial cells that are critical to the development and maturation of each oocyte (Fig. 1.4). Theca and granulosa cell layers work together to synthesize various steroids needed for growth and maturation. The conversion of cholesterol to androgen, and androgen to estradiol is a process regulated by various cytochrome P450 enzymes and regulatory proteins, such as the steroidogenic acute regulatory (StAR) protein which translocates cholesterol from outer to inner mitochondrial membranes (Stocco, 2001). Cytochrome P450 cholesterol side chain cleavage enzymes (P450scc/CYP11A) and various hydroxylase and dehydrogenase enzymes are also important for androgen metabolism. Thecal cells produce and secrete androgens, which are converted by ovarian aromatase (cytochrome P450 19A1) into 17β–estradiol in adjacent granulosa cells. The secretion of 17β–estradiol by the granulosa cells and surge of circulating levels stimulates vitellogenesis in the liver. Exposure to various aryl hydrocarbon receptor agonists has been shown to decrease expression of StAR and P450scc (Aluru et al., 2005). Impacts on gene expression of enzymes involved in steroidogenesis may have impacts on vitellogenesis and ultimately, egg development.

Vitellogenin proteins are large glycolipoproteins that are precursors to egg–yolk proteins in the developing oocyte of nearly all oviparous organisms (e.g. birds,

amphibians, reptiles, fish, insects). During oogenesis, vitellogenins are exclusively synthesized in the hepatocytes of the liver of all teleosts, and are transported through the blood to the developing follicles (Fig. 1.3) (Hiramatsu et al., 2006). Vitellogenin gains access to the oocyte through intercellular channels and absorbed by receptor–mediated endocytosis beginning at the pre–vitellogenic (late) and early–vitellogenic stages (Fig. 1.3). Inside the developing oocyte, vitellogenins are cleaved by cathepsin enzymes into phosvitin and lipovitellin proteins, and the smaller β′ components (Kanungo et al., 1990; LaFleur et al., 2005). Multiple vitellogenin isoforms exist in teleosts, which are usually structurally similar and composed of lipovitellin I, phosvitin and lipovitellin II domains, which are the yolk–proteins produced by cleavage (Wahli, 1988). For example, two isoforms exist in killifish and seven in zebrafish (LaFleur et al., 2005, Wang et al., 2005). In killifish, vitellogenin 1 is the precursor for the majority of the known yolk–proteins (LaFleur et al., 2005). However, the different biological roles of each yolk–protein in embryo development are not clear. Vitellogenins are directly involved in the transport of vital biomolecules (lipids, sugars, minerals) into the egg–yolk for the development of the organism, and the derived yolk–proteins also play a role in maturation (hydration). The yolk–proteins act as a growth substrate for developing organisms through the eleutheroembryo stage. Hepatic vitellogenesis is regulated through estrogen receptor (ER) pathway activation by 17β–estradiol (Fig. 1.5). In general, circulating 17β–estradiol will enter the hepatocytes and bind to various ER isoforms, which form hetero– or homo–dimers and translocate into the nucleus to induce transcription of vitellogenin genes (Menuet et al., 2005). Several ER isoforms (α, βa and βb) are suspected to play a role in the induction of vitellogenin in teleosts, although the specific role of each isoform in

forming homo and heterodimers, and their regulation of each vitellogenin isoform *in vivo* is not well understood (Nelson and Habibi, 2010). In female oviparous organisms, circulating vitellogenin and mRNA expression levels can be useful for evaluating reproductive status. However, in males vitellogenin genes are present but are not actively expressed unless stimulated by xeno–estrogens (i.e. estrogenic contaminants), which allows vitellogenin to be a useful biomarker for exposure (Sumpter and Jobling, 1995).

1.3. Major contaminants in the NY–NJ Harbor Estuary and effects

Newark Bay, NJ, is part of an interconnected system of bays, rivers, and tributaries known as the greater New York–New Jersey Harbor Estuary (Fig. 1.1). The Raritan River, Hudson River, Passaic River, Hackensack River, Newark Bay, and Upper New York Bay are major rivers and bays of the NY–NJ Harbor Estuary, but many smaller tributaries are also included. Due to their connectivity, migratory species and contaminants (through tidal forces) have the ability to move throughout the NY–NJ Harbor Estuary. The area surrounding this ecosystem has a long history of economic and industrial development which has led to contamination and accumulation of virtually every anthropogenic pollutant, including but not limited to polychlorinated dibenzo–*p*–dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, heavy metals, brominated flame retardants, pharmaceuticals and endocrine active compounds (Assaf–Anid, 2003; Litten 2003; CARP, 2007; Bugel, 2009). Sources of contaminants are historically from local industry, although much of the contamination comes from many non–point sources from the surrounding heavily metropolitan area. Ongoing

contamination in this watershed is primarily due to municipal discharges, wastewater effluents, atmospheric deposition, storm water runoff, incinerators, waste management burning and structural fires. Because of the ubiquity of contaminants in this system, aquatic species are exposed to complex mixtures of contaminants. Reproductive impacts are therefore likely the cumulative effect of contaminants disrupting various processes throughout the HPGL. The major toxicants in Newark Bay and their impacts on fish that are reviewed here are aryl hydrocarbon receptor agonists (e.g. PCDDs, PCDFs) and endocrine active compounds, which have a widespread occurrence throughout the NY–NJ Harbor Estuary. A number of these contaminants are found at levels in sediment and water which would impact reproductive success in species inhabiting these waters (CARP, 2007; Steinberg et al., 2004).

1.3.1. Aryl hydrocarbon receptor (AhR) agonist contaminants and toxicity

The major contaminants throughout the NY–NJ Harbor estuary include PCDDs, PCDFs, PAHs and PCBs (Table 1.1). These compounds are agonists for the aryl hydrocarbon receptor (AhR), which mediates many toxic responses when activated, such as developmental and reproductive toxicity.

In Newark Bay, total PAHs and PCBs are very high in sediments and have been measured to be 44,000 and 756 ng/g (parts–per–billion, ppb), respectively (Huntley et al., 1995; Gigliotti et al., 2005; Panero et al., 2005; Valle et al., 2007). Elevated body burdens of PCBs have been measured in killifish ranging between 200 to 1596 ng/g in the muscle, liver and gonad (Monosson, 2003). However, in Newark Bay, the predominant toxicants are the PCDDs and PCDFs, which are among the highest concentrations in the world ever

reported for tissue and sediment concentrations. In 1985, 2,3,7,8–tetrachlorodibenzo–*p*–dioxin (2,3,7,8–TCDD) and 2,3,7,8–tetrachlorodibenzofuran (2,3,7,8–TCDF) were 310 and 290 parts–per–trillion (pptr) in surface sediments, and 260 pptr and 280 pptr in suspended solids in Newark Bay, respectively (Bopp et al., 1991). In 1985, the NJ Department of Environmental Protection reported tissue levels of 2,3,7,8–TCDD in fish from Newark Bay that exceeded the 50 parts–per–trillion (pptr) level of concern set by the Food and Drug Administration (Belton et al., 1985). Over twenty years later, Dimou and Pecchioli (2006) reported that concentrations of 2,3,7,8–TCDD were as high as 210 pptr in Newark Bay, and found 2,3,7,8–TCDD to be at parts per billion levels elsewhere in the NY–NJ Harbor Estuary (Elizabeth River, Rahway River, Raritan River). Muñoz et al. (2006) estimates that the 3 most toxic congeners were 2,3,7,8–TCDD, 2,3,7,8–TCDF and 2,3,4,7,8–pentachlorodibenzofuran (2,3,4,7,8–PeCDF), which account for > 90% of the total sediment toxicity and that these three congeners are being removed from the NY–NJ Harbor Estuary although total PCDD/PCDFs are accumulating. Total PCDDs and PCDFs in Newark Bay sediments have been demonstrated to be 0.8 to 9.3 ng/g (ppb) and 0.2 to 3.7 ng/g (ppb), respectively (Dimou and Pecchioli, 2006; Muñoz et al., 2006). Tissue concentrations of total PCDDs in Atlantic Tomcod (*Microgadus tomcod*) (1,109 to 1,736 pptr) and blue crab (*Callinectes sapidus*) (6,000 pptr) from the Newark Bay area were also amongst the highest ever reported in wildlife (Rappe et al., 1991; Fernandez et al., 2004; Muñoz et al., 2006). Soft–shell clams (*Mya arenaria*) in Newark Bay have been reported to have 2,3,7,8–TCDD levels ranging from 11 to 20 pptr (Brown et al., 1993). These studies demonstrate that PCDD/PCDF concentrations in sediments have remained high for several decades, and that in particular 2,3,7,8–TCDD bioaccumulates

to high concentrations in various aquatic species leading to chronic exposure to relatively high concentrations.

Toxicity of AhR agonists (PCDDs, PCDFs, PAHs, and PCBs), is largely mediated by activation of the aryl hydrocarbon receptor pathway (Fig. 1.6), which is reviewed by Hahn (1998, 2002) and Denison and Nagy (2003). The inactive AhR is bound to heat shock protein 90 (HSP90) in the cytosol and exogenous ligands bind to the cytosolic AhR through competition with HSP90. The ligand–bound AhR translocates into the nucleus and forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT). The ligand–bound AhR competes for binding to ARNT with the aryl hydrocarbon receptor repressor (AHRR), which is an inhibitory protein in this pathway that inactivates the ARNT by binding to it (Evans et al., 2008). The activated ligand–AhR–ARNT recruits various cofactors and the complex binds to DNA via xenobiotic responsive elements (recognition motif) and induces a variety of genes, such as cytochrome P450 genes (e.g. CYP1A). Under normal conditions, the AhR pathway is involved in a myriad of biological activities. Many cytochrome P450 enzymes regulated by the AhR pathway are involved in the anabolic metabolism of many biomolecules, such as steroids (e.g. CYP1A, CYP11A, CYP19A1, CYP19A2). Many cytochrome P450 enzymes also carry out phase I hydroxylation reactions, which work in conjunction with phase II conjugation enzymes (e.g. glutathione S–transferase, sulfotransferases) to transform chemicals and facilitate elimination. The AhR pathway also has an endogenous role in cell cycle signaling, cell proliferation and the regulation of transforming growth factor β1 (Chang et al., 2007; Kawajiri and Fujii–Kuriyama, 2007). In addition to regulating processes involved in maintaining cell function, metabolism and homeostasis, a wide array of toxic

responses are mediated by activation of the AhR pathway. The AhR pathway has been demonstrated to mediate developmental abnormalities (circulation failure, craniofacial malformations, pericardial edema, yolk sac edema) and mortality in several teleosts such as killifish and zebrafish (Henry et al., 1997). In teleosts there are several AhR isoforms (e.g. zebrafish: AhR 1a/b, 2; killifish: AhR 1, 2) and multiple isoforms of ARNT (e.g. ARNT 1, 2). In killifish and zebrafish, AhR2 and ARNT 1 have been demonstrated to mediate CYP1A induction and developmental toxicity while the roles of AhR 1 and ARNT 2 are unclear (Dong et al., 2004; Prasch et al., 2003; Prasch et al., 2004; Prasch et al., 2006; Clark et al., 2010). In zebrafish, the induction of CYP1A is correlated with the toxic effects of AhR agonists, but is not thought to be a causative factor (Prasch et al., 2003; Carney et al., 2004). However, it is not clear whether CYP1A induction contributes to developmental toxicity because of conflicting reports by Teraoka et al. (2003). Nevertheless, elevated CYP1A expression and induction in the liver is a commonly used molecular biomarker for exposure to AhR contaminants and activation of AhR 2 (James and Bend, 1980; Binder and Stegeman, 1984; Billiard et al., 2002).

In addition to developmental toxicity, activation of the AhR pathway has been implicated in the disruption of estrogen signaling. In general, there is more information regarding AhR–ER cross–talk in higher vertebrates than in teleosts, and most cross–talk studies in fish are *in vitro* and relatively few take advantage of *in vivo* models. Exposure to AhR agonists have been shown to down–regulate estrogenic processes. There are six proposed mechanisms of AhR–ER cross–talk (Fig. 1.7). These include (1) direct inhibition of the estrogen receptor pathway by AhR binding to cis–regulatory elements near estrogen responsive elements (regulatory motifs) required for ER binding (inhibitory

xenobiotic response elements), (2) interaction of the AhR with common cofactors required by the ER (squelching), (3) AhR–induced synthesis of an inhibitory factor, 4), proteasomic degradation of estrogen receptors, (5) induction of enzymes involved in estrogen metabolism/clearance, and (6) competition for resources necessary for synthesis of AhR and ER–mediated mRNA and proteins (Safe and Wormke, 2003; Wormke et al., 2003; Matthews and Gustafsson, 2006; Ohtake, 2008). In rodents, exposure to 2,3,7,8–TCDD decreases circulating 17β–estradiol and induces an early transition into reproductive senescence (Petroff et al., 2000; Franczak et al., 2006; Shi et al., 2007; Valdez et al., 2009). Ovarian toxicity by AhR agonists and the impact on reproductive success has been a much greater focus than relating impacts on the liver and impacts on fecundity. In fish, vitellogenesis in the liver is an estrogen receptor mediated process that is thought to be directly affected by AhR activation. Zebrafish exposed to 2,3,7,8–TCDD exhibit a decreased sensitivity to gonadotropins, decreased 17β–estradiol levels, reduced vitellogenin synthesis, and an inhibition of follicle development (King Heiden et al., 2006; King Heiden et al., 2008; King Heiden, 2009). Furthermore, studies by King Heiden (2009) showed that zebrafish exposed to 2,3,7,8–TCDD decreased fecundity in the F1 generation, demonstrating the potential for transgenerational reproductive effects. These studies concluded that the impacts on follicle development, and vitellogenin production, were primarily due to impacts on steroidogenesis in the ovary. However, there is mounting evidence from *in vitro* studies that AhR agonists can directly affect vitellogenin regulation by inhibiting induction by 17β–estradiol in teleost hepatocytes (Anderson et al., 1996a; Navas and Segner, 2000; Bemanian et al., 2004; Mortensen and Arukwe, 2007; Gräns et al., 2010). However, very little evidence exists regarding AhR–

ER interactions *in vivo* and the effect of AhR activation on vitellogenesis gene regulation resulting from acute exposures. Vaccaro et al. (2005) and Anderson et al. (1996b) demonstrated that acute exposure to PCB 126 and PAHs can inhibit E2–induced vitellogenin synthesis in sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*), *in vivo*. The relationship between exposure to AhR agonists resulting in AhR activation, and reproductive impacts in aquatic species in Newark Bay and elsewhere in the NY–NJ Harbor Estuary is a topic explored *in vivo* by the current studies using the killifish and zebrafish models.

1.3.2. Altered AhR signaling in Newark Bay killifish and acquired AhR resistance

Aquatic species in Newark Bay are chronically exposed to complex mixtures of AhR agonists and activation of the AhR pathway may have reproductive impacts due to AhR–ER cross–talk. Studies by Prince and Cooper (1995a,b) have previously shown that a population of killifish inhabiting Newark Bay expresses induced levels of CYP1A activity relative to the reference population from Tuckerton, which is indicative of an activated AhR pathway from exposure to AhR agonists. This population also has an inability to induce pathological lesions, CYP1A activity and CYP1A mRNA when challenged with PCDDs, PAHs, and PCBs (Prince and Cooper, 1995a,b; Arzuaga and Elskus, 2002). This chemical resistance is an adaptation due to chronic exposure to AhR agonists over several decades and generations, which has resulted in an attenuated AhR pathway (i.e. refractive to induction). A separate study demonstrated that the depuration of killifish from Newark Bay resulted in an 80% reduction of total embryo PCB levels, yet embryos were still resistant to CYP1A activity and protein induction by a PCB

challenge (Elskus et al., 1999). This suggests that biochemical and molecular changes to AhR signaling are persistent even after the toxicants have been depurated out of the organism. However, the mechanism for this resistance is currently unknown. The population of Newark Bay killifish used within this Dissertation is not the same as the previously reported resistant population, and it is not known whether they too are resistant to AhR–mediated toxicity. The resistant population in Newark Bay (West bank) is directly across the Bay from the population used in the current studies (East bank), which are approximately 1.3 km apart. In addition to the resistant Newark Bay population, several other populations of killifish along the east coast have been shown to be resistant to AhR activation, including: Bridgeport, CT; New Bedford Harbor, MA; Elizabeth River (Portsmouth), VA (Nacci et al., 1999; Bello et al., 2001; Arzuaga, 2010; Nacci et al., 2010). In the Elizabeth River population, the resistant AhR pathway was demonstrated to be heritable and resistance to PAHs carried for several generations raised under laboratory conditions (Meyer et al., 2002). However, the resistance was evident in three generations of field–caught offspring, although the second and third generation's resistance was less than that of the parental generation which suggests that the etiology of the refractory phenotype although long–lasting, is not permanent. The AhR2 has been demonstrated to mediate PAH and PCB developmental toxicity in killifish, therefore impacts on the AhR2 are suspected to be the mechanism for this resistance (Clark et al., 2010). It is clear that resistance to AhR agonists is a condition resulting from chronic exposure to AhR agonists. In Newark Bay and other populations, the relationship between impacts on AhR the AhR pathway and cross–talk with the estrogen receptor pathway are not yet clear.

A population of Atlantic Tomcod (*Microgadus tomcod*) has also been shown to be resistant to AhR mediated toxicity in the Hudson River in the NY–NJ Harbor Estuary (Roy and Wirgin, 1997; Courtenay et al., 1999). The Hudson River is heavily polluted by PCBs and PAHs and interestingly, this population is resistant to PCBs, but not PAHs, demonstrated by decreased CYP1A inducibility, and altered CYP1A kinetic profiles. CYP1A (mRNA) in Hudson River tomcod is inducible by high doses of AhR agonists, with maximum induction values comparable to fish from the reference site (Yuan *et al*., 2006). This suggests that the AhR pathway is functional but much less sensitive to activation by agonists. Recently, it has been demonstrated that the basis for resistance in this population of tomcod is due to a six base pair deletion in a critical exon of the AhR2 gene (Wirgin et al., 2011).

1.3.3. Endocrine active compounds

In addition to historical sources of AhR agonist contaminants, the NY–NJ Harbor Estuary ecosystem is threatened by emerging contaminants of concern, such as pharmaceuticals and endocrine active compounds. This ecosystem is at the center of the most densely populated region in the country with approximately 13 million people living within a 10 mile radius. This aquatic system receives discharge from approximately 30 wastewater treatment plants serving this metropolitan population (NY–NJ HEP, 2006). Wastewater effluent has emerged as a significant source of endocrine active compounds such as pharmaceuticals and 17α–ethynylestradiol (Kolpin et al., 2002; Zogorski et al., 2006; Barnes et al., 2008; Focazio et al., 2008). Endocrine active compounds are contaminants that are not characterized by being structurally similar, but by having

hormonal biological activity. Endocrine active compounds known to be present in the NY–NJ Harbor Estuary include: various pesticides, tributyltin from shipping, 17β–estradiol and ethinylestradiol (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, 2005), bisphenol A from secondary water treatment facilities, brominated flame retardants (Hamers et al., 2006) and various PCDDs/PCDFs.

Exposure to endocrine active compounds can disrupt reproductive physiology in fish and other aquatic species leading to compromised reproductive health, and ultimately threaten the sustainability of a population (Arcand–Hoy and Benson, 1998; Tyler et al., 1998; Nash et al., 2004). Reproductive impacts from low–dose, chronic exposures manifest over many years and generations. Despite the presence of known xeno–estrogens within Newark Bay, studies by McArdle et al (2004) have shown that sediment extracts injected into killifish do not induce an estrogenic response, measured by vitellogenin induction. Therefore, endocrine responses may not be classical due to the inhibition or antagonism of other contaminants present in the complex mixtures. Studies by McArdle et al (2000) and Todorov et al (2002) demonstrated that effluents from wastewater treatment plants entering the NY–NJ Harbor Estuary are estrogenic and induce vitellogenin in sunshine bass (*Morone saxatilis* × *Morone Chrysops).* Studies by Baldigo et al. (2006) reported elevated vitellogenin levels and altered 17β–estradiol/11–ketotestosterone ratios in common carp (*Cyprinus carpio*), bass (*Micropterus salmoides* and *Micropterus dolomieui*) and bullhead (*Ameiurus nebulosus*) in the Hudson River (NY). Taken together, these studies show that endocrine disrupting contaminants are entering the NY–NJ Harbor Estuary and are likely widespread. Therefore, endocrine

active contaminants, even at low doses, may be impacting the reproductive success of fish populations that are chronically exposed. For example, exposures to low doses of estrogen have been shown to impair both male and female fish. Kidd et al (2007) reported that fathead minnows (*Pimephales promelas*) chronically exposed to environmentally relevant concentrations of 17α–ethynylestradiol (5–6 ng/L) over seven years resulted in feminization of males, inhibition of oogenesis in females, and the collapse of the population. In males, exposure to estrogen mimics impairs gonadal development and testis function (Jobling et al., 1996; Nash et al., 2004; Kidd et al., 2007).

1.3.4. Killifish population selection in Newark Bay and Tuckerton

The killifish sub–population sampled within Newark Bay for studies conducted in this Dissertation was on the Eastern shore of the bay in a restored wetland in Bayonne, NJ, known as the "North 40 Park", also known as the "Richard Rutkowski Park" (Fig. 1.1). This sub–population is directly across from the sub–population that was previously characterized by Prince and Cooper (1995a,b) to be resistant to AhR–mediated toxicity (western shore). These two populations are separated by approximately 1.3 km of open water, and it is unknown whether the sub–population from the Bayonne wetland site is resistant to AhR agonists. This sub–population was selected due to its readily available access. Restoration of this 10 acre wetland was completed 2006 and dedicated as a wetland preserve by the City of Bayonne. Approximately 32 upland acres and 10 wetland acres were restored by widening and deepening the small channels innervating the wetland that were previously present to increase tidal flow into the wetland. This

wetland was restored to additional habitat for aquatic species, including migratory fish and birds, and shellfish that may 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 (Fig. 1.1). 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.

1.4. Research objectives and hypothesis

The global hypothesis tested was that contaminants in Newark Bay (AhR agonists such as PCDDs) alter the gene regulation of vitellogenesis in the liver and down–regulate the pathway through AhR–ER interactions, resulting in reproductive dysfunction. The specific aims of this study were to (1) evaluate the effects of contaminants on the reproductive success of killifish from Newark Bay, (2) investigate chemical impacts on the gene regulation of vitellogenesis and steroid signaling pathways in killifish from Newark Bay, and (3) use the zebrafish as a comparative model to demonstrate how AhR agonists directly down–regulate vitellogenesis through AhR–ER pathway cross–talk. Many studies focus on the impacts of contaminants on the ovary, but the goal of this work was to establish a stronger link between contaminant impacts directly on the liver and reproductive dysfunction.

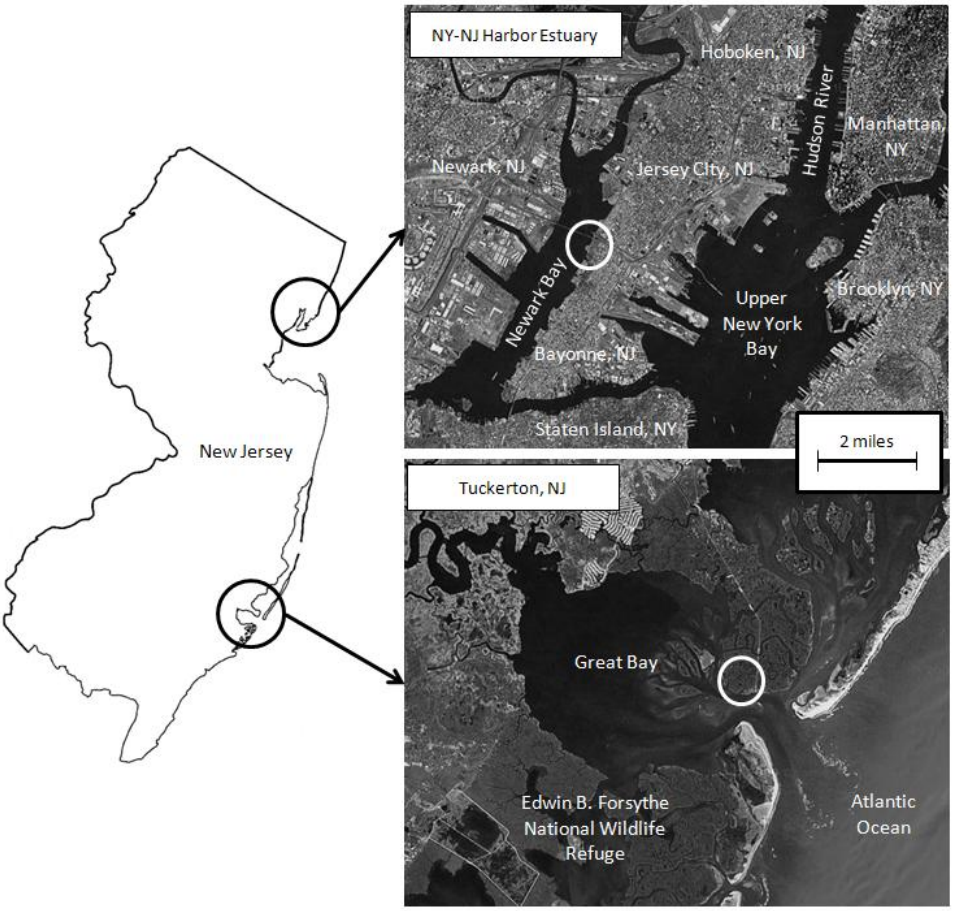


Fig. 1.1. Satellite images of the killifish sampling sites (circled) are shown at Newark Bay (part of the NY–NJ Harbor Estuary), and the reference site at Tuckerton, NJ.

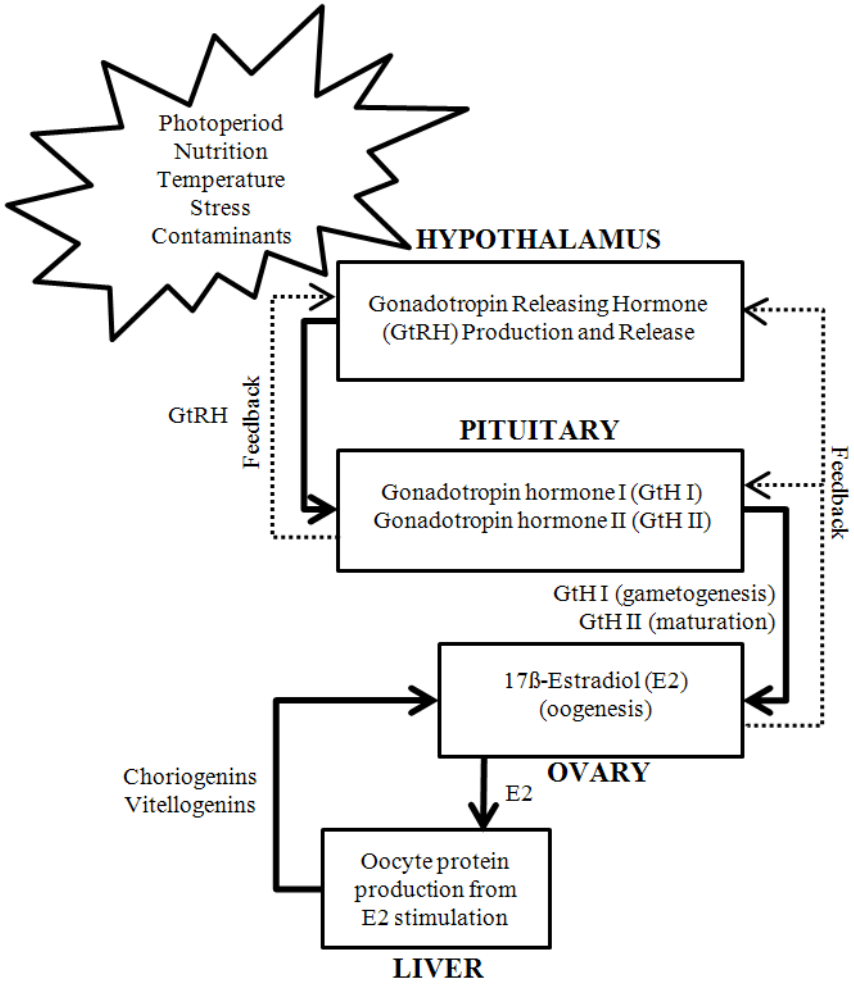
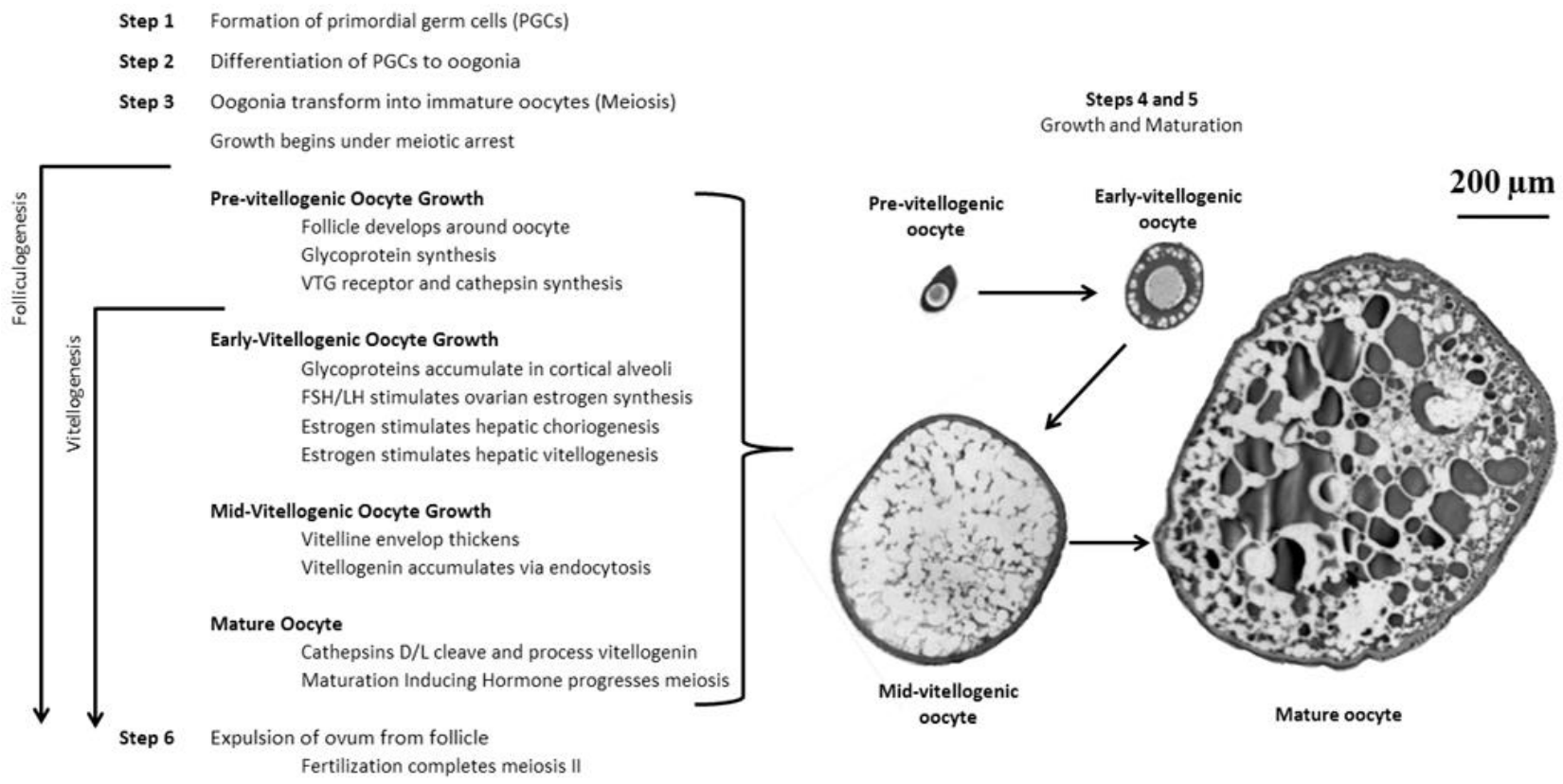


Fig. 1.2. The hypothalamus–pituitary–gonad–liver (HPGL) axis in teleosts is highly conserved and regulates reproduction through neurotransmitter, protein and steroidal hormones.

Fig. 1.3. Oogenesis events in teleosts.



Sizes of follicles are relative. Magnification is 40X.

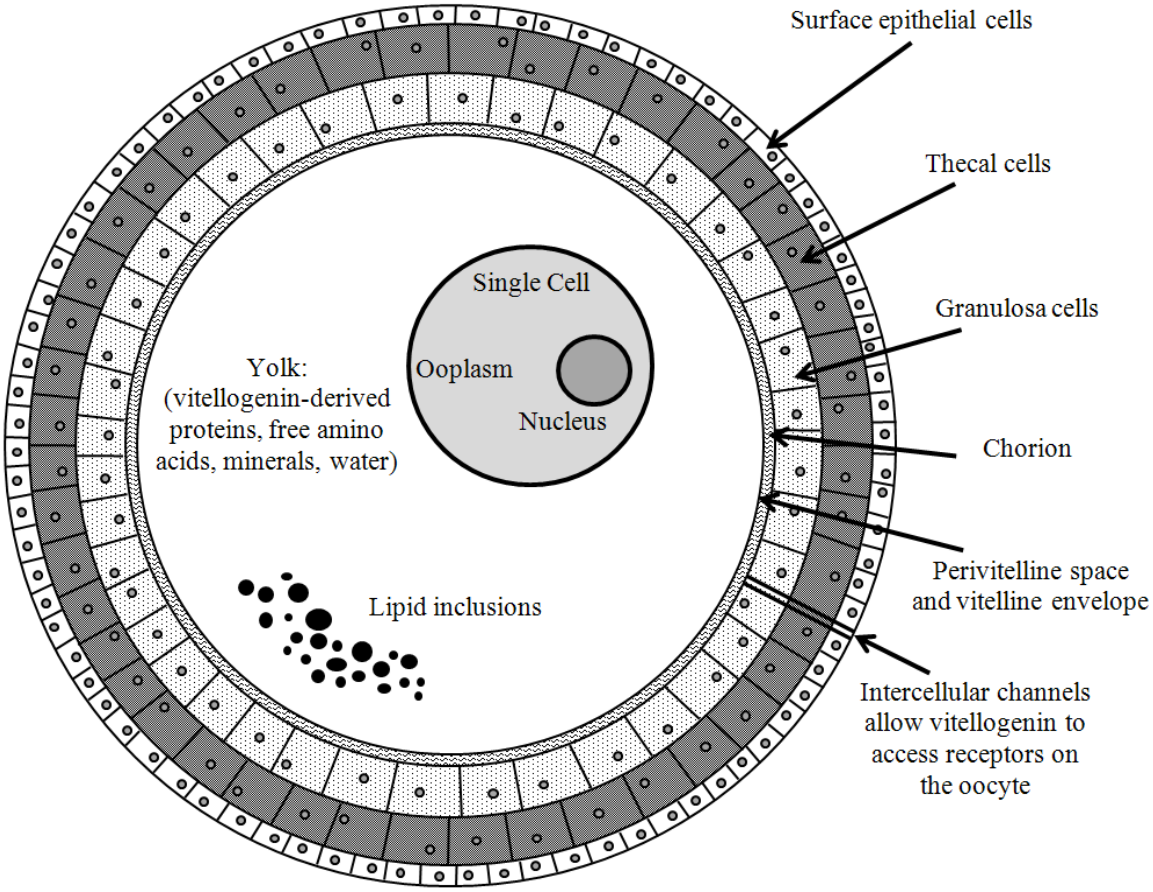


Fig. 1.4. General schematic of a mature follicle in females. The mature egg is surrounded by many layers of various steroid secreting cells and epithelium.

Table 1.1. Sediment concentrations of various AhR agonists in Newark Bay, NJ

Contaminant	Sediment Concentration	Sources
PCDDs	ng/kg (dry weight)	
2,3,7,8-TCDD	49.9, 6-210	Muñoz et al., 2006; Dimou and Pecchioli, 2006
1,2,3,7,8-PeCDD	2.8	Muñoz et al., 2006
1,2,3,4,7,8-HxCDD	3.2	Muñoz et al., 2006
1,2,3,6,7,8-HxCDD	13.5	Muñoz et al., 2006
1,2,3,7,8,9-HxCDD	8.3	Muñoz et al., 2006
1,2,3,4,6,7,8-HpCDD	213	Muñoz et al., 2006
OCDD	2370	Muñoz et al., 2006
Total PCDD	800-9300	Dimou and Pecchioli, 2006
PCDFs	ng/kg (dry weight)	
2,3,7,8-TCDF	15.6	Muñoz et al., 2006
1,2,3,7,8-PeCDF	7.2	Muñoz et al., 2006
2,3,4,7,8-PeCDF	14.4	Muñoz et al., 2006
1,2,3,4,7,8-HxCDF	41.7	Muñoz et al., 2006
1,2,3,7,8,9-HxCDF	2.9	Muñoz et al., 2006
1,2,3,4,7,8,9-HpCDF	10.2	Muñoz et al., 2006
OCDF	419	Muñoz et al., 2006
Total PCDF	200-3700	Dimou and Pecchioli, 2006
PCBs	ng/kg (dry weight)	
3,3´,4,4´-TeCB (PCB 77)	11560	Muñoz et al., 2006
2,3,3´,4,4´-PeCB (PCB 105)	12516	Muñoz et al., 2006
2,3´,4,4´,5- PeCB (PCB 118)	28147	Muñoz et al., 2006
3,3´,4,4´,5- PeCB (PCB 126)	5040	Muñoz et al., 2006
Total PCB	756000 ± 270000	Panero et al., 2005
PAHs	mg/kg (dry weight)	
Anthracene	2.4±5.57	Huntley et al., 1995
Benzo(a)pyrene	1.7±4.22	Huntley et al., 1995
Chysene	2.2±4.90	Huntley et al., 1995
Dibenzofuran	2.0±4.60	Huntley et al., 1995
Fluoranthene	5.3±15.0	Huntley et al., 1995
Naphthalene	1.8±4.44	Huntley et al., 1995
Phenanthrene	4.7±15.8	Huntley et al., 1995
Pyrene	3.9±9.76	Huntley et al., 1995
Total PAHs	44±98.5	Huntley et al., 1995

Note: Not all contaminants shown. Totals do not reflect the sum compounds shown. Values are presented as means or ranges of measurements.

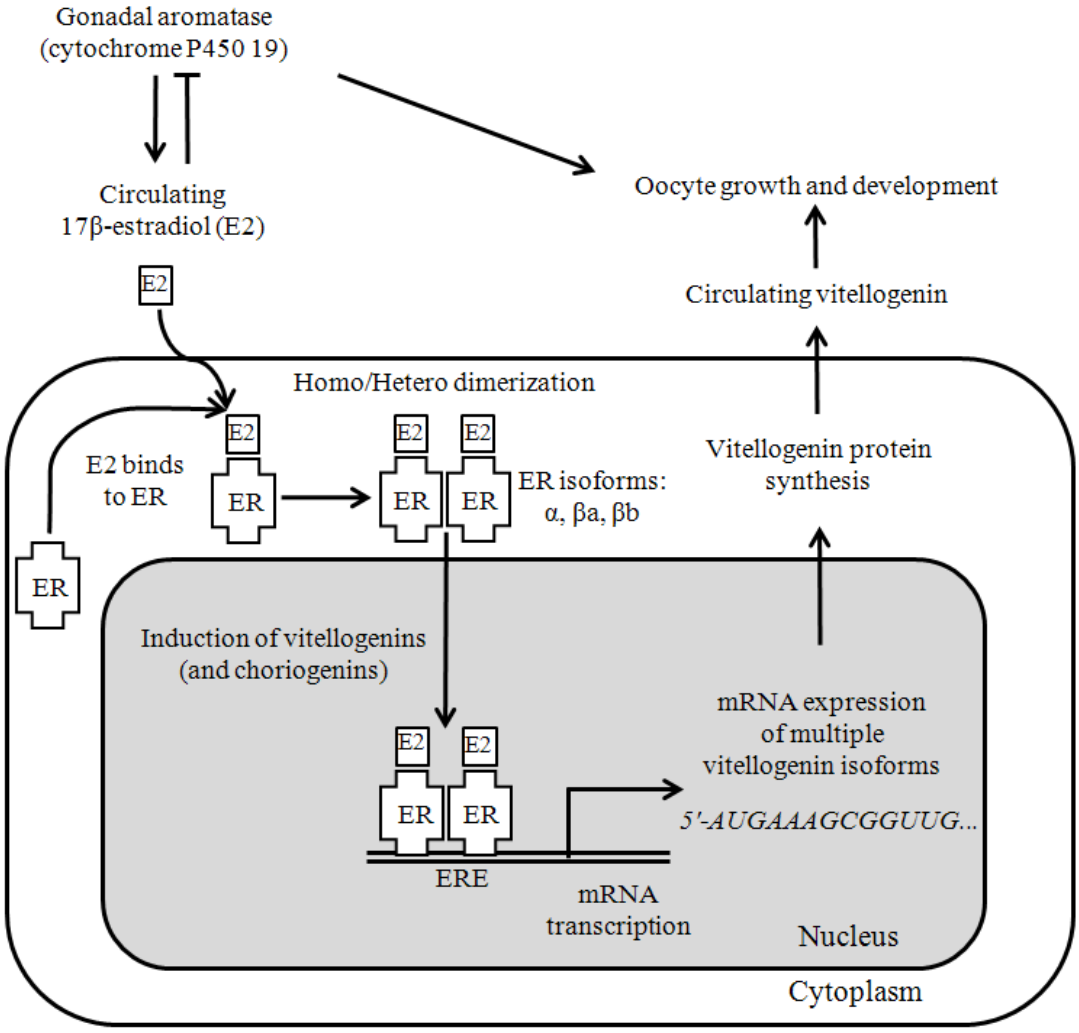


Fig. 1.5. General schematic of the estrogen receptor (ER) pathway regulation of vitellogenesis in teleosts. The role of each individual isoforms in forming homo and hetero dimers and their regulation of various vitellogenin and choriogenin genes are poorly understood. Abbreviations: ERE, estrogen responsive element.

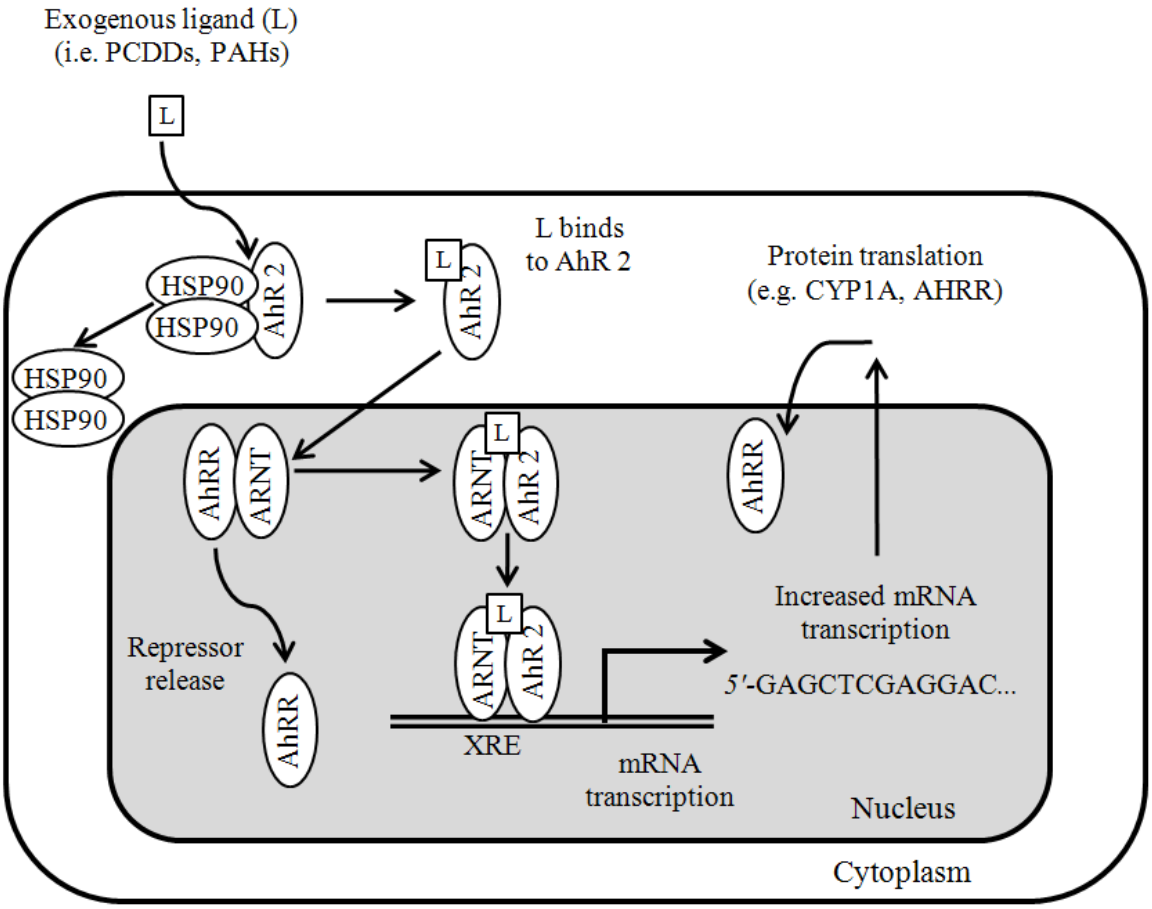


Fig. 1.6. General schematic of ligand activation of the aryl hydrocarbon receptor (AhR) pathway. Abbreviations: HSP90, heat shock protein 90; AHRR, aryl hydrocarbon receptor repressor; ARNT, aryl hydrocarbon receptor nuclear translocator; CYP1A, cytochrome P450 1A; PCDD, polychlorinated dibenzo–*p*–dioxins; PAHs, polycyclic aromatic hydrocarbons; XRE, xenobiotic response element.

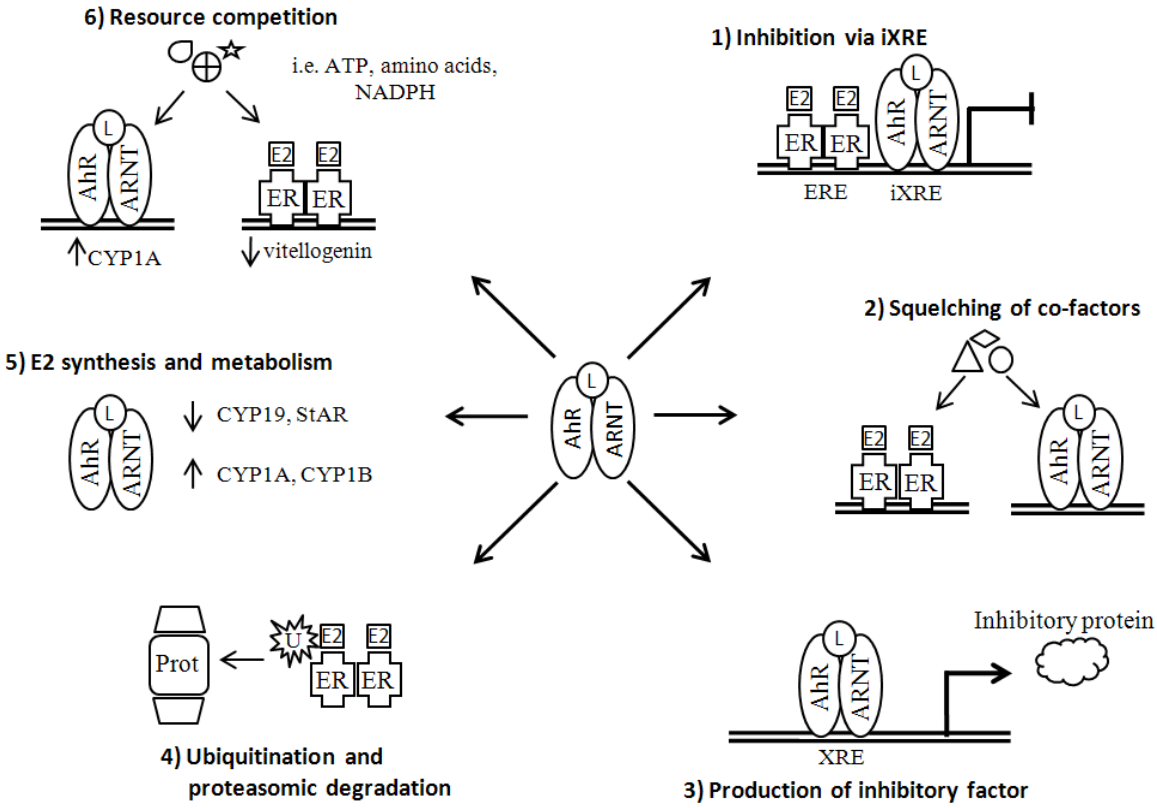


Fig. 1.7. AhR–ER cross–talk is mediated through six proposed mechanisms. Figure adapted from Matthews and Gustafsson (2006). Abbreviations: ERE, estrogen responsive element; XRE, xenobiotic responsive elements; iXRE, inhibitory xenobiotic responsive elements.

CHAPTER 2

Impaired reproductive health of killifish (*Fundulus heteroclitus*) inhabiting Newark Bay, NJ, a chronically contaminated estuary

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This manuscript appeared in: Aquatic Toxicology, 2010. 96, 182–193.
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Abstract

A battery of biomarkers were used to evaluate the reproductive health and contaminant exposure of Atlantic killifish (*Fundulus heteroclitus*) inhabiting the heavily industrialized Newark Bay and a reference population from Great Bay, Tuckerton, NJ. The biomarkers investigated included classical endpoints (gonad and liver histopathology, body and tissue morphometrics), hepatic mRNA expression (CYP1A and vitellogenin I), hepatic protein levels (CYP1A and vitellogenin), gonadal aromatase mRNA expression, and chemical exposure analyses (bile PAHs). Our data showed no significant differences between populations for body size and body weight. However, Newark Bay killifish exhibited molecular and morphological changes indicative of impaired reproductive health and endocrine disruption compared to the reference population. Newark Bay males had decreased gonad weight, altered testis development and decreased gonadal aromatase mRNA expression. Newark Bay females had decreased gonad weight, inhibited gonadal development, decreased hepatic vitellogenin production (mRNA and protein) and increased mRNA expression of gonadal aromatase. In addition, Newark Bay females had 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% mature at Tuckerton and 3% at Newark Bay). In addition to reproductive endpoints, killifish at Newark Bay exhibited high basal levels of CYP1A mRNA and protein expression which indicated exposure to aryl hydrocarbon receptor (AhR) agonists. An inverse relationship between hepatic CYP1A protein and hepatic vitellogenin mRNA expression was established suggesting a possible link between AhR agonist exposure and vitellogenesis. Killifish in the NY–NJ Harbor Estuary are exposed to a number of chemicals that can interact with the AhR pathway and stimulate enzymatic activity along with chemicals that can modify reproductive success in this indigenous species. Similar effects on the reproductive development in less resilient species may limit their ability to repopulate the NY–NJ Harbor Estuary and similarly contaminated water systems.

2.1. Introduction

The purpose of this study was to evaluate the reproductive health of Atlantic killifish (*Fundulus heteroclitus*) inhabiting Newark Bay, NJ, a heavily contaminated bay compared to a reference site in Great Bay, Tuckerton, NJ (Fig. 2.1). The Atlantic killifish is a commonly studied sentinel teleost species (Burnett et al., 2007) and was chosen for this study because of its role in the food web as both a predator and prey (Able et al., 2007), for its non–migratory behavior (Skinner et al., 2005), and for its abundance throughout the NY–NJ Harbor due to an acquired resistance to contaminants when compared to reference populations (Weis et al., 1981; Prince and Cooper, 1995a,b; Elskus et al., 1999; Arzuaga and Elskus, 2002). An integrated biomarker assessment was

used to evaluate the reproductive health of killifish, which included a number of traditional markers of exposure and markers of effect at different levels of organization (molecular, cellular, and tissue) (Bartell, 2006; Burger, 2006; NRC, 1989). This approach allowed for the determination of each individual's reproductive status in a field population of killifish that has been impacted by multiple chemical exposures. Biomarkers examined included various reproductive endpoints (hepatic vitellogenin production, gonadal aromatase expression, and gonad histology), a metabolism enzyme (hepatic cytochrome P4501A) and bile chemistry for select polycyclic aromatic hydrocarbons. This integrated biomarker assessment does not allow for the determination as to which chemicals are primarily responsible, but integrates the impacts of complex mixtures on the organism of interest.

Ecoepidemiological studies of fish populations in decline (i.e. lake trout in the Great Lakes, English sole in Puget Sound) have shown there to be a correlation between tissue levels of organic pollutants and reproductive failure and early life stage mortality (Cook et al., 2003; Fitzsimons, 1995; Casillas et al., 1991). The reproductive health of fish living within the heavily industrialized NY–NJ Harbor Estuary may be impacted by exposure to a wide variety of legacy compounds known to be present (Assaf–Anid, 2003; Litten, 2003). The NY–NJ Harbor Estuary is widely contaminated by persistent organic pollutants including dioxins/furans (Muñoz et al., 2006), polycyclic aromatic hydrocarbons (Gigliotti et al., 2005; Valle et al., 2007), polychlorinated biphenyls (Panero et al., 2005) and heavy metals (de Cerreño et al., 2002; Boehme and Panero, 2003). For example, sediment concentrations of total polychlorinated dibenzo–*p*–dioxins (PCDDs) in Newark Bay were reported up to 3425 parts per trillion (pptr) and were

amongst the highest concentrations reported in the world (Muñoz et al., 2006). Tissue concentrations of total PCDDs in Atlantic tomcod (1109–1736 pptr) and blue crab (6000 pptr) from the Newark Bay area were also amongst the highest ever reported in wildlife (Rappe et al., 1991; Fernandez et al., 2004; Muñoz et al., 2006). A number of these contaminants are still at levels which would impact reproductive success in species inhabiting these waters (CARP, 2007; Steinberg et al., 2004).

The reproductive health of wildlife in the NY–NJ Harbor Estuary maybe impacted by the historically documented contaminants discussed above as well as endocrine disrupting compounds (EDCs), which have emerged as significant anthropogenic contaminants that enter rivers and estuaries from wastewater treatment facilities (Kolpin et al., 2002; Zogorski et al., 2006; Barnes et al., 2008; Focazio et al., 2008). There are approximately 30 wastewater treatment plants discharging EDC containing effluents into the NY–NJ Harbor Estuary. EDCs can alter reproductive processes and lead to deleterious effects in the organism, its offspring,
and ultimately the population (Arcand–Hoy and Benson, 1998; Tyler et al., 1998; Nash et al., 2004). For example, Kidd et al. (2007) reported the collapse of a population of fathead minnows (*Pimephales promelas*) following a seven–year chronic exposure to 17α–ethynylestradiol (5–6 ng/L) due to the feminization of males and inhibition of oogenesis in females. Chronic chemical exposure can result in subtle changes in a population's ability to reproduce which are often manifested over many years. The impaired ability of the species to successfully reproduce may result in local extinction or reduced species fitness. EDCs that are known to be present in the NY–NJ Harbor Estuaries include the following: various pesticides, tributyltin from shipping, 17β–

estradiol and ethynylestradiol (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, 2005), bisphenol A from secondary water treatment facilities, brominated flame retardants (Hamers et al., 2006) and dioxin like compounds.

The markers of exposure used in this study included hepatic cytochrome P4501A (CYP1A) production as a biomarker for exposure to aryl hydrocarbon receptor (AhR) agonists (James and Bend, 1980; Binder and Stegeman, 1984; Billiard et al., 2002) and chemical analyses of bile for assessing polycyclic aromatic hydrocarbons (PAHs) exposure (Aas et al., 2000). Cytochrome P450 (CYP) enzymes are a diverse class of oxidative enzymes that are commonly induced by PAHs, polychlorinated biphenyls (PCBs), polychlorinated dibenzo–*p*–furans (PCDFs) and PCDDs through the AhR pathway (Hahn, 2002; Zanette et al., 2009). PAHs were selected for analysis due to their ubiquitous occurrence in urbanized systems and for their ease of detection in bile. Various biochemical markers were used to evaluate the reproductive status of the two populations. Gametogenesis in teleosts is regulated by a number of environmental and chemical cues and is too complex to be described in detail in this paper, therefore reviews by Patiño and Sullivan (2002), Rempel and Schlenk (2008), and Thomas (2008), should be consulted. Cytochrome P450 19A1 (gonadal aromatase) mRNA expression was used in this study as an indicator of the capacity of the fish to convert testosterone to 17β–estradiol. However, circulating sex steroids were not determined. CYP19A1 expression and protein are normally high in females and relatively low in males. CYP19A1 converts androgen steroids to 17β–estradiol in granulosa cells of immature oocytes which then circulates to the liver and stimulates vitellogenin production (egg–yolk precursor proteins

required for follicle growth) through an estrogen receptor (ER) mediated pathway (Tchoudakova and Callard, 1998; Greytak et al., 2005; Dong and Willett, 2008). Vitellogenin proteins circulate to developing follicles in the ovaries and are cleaved by cathepsins into approximately a dozen unique vitellin proteins during growth and maturation (LaFleur et al., 2005). Hepatic vitellogenin production in males has been used extensively as a biomarker of exposure to contaminants that can activate the ER pathway and was therefore employed in this study (Pait and Nelson, 2003). However, hepatic vitellogenin expression has not been used as a common biomarker in females since vitellogenin is produced in high quantities during egg development, but was used in this study for evaluating the reproductive status of females. Histology of the gonads was also investigated in this study, which allows for the evaluation of organ level alterations and follicle development (Blazer, 2002). Follicle development, which consists of 4 stages of vitellogenin–dependent follicle growth, can serve as a biomarker of oogenesis activity by demonstrating alterations in the abundance of each vitellogenin–dependent stage (Blazer, 2002; Wolf et al., 2004). Failure of follicles to mature fully may result in decreased fecundity that may be manifested at the population level.

The purpose of this biomarker study was to evaluate the reproductive health of killifish inhabiting the NY–NJ Harbor Estuary. This study demonstrated evidence for endocrine disruption and a decreased capacity for reproduction in both genders of killifish inhabiting the Newark Bay relative to reference fish at Great Bay, Tuckerton, NJ. Males exhibited altered testis development and decreased gonad weight. Females exhibited an inability to be successfully strip spawned in the field, decreased gonad weight, altered follicle development, decreased hepatic vitellogenin production (mRNA

and protein) and an up–regulation of gonadal aromatase expression. This study is the first biomarker study, to our knowledge, to demonstrate alterations in stage–specific egg development between a killifish population from a heavily contaminated site and a reference site. Additionally, killifish at Newark Bay exhibited significantly increased basal levels of hepatic CYP1A (mRNA and protein), and increased levels of bile PAHs (known AhR agonists). This study is also one of the first field studies to demonstrate an inverse relationship between the AhR (elevated CYP protein) and ER pathways (decreased VTG expression) in females, which has only previously been demonstrated *in vitro* (Anderson et al., 1996).

2.2. Materials and methods

2.2.1. Fish model, site selection, collection and necropsy protocols

Killifish were collected from two estuaries in New Jersey, USA, using baited minnow traps (Fig. 2.1). Specifically, 25 fish of breeding size (4.0 cm or greater) were collected from the heavily contaminated 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) on June 30th, 2007. Water temperature was 21.7 °C at Tuckerton and 21.5 °C at Newark Bay. To ensure valid biomarker comparisons for assessing reproductive health, killifish were collected from both sites at the peak of the full moon, which is the peak of the killifish's lunar–dependant spawning cycle (Taylor et al., 1979; Taylor and DiMichele, 1980; Taylor,

1986; Hsiao et al., 1994; Cerdá et al., 1996). Fish were transported immediately back to the laboratory in aerated containers to reduce stress with water from the collection site.

All animal protocols were approved through the Rutgers Animal Care and Facilities Committee (Protocol #08–025). Upon arrival at the laboratory, each fish was euthanized with an overdose of MS–222 (tricaine methanesulphonate), weighed, and measured for body length (N = 9 for Tuckerton males, N = 10 for Newark Bay males, N = 16 for Tuckerton females and N = 15 for Newark Bay females). The liver was then removed, weighed and divided into three portions, one for histopathology and two for CYP1A and vitellogenin analyses (100mg each). Samples saved for molecular analyses were stored in RNAlater® (Ambion®, Austin, TX), snap frozen and maintained at −80 °C. Bile was collected from the gall bladder, snap frozen, and stored at −80 °C for PAH analyses. The gonads were then removed, weighed, and also divided into portions for histopathology and gonadal aromatase analysis (100 mg).

2.2.2. Bile PAH determination

Synchronous fluorescence spectrophotometry (SFS) methods were used to quantify naphthalene, pyrene and benzo[a]pyrene as surrogate measurements of exposure to low, medium and high molecular weight PAHs, respectively. Bile was diluted 1:1500 in 50% ethanol (3 mL final volume) and scanned from 250 to 450 nm using a Cary Eclipse Fluorescent Spectrophotometer using an excitation/emission wavelength offset of 44 nm (adapted from Aas et al., 2000). Naphthalene (290 nm), pyrene (346 nm) and benzo[a]pyrene (380 nm) were semi–quantitated as standard equivalent masses per microliter of bile using 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). Detection limits were set at 5 ng/µL for naphthalene, 0.5 ng/µL for pyrene and 50 pg/µL for benzo[a]pyrene. Samples below these levels were recorded at the detection limit.

2.2.3. Gonad histology

Gonadal tissues were fixed in 10% buffered formalin, embedded in paraffin, cut into 6 µm sections and stained with hematoxylin and eosin. The tissues were examined by light microscopy without knowing the location from which the animal was collected. Testis were evaluated for interstitial fibrosis (thickened walls), empty follicles, macrophage aggregates, hemosiderin/lipofuschin deposits and sperm developmental stages. Ovaries were evaluated for gonadal development by staging 150 follicles from each individual. Follicles were counted as pre–vitellogenic, early vitellogenic, mid–vitellogenic or mature. Pre–vitellogenic follicles were those having cortical alveoli, early vitellogenic follicles 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 having fused hyaline yolk material. Each stage of the follicle was approximately two times the size of the previous stage.

2.2.4. RNA isolation and quantitative polymerase chain reaction (qPCR)

RNA was isolated from 100 mg liver and gonadal tissue and prepared for qPCR as previously described (Hillegass et al., 2007). Genes targeted for quantification

included β–actin (GenBank AY735154), hepatic microsomal cytochrome P4501A (CYP1A, Gen–Bank AF026800), hepatic cytosolic vitellogenin I (VTG, GenBank U07055) and gonadal aromatase (CYP19A1, GenBank AY428665). Each gene was analyzed in triplicate to use average threshold cycle (CT) values for quantification with standard curves of each gene. Each individual was normalized to the population's median β–actin CT value and then quantified as nanograms of target mRNA per microliter of template. Threshold concentrations for VTG and CYP19A1 were set at detection limits of 1.5×10^{-7} and 2.0×10^{-8} ng/μL, respectively (threshold of linear range). Data below these values were set as the detection limit.

The β–actin forward primer was 5′–GCT CTG TGC AGA ACA ACC ACA CAT–3′ and the reverse primer was 5′–TAA CGC CTC CTT CAT CGT TCC AGT–3′ giving a product size of 136 base pairs (bp). The CYP1A forward primer was 5′–TGT TGC CAA TGT GAT CTG TG–3′ and reverse primer was 5′–CGG ATG TTG TCC TTG TCA AA–3′ giving a product size of 258 bp. The VTG forward primer was 5′–AGG ATT CGT CCG AAC AAC AC–3′ and reverse primer was 5′–TTT CAG ACG GCA CTC AGA TG–3′ giving a product size of 416 bp. The gonadal aromatase forward primer was 5′–ACG AGA AAG AGC TGC TGC TGA AGA–3′ and reverse primer was 5′–TGA TGT CCA GCT TAT CTG CCT GCT–3′ giving a product size of 198 bp. Primers were developed to amplify small fragments (100–400 bp) of each gene. Primer sequences were searched using BLAST (National Center for Biotechnology Information) to ensure specificity to the target gene. OligoAnalyzer (Integrated DNA Technologies) was used to evaluate the primer sets for hetero/homo–dimers ($\Delta G > -9$), formation of hairpins (Tm of hairpin << Tm of oligo) and Tm of primers (60 °C). Melt curves were

generated for each qPCR run to ensure formation of a single product, and product size was verified by gel electrophoresis.

2.2.5. Protein isolation and immunoblotting

Liver tissue (100 mg) was homogenized with 150 µL RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5 M EDTA, 0.5 M EGTA, 1% igepal, 0.1% SDS, 10 mM $NaMoO_4$, 1 mM Na_3VO_4, 40 mM NaF, 1 mM PMSF and Sigma P8340 Protease Inhibitor cocktail). Tissues were incubated on ice for 10 min and then centrifuged at 10,000 × g for 10min at 4°C. The resulting supernatant was removed and centrifuged at 108,000 × g for 90 min at 4 °C. The supernatant (cytosolic proteins) was saved for vitellogenin analysis and the microsomal pellet was resuspended in RIPA buffer and saved for CYP1A analysis.

Protein samples were quantified by the modified Lowry protein assay (Pierce Biotechnology) immediately prior to immunoblotting. For CYP1A analysis, 10 µg of hepatic microsomal protein was used and 75 µg of hepatic cytosolic protein was used for vitellogenin analysis. Samples were reduced with dithiothreitol at 80 °C for 5 min. CYP1A samples were separated using Novex 4–12% Bis–Tris SDS polyacrylamide gels (Invitrogen) and vitellogenin samples were separated using Novex 3–8% Tris–acetate SDS polyacrylamide gels (Invitrogen). Protein was then transferred to an Immobilon PVDF membrane for 3 h using 150 mA (constant) in 1 transfer buffer (25 mM Tris–base, 192 mM glycine, 20% methanol) and stained with ponceau S to verify equal sample loading. Membranes were blocked for 60 min in 5% non–fat milk in 1× TBST (200 mM NaCl, 50 mM Tris, 0.1% Tween–20). Membranes were incubated at 4 °C for

approximately 20 h in 5% non–fat milk in 1× TBST with either 1.0 µg/mL CYP1A (scup) monoclonal antibody 1–12–3 (gift of Dr. John J. Stegeman, Woods Hole, MA) for CYP1A or vitellogenin (striped–bass) monoclonal antibody ND–1C8 diluted 1:500 (Cayman Chemical) for vitellogenin analysis. Membranes were washed 3 times for 10 min in TBST (350 mM NaCl, 50 mM 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 1× TBST. Membranes were finally washed 2 times for 10 min in TBST (350 mM NaCl, 50 mM Tris, 0.1% Tween–20) and once for 10 min in deionized water. The Amersham 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, all samples were normalized to the signal of a single individual from Newark Bay that was run on all gels. For vitellogenin, two samples of purified rainbow trout vitellogenin (Vtg–51, Biosense Laboratories, Bergen, Norway) were run on each gel (1:5 and 1:10 dilution of stock) and each individual was normalized to the 1:5 stock sample signal.

2.2.6. 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 (Tuckerton). Unpaired t–tests were used to compare two different treatments. When normality failed the Student's Neuman–Keuhls test was used. When equal variance

failed the Mann–Whitney Rank Sum test was used. Discrete data was compared using the Chi Square Analysis or Fisher's Exact Test.

2.3. Results

2.3.1. Morphometric measurements

There were no significant differences between sites for either gender when comparing average snout to peduncle length, average snout to tail length, average body weight, average liver weight, average length to body weight ratio and average liver to body weight ratio (Table 2.1). Males at Newark Bay exhibited significantly decreased average gonad weight (147 ± 53 mg) and average gonad to body weight ratio (0.023 ± 0.005) in comparison to the Tuckerton male average gonad weight (266.9 ± 131.7 mg) and average gonad to body weight ratio (0.035 ± 0.007). Females at Newark Bay exhibited significantly decreased average gonad weight (274.6 ± 147.7 mg) and average gonad to body weight ratio (0.041 ± 0.023) in comparison to Tuckerton females (708.0 ± 262.5 mg average gonad weight and 0.089 ± 0.033 average gonad to body weight ratio).

2.3.2. Bile PAH concentrations

Bile PAHs were used as a biomarker of exposure for comparisons between the Tuckerton and Newark Bay populations (Aas et al., 2000). Newark Bay fish (males and females) had higher concentrations for all compounds measured. Table 2.2 shows the concentrations of the 3 measured PAH equivalents at both sites and the fold difference between concentrations at Newark Bay over those at Tuckerton. Newark Bay males had

significantly higher concentrations of pyrene compared to Tuckerton males. There was a trend suggesting that naphthalene (p = 0.06) and benzo[a]pyrene (p = 0.07) were higher in Newark Bay males. Newark Bay females had significantly higher bile concentrations than Tuckerton females for naphthalene, pyrene and benzo[a]pyrene. There were no significant differences for intra–site gender comparisons for the examined PAHs.

2.3.3. Hepatic CYP1A expression and protein levels

CYP1A expression and protein levels are commonly used biomarkers for assessing exposure to AhR agonists present in contaminated and non–contaminated sites (Fujita et al., 2001; Billiard et al., 2002; Hahn, 2002). Basal hepatic CYP1A mRNA expression was significantly elevated in both genders of Newark Bay fish compared to expression levels observed from Tuckerton fish (Fig. 2.2A). Males from Newark Bay compared to Tuckerton males had approximately a 7–fold higher mRNA expression while females from Newark Bay had approximately a 3–fold higher mRNA expression (using median values). There were no significant gender differences inCYP1Aexpression at either site. Western blot analysis of microsomal CYP1A protein showed similar trends. CYP1A protein levels for Newark Bay males were approximately 5–fold higher (using median values) than Tuckerton males, and 8–fold higher (using median values) in Newark Bay females compared to Tuckerton females (Fig. 2.2B and C). Females at Tuckerton had significantly lower CYP1A protein than males; however, there were no gender differences in killifish from Newark Bay. The low level of CYP1A mRNA expression and protein levels at Tuckerton support the assertion that Tuckerton fish are exposed to relatively low levels of AhR agonists and are a good reference population.

2.3.4. Gonad histology and development

Histopathological evaluations of the testis showed a high prevalence of interstitial fibrosis (thickened walls), macrophage aggregates and depleted follicles at both sites. Newark Bay tended to have a higher prevalence of these lesions however this was not significant (Bugel, 2009). All animals evaluated from Tuckerton exhibited normal synchronous spermatogenesis evident by a wide margin of germinal epithelium follicles containing spermatocytes at the periphery of the testis (Fig. 2.3A). In contrast, all males from Newark Bay had a markedly reduced germinal epithelium layer (Fig. 2.3B).

Newark Bay females exhibited ovaries that were relatively undeveloped compared to the Tuckerton population which had a similar average body size (length, body weight, and length to body weight). Representative photomicrographs for Tuckerton and Newark Bay ovaries demonstrate major differences in the development of follicles between sites, and that Newark Bay females had relatively high numbers of pre–vitellogenic follicles and few mature follicles compared to Tuckerton (Fig. 2.4A and B). 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 (Fig. 2.4C).

2.3.5. Reproductive biomarkers

Expression of hepatic vitellogenin in male fish is a frequently used biomarker indicating exposure to estrogenic compounds (Pait and Nelson, 2003). Newark Bay males did not exhibit increased vitellogenin mRNA expression relative to Tuckerton males and

no detectable vitellogenin protein was detected by western blot analysis (Fig. 2.5). However, there was a significant difference between female populations. The median expression of hepatic vitellogenin mRNA in Newark Bay females was approximately 6–fold lower than that of Tuckerton females (Fig. 2.5A). The western blot analyses of hepatic cytosolic vitellogenin levels were also decreased. Newark Bay females had a significant 27–fold lower median level of hepatic vitellogenin protein relative to Tuckerton females (Fig. 2.5B and C).

Expression of gonadal aromatase (CYP19A1) mRNA was used as a surrogate measure of potential estrogen production in females and feminization of males (Tchoudakova and Callard, 1998; Greytak et al., 2005; Dong and Willett, 2008). Both genders at Newark Bay had significantly altered gonadal aromatase mRNA expression compared to Tuckerton (Fig. 2.6). The median mRNA expression in Newark Bay males was approximately 3–fold lower than Tuckerton males and approximately 210–fold higher (using median values) in Newark Bay females compared to Tuckerton females (Fig. 2.6).

2.4. Discussion

This study has shown that Newark Bay killifish exhibit an increased exposure to AhR agonists which was demonstrated by having both (1) increased levels of hepatic CYP1A mRNA expression and protein levels and (2) increased concentrations of bile PAHs, compared to the reference population. This study has also demonstrated that Newark Bay killifish (both males and females) exhibited signs of endocrine disruption and a decreased capacity for reproduction. Data showed that although there were no

differences between the killifish harvested from Tuckerton and Newark Bay with respect to the basic morphometric measurements (Table 2.1) and histopathology of the liver (Bugel, 2009), there were differences in the reproductive status of Newark Bay killifish compared to those from the reference site. Reproductive impacts are likely due to poor environmental quality and exposure to complex mixtures of contaminants known to be present in Newark Bay. The contaminants present at Newark Bay (i.e. dioxins/furans, PAHs, endocrine disrupting compounds) are present in a number of urbanized and industrialized harbor, rivers and estuaries which likely results in similar effects where concentrations are comparable to those reported for Newark Bay. Therefore, reproductive impacts observed in Newark Bay killifish may explain in part why other species have failed to successfully reestablish breeding populations in the NY–NJ Harbor Estuary.

2.4.1. PAH exposure and CYP1A levels

Results from the bile PAH analysis are reflective of exposure to the extensive PAH contamination known to be widespread throughout this ecosystem (Litten, 2003; Valle et al., 2007). All compounds measured (pyrene, naphthalene, benzo[a]pyrene) were 4–9–fold higher in Newark Bay fish, indicating that Newark Bay killifish were exposed to elevated concentrations of PAHs (Table 2.2). Although Newark Bay killifish were exposed to increased levels of carcinogenic and mutagenic PAHs, this population did not exhibit a significantly increased prevalence of pre–neoplastic and neoplastic lesions in the liver or a significantly increased prevalence of red blood cell micronuclei (Bugel, 2009). The low occurrence of hepatic lesions and micronuclei in red blood cells may be due to an adaptation to AhR agonist toxicity in this population, which has been

previously demonstrated to be resistant to AhR mediated developmental effects and hepatic CYP1A induction (Prince and Cooper, 1995a,b; Elskus et al., 1999; Arzuaga and Elskus, 2002).

Newark Bay killifish exhibited significantly higher levels of hepatic CYP1A mRNA expression and protein relative to Tuckerton killifish (Fig. 2.2). This indicated that Newark Bay killifish were exposed to elevated levels of AhR agonists, such as PAHs which were also demonstrated to be elevated in this population (Table 2.2) or dioxins which are known to be widespread throughout this system (Muñoz et al., 2006). Dioxins are potent inducers for CYP1A in fish (Fujita et al., 2001). Prince and Cooper (1995a,b) have previously demonstrated that Newark Bay killifish exhibited an elevated basal level of CYP1A activity, although they did not investigate mRNA or protein levels. Results from this study and those by Prince and Cooper (1995a,b) indicate the chronic exposure of Newark Bay killifish to AhR agonists and long term induction of the AhR pathway. Therefore, killifish continue to be exposed to AhR agonists (dioxins/furans/PAHs) and have maintained a high basal level of AhR pathway activity for many generations compared to the reference population from Tuckerton, NJ.

In contrast to results from the present study and those reported by Prince and Cooper (1995a,b), similar studies by Arzuaga and Elskus (2002) and Elskus et al. (1999) showed no significant elevation in hepatic basal CYP1A (mRNA, protein and activity) in Newark Bay killifish compared to a control population from Flax Pond, NY. These contrasting findings may be due to differences in basal CYP1A levels exhibited in the different reference populations. Killifish from a reference site in Tuckerton, NJ (Prince and Cooper, 1995a,b; the present study) may have relatively lower basal CYP1A levels

than killifish from the reference site in Flax Pond, NY (Arzuaga and Elskus, 2002; Elskus et al., 1999).

2.4.2. Reproductive health of the Newark Bay male killifish population

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). For a number of pathologic lesions in the testis there were no significant differences between sites (interstitial fibrosis, macrophage centers, empty follicles, hemosiderin deposits, parasitic loads) between sites suggesting that Newark Bay and Tuckerton males had similar gonad disease states (Bugel, 2009). Gonad development however was significantly different between sites. All Newark Bay males exhibited a decreased margin of peripheral germinal follicles at the primary spermatocyte stage compared to Tuckerton males (Fig. 2.5). Follicles containing spermatids were present in Newark Bay males, however there was an increased prevalence of empty follicles (although not significant at $p < 0.05$). Newark Bay males exhibited altered testis development and decreased gonad weights which suggested that males had a reduced ability to produce spermatozoa at Newark Bay. The specific cause of these observations are not known, but may be related to contaminant exposure.

Males at Newark Bay did not exhibit upregulated vitellogenin production (at the mRNA and protein level) relative to Tuckerton males (Fig. 2.5). This suggests that the population of Newark Bay males were either not responding or were not exposed to sufficiently high concentrations of xeno–estrogens to illicit up–regulation of hepatic vitellogenin. This was unexpected because of the presence of various estrogenic

contaminants throughout the NY–NJ Harbor such as PCBs, dioxins, bisphenol A, phthalates, and synthetic estrogens (Litten, 2003; Panero et al., 2005; Muñoz et al., 2006). Gonadal aromatase mRNA expression was 3–fold lower in Newark Bay males compared to Tuckerton males (Fig. 2.6). However, the biological significance of this is unknown because aromatase is only known to play an important role in estrogen synthesis in females. The molecular and histological changes in male killifish at Newark Bay were relatively minor, and therefore the impact of these effects on the reproductive fitness of the animal is not fully understood. This study also determined that there was no evidence of estrogenic responses in male killifish. McArdle et al. (2004) made similar conclusions in a study that failed to illicit up–regulation of hepatic vitellogenin in killifish that were injected with organic sediment extracts from Newark Bay, NJ.

2.4.3. Reproductive status and impacts on the Newark Bay female killifish population

Field observations and morphometric data served as early indicators of impaired reproductive health of females at Newark Bay. Efforts to strip spawn Newark Bay females at roughly weekly intervals throughout the breeding season (May through August, 2007 and 2008) failed to procure significant numbers of viable eggs when at the same time Tuckerton females were producing relatively large numbers of viable eggs (these observations were empirical). Young–of–the–year were however observed in the field at both sites, although in seemingly greater numbers at Tuckerton. These field observations were not quantified although are supported by histological analysis of the female gonads. Qualitatively, Newark Bay females exhibited impaired gonadal development as evident by having few mature follicles present compared to Tuckerton

females (Fig. 2.4A and B), although this study was unable to conclude that the eggs might not progress further. Quantitative analysis of the 4 follicular stages of vitellogenic growth confirmed that Newark Bay females exhibited significantly impaired gonad development. This study is one of the first field studies to report a significant shift in the stage–specific development of gonads between populations. Newark Bay females had pre–dominantly pre–vitellogenic follicles (64% of follicles in Newark Bay females compared to 43% of follicles in Tuckerton females). This indicated that formation of the follicle during early folliculogenesis had occurred normally in Newark Bay females because of the pre–dominant presence of pre–vitellogenic follicles. However, pre–vitellogenic follicles were not stimulated to develop further because Newark Bay females exhibited a significantly decreased percentage of eggs at the mid–vitellogenic and mature stages of follicular development and (Fig. 2.4C). Few Newark Bay female follicles were observed at maturity (3%) compared to Tuckerton females (25%), and several females at Newark Bay had no mature follicles (3 of 13 females).

Pre–vitellogenic follicles require vitellogenin to progress to mature eggs, therefore hepatic vitellogenin mRNA expression and protein analyses were used as biomarkers of vitellogenesis activity in Newark Bay females. Hepatic vitellogenin expression and protein levels in Newark Bay females were shown to be 6–fold lower and 27–fold lower, respectively, than Tuckerton females (Fig. 2.5) suggesting that an anti–estrogenic mechanism of vitellogenesis inhibition is the etiology for impaired oocyte development in Newark Bay females. Interestingly, gonadal aromatase expression in the Newark Bay females was significantly higher than the Tuckerton population by 210–fold (Fig. 2.6). This suggests that gonadal aromatase was active in Newark Bay fish at the

mRNA expression level but did not lead to a subsequent activation of vitellogenin transcription and protein production in the liver. This may be due to disruption of estrogen signaling in the liver leading to inhibition of vitellogenesis. It is hypothesized that due to the lack of vitellogenin protein production, the follicles in the gonads did not progress beyond the pre–vitellogenic stage, leading to few mature eggs in Newark Bay females.

Despite the overall low vitellogenin production in the Newark Bay female population there were several individuals with vitellogenin levels similar to Tuckerton females. A vitellogenin distribution plot of individual vitellogenin values (Fig. 2.7) shows that several (4 or 5 of 13 individuals) of the female killifish from Newark Bay in this study exhibited vitellogenin protein levels similar to the reference population (also true for mRNA results) that are significantly different than the remainder of the Newark Bay female population. The females with vitellogenin levels similar to Tuckerton females are considered to be normal among the Newark Bay population and may represent non–responders. This small group of females is believed to be resistant to the effects of contaminants and may function at a relatively normal capacity because of acquired resistance and/or variability in the population's hardiness. Although the average percent of follicles reaching maturity in the Newark Bay female population was 3%, several individuals had as high as 6, 7 and 10% mature follicles. Tuckerton females had 25% mature follicles on average with the lowest values being 12, 17 and 18%. It is therefore hypothesized that a small percentage of the Newark Bay female population is performing the reproductive duties to carry the population. This also explains field observations in 2007 and 2008 that noted the presence of young–of–the–year killifish at Newark Bay,

regardless of the markedly lower success with strip spawning adults at Newark Bay compared to Tuckerton. While many adults at Newark Bay produced no eggs, most killifish at Newark Bay only produced several dozen eggs compared to Tuckerton fish which had hundreds of eggs when strip spawned (these are empirical observations from the summers of 2007 and 2008).

It is well documented that *F. heteroclitus* have asynchronous follicle development that is lunar–dependent and with the egg production (number of mature follicles) peaking on the full and new moons (Taylor et al., 1979; Taylor and DiMichele, 1980; Taylor, 1986; Hsiao et al., 1994; Cerdá et al., 1996). This study compared two populations of killifish at a single time–point in their reproductive cycles (on the full moon) where development was thought to be similar. However, the possibility exists that the Newark Bay and Tuckerton females do not have reproductive cycles that are in sync with each other. Alternatively, the major differences in follicle development reported in the present study may be due to Newark Bay females lagging behind in their reproductive cycle. However, the major differences reported here (decreased ovary weight, decreased vitellogenin levels, altered follicle development) cannot be explained by a difference of a few days and a larger deviation than 1–2 days would be necessary. This explanation is considered unlikely however, because the Newark Bay females had significantly lower vitellogenin levels (hepatic mRNA and protein expression), which is opposite of what would be expected in a population that was collected in the days prior to their peak in their reproductive cycle. Vitellogenin is necessary for follicle growth and maturation, and Newark Bay females had relatively low levels. If Newark Bay truly had a delayed reproductive cycle and were off the lunar cycle by a few days, their vitellogenin levels

would be expected to be much higher because vitellogenin levels have been shown to be highest in the days preceding the peak in egg production (Cerdá et al., 1996).

Other variables that control reproduction such as temperature and diet are also unlikely site–site differences accounting for the impacts on female reproduction reported here. Water temperatures were not significantly different between sites (21.7 °C at Tuckerton and 21.5 °C at Newark Bay) nor were they significantly different in the week prior to the collection (historical data taken from the National Oceanographic Data Center for the Bergen Point buoy in Newark Bay and the National Estuarine Research Reserve System for Buoy 126 in Tuckerton). Diets were also not believed to be different because all fish from Newark Bay as well as Tuckerton were observed to have full GI tracts upon necropsy indicating that they were all well fed.

2.4.4. Potential for AhR agonist impacts on vitellogenesis

This field study is amongst the first to employ integrated biomarkers to study relationships between molecular endpoints. Based on results from this study there appears to be a relationship between hepatic CYP1A protein, a biomarker of exposure to AhR agonists, and vitellogenin mRNA expression, a biomarker of the ER–dependent vitellogenin transcription (Fig. 2.8). Fig. 2.8 shows that vitellogenin mRNA expression levels are inversely related to CYP1A protein in females which suggests that elevated AhR pathway activity may be related to low vitellogenin production. Several Newark Bay females that exhibited normal CYP1A protein levels (low when compared to Tuckerton) also exhibited similar vitellogenin mRNA expression compared to Tuckerton (Fig. 2.8).

The relationship we propose between hepatic CYP1A protein and hepatic vitellogenin mRNA is supported by other studies that have demonstrated correlations between AhR agonist exposure and low vitellogenin production *in vitro*. Several studies using primary cultures of fish hepatocytes have demonstrated an inverse relationship between AhR agonist dose and vitellogenin production which has suggested that AhR agonists antagonize vitellogenin production (Anderson et al., 1996a; Navas and Segner, 2000; Bemanian et al., 2004; Mortenson and Arukwe, 2007). *In vivo* studies using zebrafish (*Danio rerio*) demonstrated that dioxin exposure resulted in low egg production and circulating estrogen levels, decreased spawning frequency, and altered oocyte development (King Heiden et al., 2006). The prototypical AhR agonist 2,3,7,8–tetrachlorodibenzo–*p*–dioxin has also been shown to impair gametogenesis and inhibit vitellogenin production using a bivalve model (Wintermyer and Cooper, 2007). These laboratory studies have suggested that exposure to AhR agonists reduces the reproductive capacity of aquatic organisms by interfering with vitellogenin production. Other *in vitro* studies using mammalian cell lines have demonstrated and proposed several mechanisms of cross–talk between the AhR and ER pathways that can lead to down–regulation of ER–dependent genes. The nongenomic mechanism of cross–talk involves the AhR–ARNT complex binding directly to the ER in the nucleus which leads to ubiquitination and proteasomic degradation of the AhR–ER complex (Wormke et al., 2003; Ohtake et al., 2008). The genomic mechanism of cross–talk involves AhR–ARNT complex binding to DNA regions such as inhibitory xenobiotic response elements which are located both upstream of the gene and downstream of estrogen response elements, thereby interfering with ER–dependent gene translation (Safe et al., 2000). Other field and laboratory studies

using killifish have demonstrated there to be an inverse relationship between dioxin like contaminant exposure and the population's reproductive health (embryonic survival and population growth) which has helped relate AhR agonist exposure to population level effects (Munns, Jr., et al., 1997; Black et al., 1998). Due to the nature of complex mixtures of contaminants in the NY–NJ Harbor Estuary, the anti–estrogenic endocrine disruption observed in the Newark Bay population is likely the cumulative result of many mechanisms of action. Further studies need to be carried out to better understand the mechanism of endocrine disruption observed in the Newark Bay killifish population, as well as to better understand the relationship between the AhR and ER pathways.

The possibility for an interaction between the AhR pathway (when upregulated) and the ER pathway may interfere with vitellogenin being a valid biomarker for exposure to estrogenic compounds. This potential interaction may explain the low vitellogenin production in the Newark Bay male population despite the presence of known endocrine disrupting contaminants in this system. This study demonstrated an anti–estrogenic phenotype in female killifish at Newark Bay. Therefore, this study cannot eliminate the possibility of exposure to estrogenic compounds if there is a pre–dominant anti–estrogenic effect caused by contaminants in Newark Bay killifish. Due to this, vitellogenin may not be a suitable biomarker for exposure to estrogenic compounds in this population, however, further studies need to explore this.

2.5. Conclusion

Newark Bay killifish had significantly decreased gonad weight and gonad to body weight ratios in both sexes, altered morphology of the testis, altered gonad development in females, altered aromatase expression in both sexes and decreased vitellogenin production in females. The reproductive health of killifish is concluded to have been negatively impacted by exposure to contaminants in the NY–NJ Harbor Estuary leading to a significantly decreased reproductive capacity. It is also likely that the biological effects observed in the Newark Bay killifish population occur in other fish species within the NY–NJ Harbor, and other urban harbors with similar anthropogenic inputs. AhR agonist exposure leading to chronic up–regulation of the AhR pathway may be regulating the ER–signaling pathway of Newark Bay killifish in an antagonistic (anti–estrogenic) way by interfering with ER activation of the vitellogenin gene. Further studies need to be carried out to better characterize the interactions between these two pathways in these populations. Monitoring the reproductive health of finfish in contaminated estuaries using an integrated biomarker assessment is essential for evaluating the effectiveness of efforts to reduce sediment toxicant loads (i.e. sediment remediation) and for re–establishing healthy and viable populations of ecologically and commercially important species (i.e. finfish and shellfish). The endocrine disruption effects demonstrated in Newark Bay killifish are important considering *F. heteroclitus* are much more resistant to contaminant effects than other aquatic organisms, suggesting that ecologically and commercially important species that are less tolerant may have difficulty in surviving and reproducing.

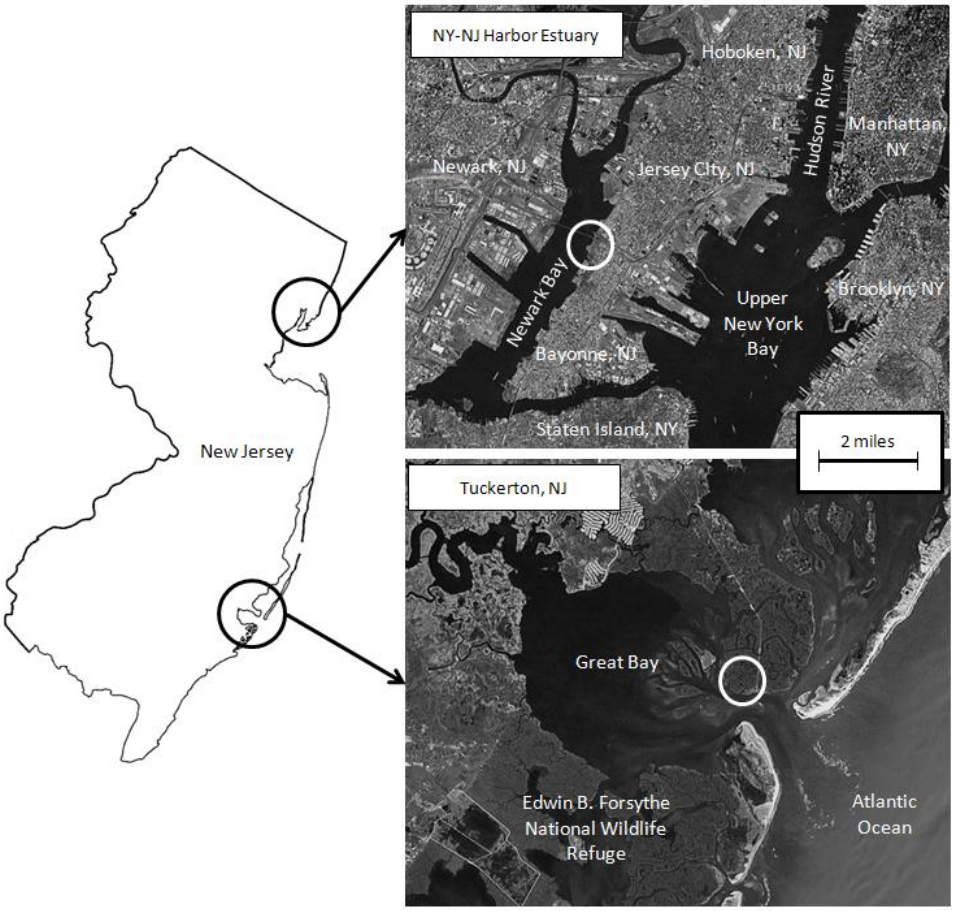


Fig. 2.1. Satellite images of the sampling sites (circled) are shown within the NY–NJ Harbor Estuary (Newark Bay) and at the reference site at Tuckerton, NJ.

Table 2.1. Morphometric measurements and organ to body weight ratios (mean and standard deviation).

	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)[#]
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)[#]
Gonad to body weight	0.035 ± 0.007 (8)	0.023 ± 0.005 (10)[*]	0.089 ± 0.033 (15)[#]	0.041 ± 0.023 (15)[*]

Values in parenthesis are N values for each group.
[*] Significantly different from Tuckerton for respective gender ($p < 0.05$).
[#] Significantly different between genders of the respective site ($p < 0.05$).

Table 2.2. Average bile PAH equivalent concentrations and fold–increases for Newark Bay concentrations over Tuckerton.

	Tuckerton males	Newark Bay males	Fold (males)	Tuckerton females	Newark Bay females	Fold (females)
Bile naphthalene (ng/μL)	189.8 ± 143.2 (4)	604.6 ± 370.2 (8)	4.2 ($p = 0.06$)	199.0 ± 108.9 (10)	504.4 ± 210.7 (11)*	2.6*
Bile pyrene (ng/μL)	1.07 ± 0.87 (4)	5.11 ± 2.74 (8)*	7.2*	0.93 ± 0.51 (11)	4.18 ± 1.77(11)*	5.9*
Bile benzo[a]pyrene (pg/μL)	65.0 ± 29.9 (4)	406.6 ± 323.4 (8)	9.3 ($p = 0.07$)	66.0 ± 28.4 (10)	174.8 ± 135.8 (10)*	3.5*

Fold values are calculated using median values of Newark Bay over Tuckerton.
* Newark Bay significantly higher than Tuckerton at $p < 0.05$.

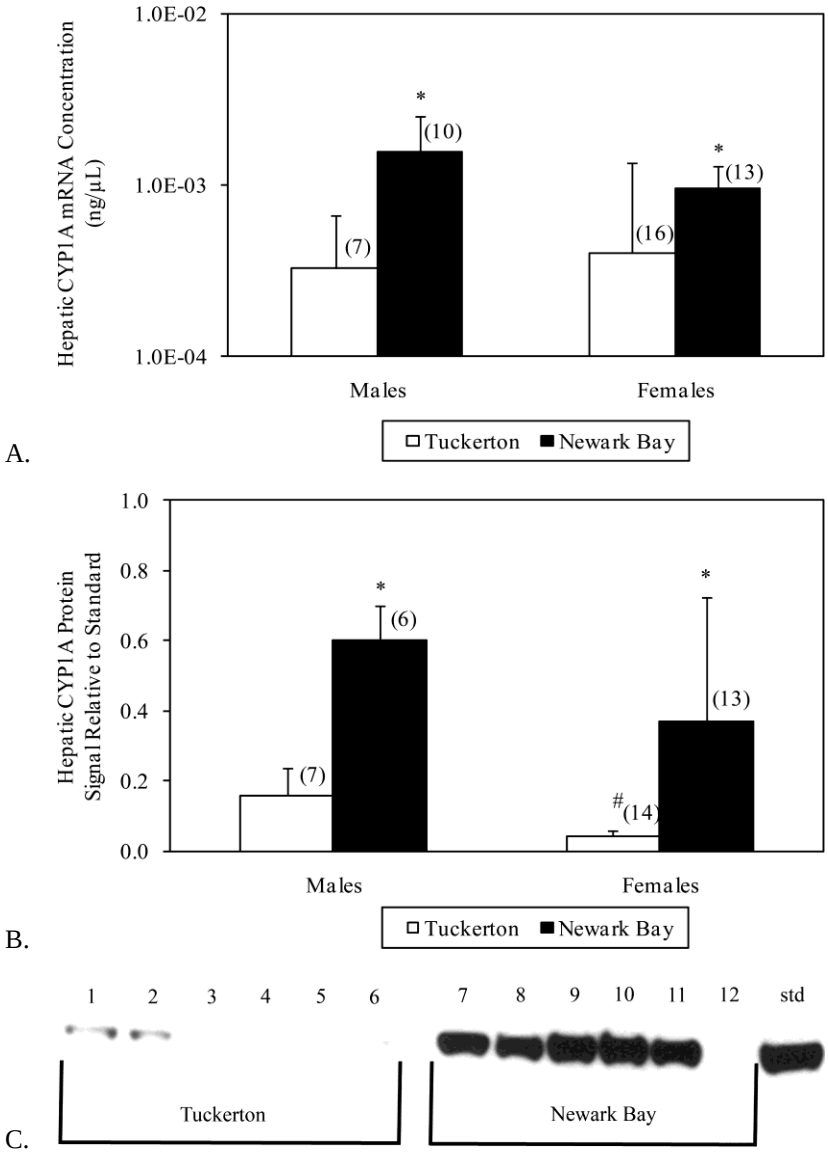
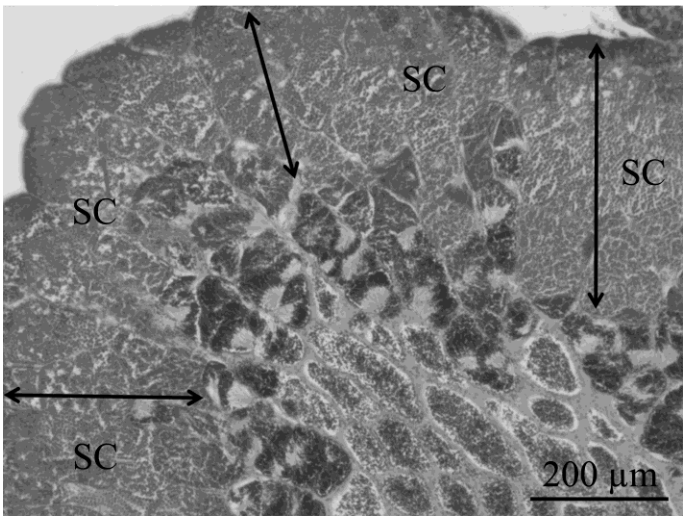


Fig. 2.2. Average hepatic CYP1A (A) mRNA expression and (B) relative microsomal protein signal. (C) Representative CYP1A immunoblot where samples 1–2 were Tuckerton males, samples 3–6 were Tuckerton females, samples 7–8 were Newark Bay males and samples 9–12 were Newark Bay females. A constant mass of a standard sample (std, which was a Newark Bay sample chosen) was loaded on all gels for calculating relative intensity of each sample. CYP1A bands were approximately 55 kDa. Sample 12 did not react for unknown reasons despite having no protein degradation determined by ponceau S and coomassie staining. Error bars indicate the standard deviation. Values in parenthesis are N values for each group. *Significantly different from Tuckerton for respective gender ($p < 0.05$). #Significantly different between genders of the respective site ($p < 0.05$).

A.



B.

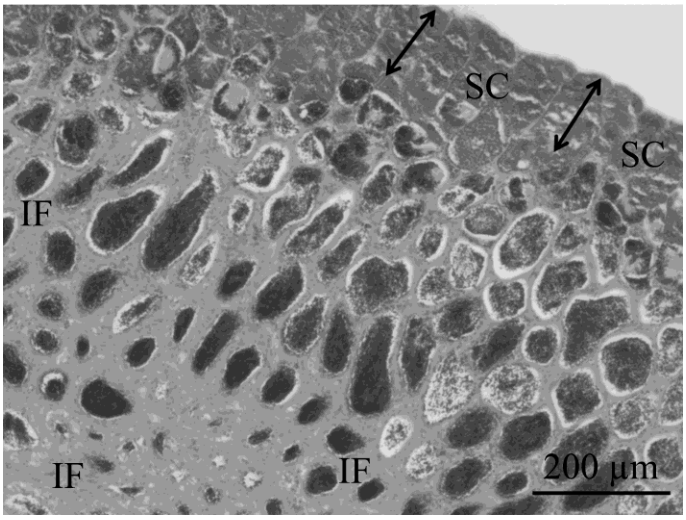
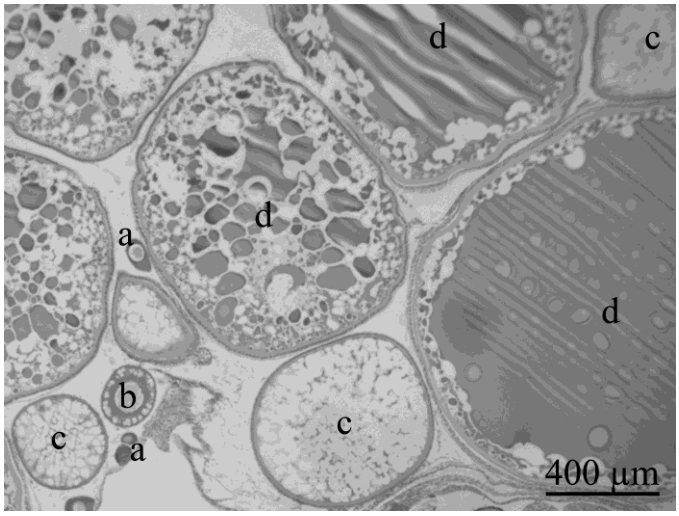
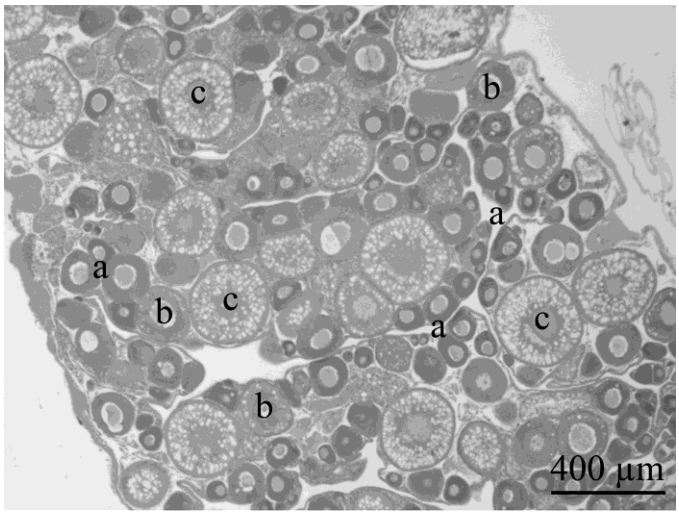


Fig. 2.3. Representative photomicrographs of testis morphology for (A) Tuckerton and (B) Newark Bay males. Spermatocytes (SC) and interstitial fibrosis (IF) are shown. Arrows indicate the germinal epithelium layer. Magnification is 100×.

A.



B.



C.

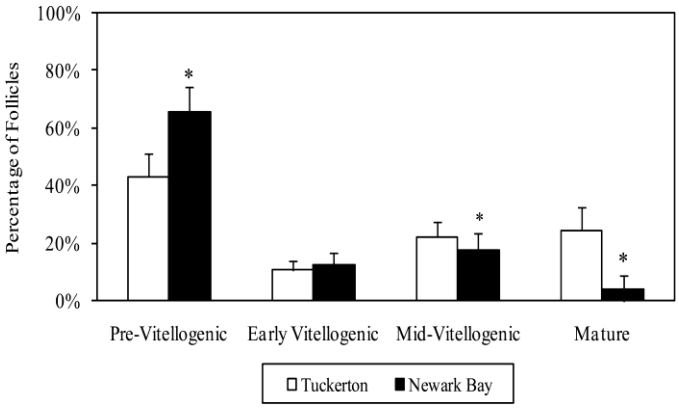


Fig. 2.4. Representative photomicrographs of (A) Tuckerton and (B) Newark Bay ovaries. Pre–vitellogenin (a), early vitellogenic (b), mid–vitellogenic (c) and mature follicles (d) are shown. Magnification is 40×. (C) Ovarian follicle developmental stages. Data are reported as mean ± standard deviation. *Significantly different from Tuckerton at $p < 0.05$. N = 13 for Tuckerton and N = 13 for Newark Bay.

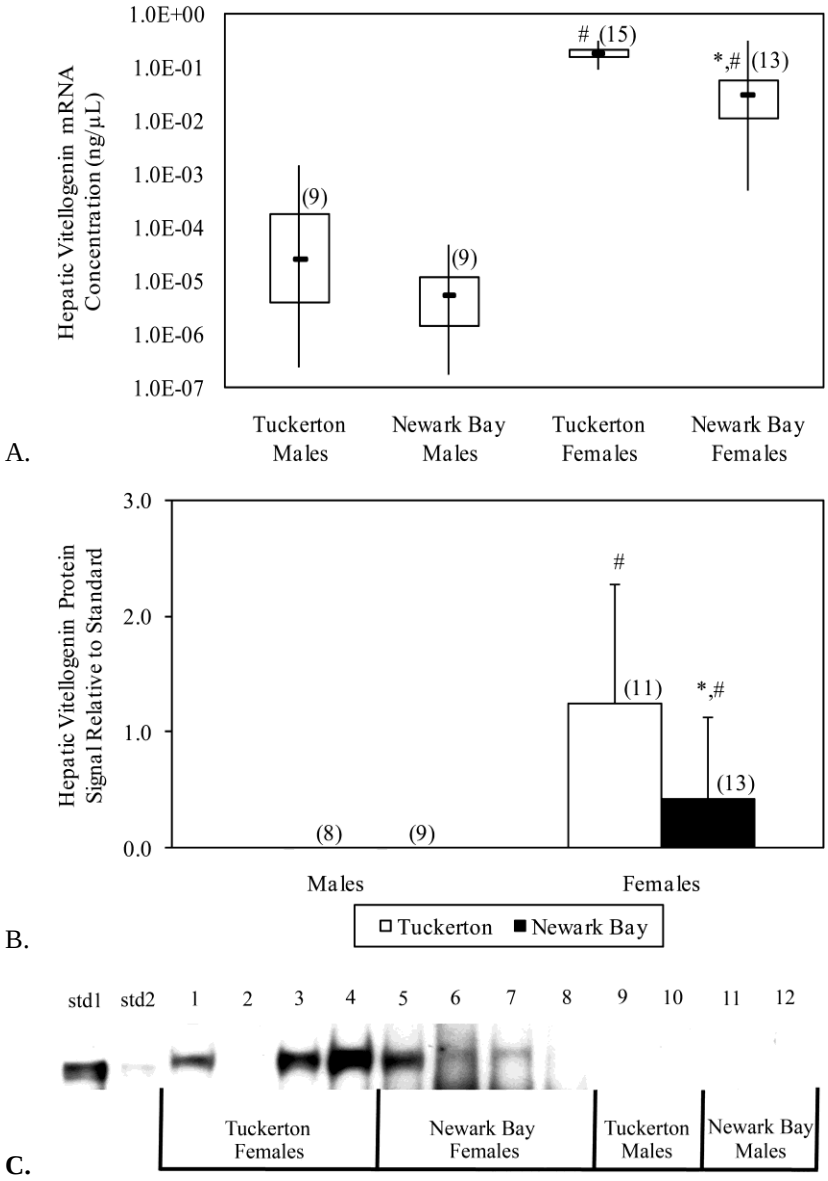


Fig. 2.5. (A) Box–and–whisker plot of mRNA expression (whiskers represent the minimum and maximum values). (B) Average relative hepatic vitellogenin cytosolic protein signal and (C) representative vitellogenin immunoblot. Sample 2 in (C) was excluded from analysis for not having enough protein loaded (determined by ponceau S staining). Standard samples (std) of purified rainbow trout vitellogenin were loaded on each gel for calculating relative intensity of each sample. Vitellogenin bands were approximately 255 kDa. Error bars indicate the standard deviation. Values in parenthesis are N values for each group. *Significantly different from Tuckerton for respective gender ($p < 0.05$). #Significantly different between genders of the respective site ($p < 0.05$).

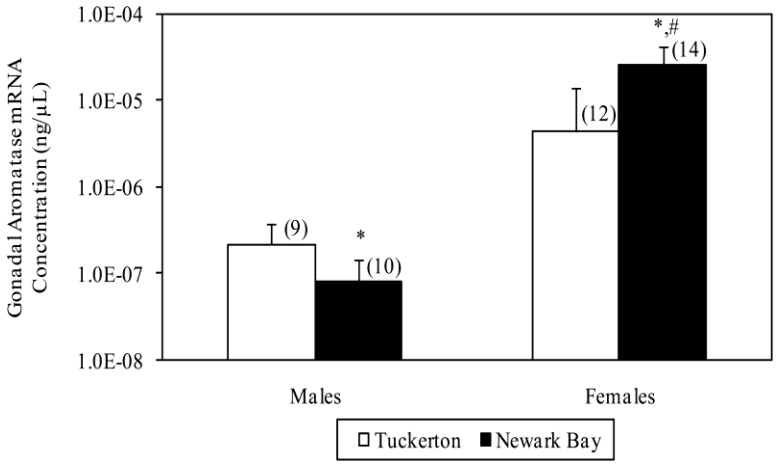


Fig. 2.6. Average gonadal aromatase mRNA expression. Error bars indicate the standard deviation. Values in parenthesis are N values for each group. *Significantly different from Tuckerton for respective gender ($p < 0.05$). #Significantly different between genders of the respective site ($p < 0.05$).

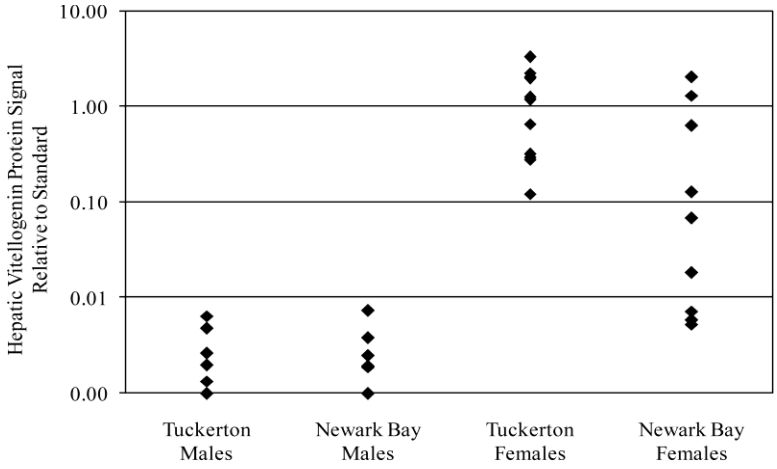


Fig. 2.7. Distribution plot of hepatic vitellogenin protein values. Each point represents an individual fish.

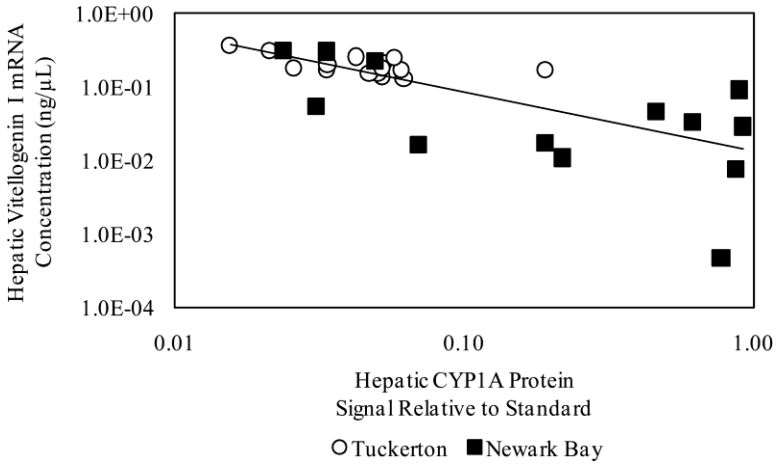


Fig. 2.8. Relationship between hepatic CYP1A protein and hepatic vitellogenin mRNA using females from both Tuckerton and Newark Bay ($R^2 = 0.522$; $y = 0.011x^{-0.845}$). Each point represents a single individual and all samples were within detectable ranges for each endpoint.

CHAPTER 3

Decreased vitellogenin inducibility and 17β–estradiol levels correlated with reduced egg production in killifish (*Fundulus heteroclitus*) from Newark Bay, NJ

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This manuscript appeared in: Aquatic Toxicology, 2011. 105 (1–2), 1–12.
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Abstract

Aquatic species inhabiting polluted estuaries are exposed to complex mixtures of xenobiotics which can alter normal reproduction. We previously reported that female Atlantic killifish (*Fundulus heteroclitus*) from the highly contaminated Newark Bay, NJ (USA) exhibited an inhibition of oocyte development due to reduced vitellogenin (egg–yolk precursor) levels. Our hypothesis was that the inhibition of oocyte development in Newark Bay killifish is due to (1) deficient levels of circulating 17β–estradiol, and (2) a decreased sensitivity of the vitellogenin pathway to physiological doses of 17β–estradiol. In the first study, adult naïve killifish from Tuckerton, NJ (reference) were caged at Tuckerton and Newark Bay. After 1 month, males caged at Newark Bay exhibited inductions of hepatic vitellogenin and estrogen receptor α, which were transient and returned to basal levels after 2 months ($p \leq 0.05$). In the second study, fecundity and 17β–estradiol levels were measured in reproductively active adult females from Tuckerton and Newark Bay. Tuckerton females produced 140 eggs per female and Newark Bay females produced 11 eggs per female. Embryos from Newark Bay had 34% greater mortality and 28% less hatch, relative to Tuckerton. In addition, embryo mass and yolk–volume of

Newark Bay embryos compared to Tuckerton embryos was 16% and 25% lower, respectively. Circulating 17β–estradiol levels in Newark Bay females (0.26 ng/mL) were measured to be 8–fold lower than Tuckerton females (2.25 ng/mL). In the third study, adult killifish from both sites were dosed with 17β–estradiol to assess the sensitivity of the vitellogenin pathway. At doses of 0.01, 0.1, 1 and 10 ng/g body weight, induction levels of circulating vitellogenin in Newark Bay males were significantly inhibited by 97, 99, 98 and 44%, respectively, compared to Tuckerton males. At doses of 0.01, 0.1, 1, 10 and 100 ng/g body weight, induction levels of circulating vitellogenin in Newark Bay females were inhibited by 89, 79, 61, 40 and 30%, respectively, compared to Tuckerton females. These differences in inducibility could not be explained by altered hepatic expression of estrogen receptors α, βa or βb. Based on the caged and dose–response studies, contaminants that down–regulate vitellogenin would interfere with its ability to be used as a biomarker for xeno–estrogen exposures. These studies demonstrate that contaminants within Newark Bay exert both estrogenic and anti–estrogenic responses which results in an overtly anti–estrogenic phenotype (reduced egg production due to inhibition of vitellogenesis).

3.1. Introduction

Previous studies in our laboratory have demonstrated that female Atlantic killifish (Fundulus heteroclitus) inhabiting Newark Bay, NJ (USA) exhibited anti–estrogenic reproductive effects, when compared to a reference population from Tuckerton, NJ (Bugel et al., 2010). Decreased hepatic expression of vitellogenin (egg–yolk protein precursor) was proposed to be the etiology for the inhibition of normal oocyte

development in Newark Bay female killifish. The purpose of the current study was to determine whether inhibition of oocyte development resulted in decreased fecundity, and to characterize contaminant effects on the regulation of circulating 17β–estradiol and vitellogenin. Atlantic killifish (*F. heteroclitus*) were chosen for our studies because of its important role in the estuarine food web as a both predator and prey, and because it has a limited home range allowing for impacts to be related to locally found contaminants (Burnett et al., 2007).

Newark Bay and adjoining estuaries in the NY–NJ Harbor Complex contain mixtures of historical and emerging contaminants at elevated levels that pose a continued risk to the sustainability of aquatic populations. Newark Bay sediment concentrations of total polychlorinated dibenzo–*p*–dioxins and furans were reported to be 0.8–9.3 ng/g (parts per billion) and 0.2–3.7 ng/g, respectively (Dimou and Pecchioli, 2006). Total polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) in sediments have been measured to be 44,000 and 756 ng/g, respectively (Huntley et al., 1995; Panero et al., 2005). Other contaminants of concern include pesticides, heavy metals, nonionic alkylphenol ethoxylate surfactants, flame retardants, bisphenol A, phthalates, and synthetic estrogens from effluent of approximately 30 wastewater treatment plants discharging into the NY–NJ Harbor Estuary (Litten, 2003; Muñoz et al., 2006; NY–NJ HEP, 2006; Wilson and Bonin, 2007; Iannuzzi et al., 2008). Little evidence to date has supported that the Newark Bay environment is estrogenic, although previous studies suggested that Newark Bay contaminants exert anti–estrogenic effects on killifish (McArdle et al., 2004; Bugel et al., 2010). For example, aryl hydrocarbon receptor (AhR) agonists modulate hormone signaling by cross–talk with the estrogen receptor and can

affect vitellogenin gene regulation (Safe and Wormke, 2003; Wormke et al., 2003; Ohtake et al., 2008). Newark Bay is heavily contaminated with AhR agonists (dioxins, furans, PAHs, PCBs) that have resulted in elevated cytochrome P4501A (CYP1A) expression and a resistant AhR pathway in Newark Bay killifish (Prince and Cooper, 1995a,b; Elskus et al., 1999; Arzuaga and Elskus, 2002; Bugel et al., 2010; Nacci et al., 2010). Reproductive impacts by AhR agonists in Newark Bay killifish and other similarly impacted populations (New Bedford Harbor, MA; Elizabeth River, VA) are not clear.

Reproductive impacts in aquatic species exposed to complex mixtures are due to the dysregulation of multiple events in the hypothalamus–pituitary–gonad–liver axis (Arcand–Hoy and Benson, 1998; Rempel and Schlenk, 2008). Estrogen signaling and vitellogenin regulation are critical to oocyte development and are discussed below, although detailed reviews of teleosts oogenesis by Tyler and Sumpter (1996), Patiño and Sullivan (2002), and Thomas (2008), should be consulted. Gonadotropin hormone I secretion by the pituitary stimulates granulosa cells in the ovary to induce aromatase (cytochrome P450 19A1) and produce 17β–estradiol. Estrogen receptors (ER) in hepatocytes bind circulating 17β–estradiol, dimerize and translocate into the nucleus to induce transcription of vitellogenin genes (Menuet et al., 2005). Three killifish estrogen receptors have been identified: ER α, ER βa and ER βb (Greytak and Callard, 2007). All three ER isoforms are suspected to play a role in the induction of vitellogenin in teleosts, although the specific role of each isoforms in killifish vitellogenesis is not yet known (Nelson and Habibi, 2010). Vitellogenin proteins are large glycolipoproteins that are precursors to egg–yolk proteins. Two vitellogenin genes are known to exist in *F. heteroclitus*: vitellogenins I and II (LaFleur et al., 1995a,b). Vitellogenin I is the

precursor for the majority of the known yolk–proteins in killifish. Circulating vitellogenin proteins are endocytosed into developing oocytes and cleaved by cathepsins into yolk–proteins required for oocyte growth and maturation (Kanungo et al., 1990; LaFleur et al., 2005). Yolk–proteins also act as a source of nutrients (lipids, inorganic ions, energy and free amino acids) during embryogenesis and eleuthero–embryo development. Estrogen and vitellogenin levels can be used for evaluating reproductive status in females. In male fish, vitellogenin genes are present but not expressed and therefore can serve as a biomarker of exposure to estrogenic contaminants (Sumpter and Jobling, 1995).

Three studies are presented within this paper. The first study was a caged study (2 month duration) designed to investigate impacts of Newark Bay contaminants on hepatic expression of ER α, ER βa, ER βb and vitellogenin using naïve killifish from Tuckerton, NJ. We hypothesized that transplantation of naïve killifish from the reference population (Tuckerton, NJ) to Newark Bay would result in estrogenic and anti–estrogenic responses in males and females, respectively. The caged study was used to evaluate sensitive responses in naïve killifish re–located to Newark Bay as an alternative approach to measuring impacts in the indigenous population that may have adapted responses. The second study assessed fecundity (egg production and embryo survival) as a measure of the reproductive capacity of killifish collected from Newark Bay and a reference site at Tuckerton. Circulating 17β–estradiol levels were also measured in killifish from both sites as an integrated measure of contaminant impacts on hormone signaling. In the third study, adult killifish from Newark Bay and Tuckerton were challenged with 17β–estradiol to assess the sensitivity and regulation of the vitellogenin pathway. Our working hypothesis was that impacts on egg development in Newark Bay female killifish were the

result of decreased 17β–estradiol signaling and decreased responsiveness of the vitellogenin pathway to 17β–estradiol. These studies are the first to demonstrate that contaminants within Newark Bay exert both estrogenic and anti–estrogenic responses in killifish. We propose that contaminant exposure has led to the inhibition of egg development, which due to decreased steroid signaling and a decreased sensitivity of the vitellogenin pathway to 17β–estradiol.

3.2. Materials and methods

3.2.1. Site selection and necropsy and husbandry protocols

Killifish were collected from two estuaries in New Jersey, USA. Killifish were collected using baited minnow traps from a reference site in Tuckerton (Rutgers Marine Station, 39°30´32.52″N, 74°19´26.50″W) and the heavily contaminated Newark Bay (Richard Rutkowski Park, Bayonne, 40°41´17.0″N, 74°06´42.0″W). Fish were transported on the day of collection back to the laboratory in aerated water from each site. Studies coincided with the new or full moons, which are the peaks of the killifish's lunar dependent reproductive cycle (Taylor et al., 1979; Hsiao et al., 1994; Cerdá et al., 1996).

Killifish used for laboratory controlled study were housed in a recirculating system (500 gallon total, 20 ppt seawater, 25% water change daily, 20 °C), maintained on a 14:10 light:dark photoperiod and fed frozen ground squid muscle daily. System water was continually filtered through activated carbon, UV and bio–filters. Animals to be sacrificed and undergo necropsy were euthanized with an overdose of MS–222 (tricaine methanesulphonate), weighed and measured for body length. Blood was obtained by

caudal severance and collected into heparinized tubes that were immediately centrifuged briefly at 14,000 × g. Plasma was transferred into a new tube and stored at −80 °C. The liver was removed, weighed, snap frozen in liquid nitrogen and maintained at −80 °C. All animal protocols were approved through the Rutgers Animal Care and Facilities Committee (Protocol #08–025).

3.2.2. Study 1: biomarker study of naïve killifish caged at Newark Bay, NJ

Adult killifish from Tuckerton, NJ (5–10 g, 7–10 cm) were caged at both Tuckerton and Newark Bay for up to 2 months. The beginning of the experiment coincided with a new moon on May 24, 2009, and two collections were made at 1 and 2 months time, each corresponding with a new moon (June 22, 2009, and July, 21, 2009). Three sets of thirty killifish (15 males, 15 females) were placed into stainless steel cages at both Tuckerton and Newark Bay. Cages were 2 cubic feet (30.5 cm × 30.5 cm × 61 cm) with 1/4″ (0.64 cm) mesh and were anchored just below the low tide line so that cages would be 1 foot (30.5 cm) below the surface at low tide. At 1 and 2 months, 12–15 males and females were sacrificed from each site. Vitellogenin (hepatic mRNA and circulating protein) and hepatic estrogen receptor mRNA expression (ER α, ER βa, ER βb) was evaluated.

3.2.3. Study 2A: fecundity study and embryo morphometric measurements

Adult male and female killifish (4–7 g, 6–7 cm) were collected and strip spawned from Tuckerton and Newark Bay to assess fecundity (number of mature eggs per female, viability, hatching success) and embryo morphometrics (mass and yolk–volume). Fish

were collected less than 48 hr before a full moon spawning (May 27, 2010), separated by gender and held for 24 h to prevent early spawning. Twenty females from each site were strip spawned into individual Petri dishes containing egg water (20 ppt sea water, 0.22 µm filtered, 20 °C). Eggs were fertilized using minced testis from a pool of 5 males from the respective site. Egg water was replaced after 3 h and was replaced daily until the end of the experiment. Dead embryos and newly hatched larvae were recorded and removed daily. Yolk–volume and embryo mass were determined at 24 h post fertilization (hpf) so that measurements were made only on fertilized and viable embryos. Photomicrographs were taken of 21 embryos from each site (7 per female, 3 females per site) to measure the diameter of each yolk–sac for calculating volume. The mass of 24 hpf embryos were also measured using pools of 10 embryos from 6 females per site.

3.2.4. Study 2B: 17β–estradiol analysis

Blood was collected from 50 adult females (4–7 g, 6–7 cm) each from Tuckerton and Newark Bay to assess plasma levels of 17β–estradiol 3 days prior to a peak in the reproductive cycle on a new moon (July 8, 2010). Blood plasma levels of 17β–estradiol have been shown to be highest 3 days prior to the peak of spawning (Cerdá et al., 1996). 17β–estradiol was quantified using Coat–A–Count Estradiol Radioimmunoassay (Siemens Medical Solutions Diagnostics, Los Angeles, CA). Plasma from5 individuals were combined in equal volumes to make 10 composite samples per site, which were diluted 20–fold and analyzed. The detection limit was 0.02 ng/mL of circulating 17β–estradiol in blood plasma. Samples below detection were recorded at the detection limit.

3.2.5. Study 3: dose–response study – vitellogenin sensitivity to a 17β–estradiol challenge

Reproductively inactive adult killifish (3–5 g, 5–7 cm) were collected from Tuckerton and Newark Bay outside of the breeding season (October, 2009) and acclimated for 1–2 weeks to laboratory conditions. Fish were injected intra–peritoneally with graded doses of 17β–estradiol (≥ 98%, Sigma–Aldrich, St. Louis, MO) to assess site–specific differences in vitellogenin regulation. Six males and females each were injected with 17β–estradiol (0.01, 0.1, 1, 10 and 100 ng/g body weight) dissolved in corn oil (10 µL/g body weight). Killifish were sacrificed 4 days post–injection because circulating vitellogenin levels have been shown to peak 4–8 days post–injection when dosed with 17β–estradiol (Pait and Nelson, 2003). Controls for this experiment included a group that was sacrificed on day 0 (non–injected) and a group that was sacrificed on day 4 (injected with corn oil alone). Hepatic mRNA expression of ER α, ER βa and ER βb were evaluated for the day 0 control group and circulating vitellogenin protein was evaluated for all treatment groups.

3.2.6. Protein and mRNA expression analysis

Total RNA was isolated from liver using TRIzol® (Invitrogen, San Diego, CA), treated with DNA–free (DNA–free, Ambion, Austin, TX), and reverse transcribed using a High Capacity cDNA Reverse Transcript Kit (Applied Biosystems, Foster City, CA). Quantitative polymerase chain reaction was performed on 50 ng cDNA using Bio–Rad iQ SYBR Green Supermix with a Bio–Rad iCycler and iCycler iQ Detection System (Bio–Rad, Hercules, CA). Samples were analyzed in triplicate for each gene and normalized to

the population's median β–actin value. After normalization, expression was quantified using a standard curve to calculate the nanogram amount of gene template per 50 ng RNA. Primers for β–actin are the same as those used previously and new primer sets for vitellogenin I and three estrogen receptors in killifish (ER α, ER βa and ER βb) were designed using criteria described previously (Bugel et al., 2010). Vitellogenin I (GenBank U07055.2) primers (234 bp product) were F: 5′–CAG CAC CAG GAA TAT CTC AG–3′ and R: 5′–GTG TAG AGT GTG TCT TCG AC–3′. Estrogen receptor α (ER α, GenBank AY571785.1) primers (195 bp product) were F: 5′–TTT CTT TCT GCA CCG GCA CAA TGG–3′ and R: 5′–GCT CCA TGC CTT TGT TGC TCA TGT–3′. Estrogen receptor βa (ER βa, GenBank AY570922.1) primers (112 bp product) were F: 5′–ATC TTT GAC ATG CTA ATC GCC GCC–3′ and R: 5′–TCA GGC ACA TGT TGG AGT TGA GGA–3′. Estrogen receptor βb (ER βb, GenBank AY570923.1) primers (162 bp product) were F: 5′– TTG ACG CTC TGG TTT GGG CTA TCT–3′ and R: 5′–ACA CAA GCA CCA CGT TCT TCC TCT–3′. The detection limit for vitellogenin was 1.0×10^{-8} ng per 50 ng total RNA. Data below these values were set as the detection limit.

Vitellogenin was detected in blood plasma using a modified immunoblotting technique described previously (Bugel et al., 2010). Blood plasma was diluted 1:50 or 1:100 and separated by electrophoresis using Novex 3–8% Tris–acetate SDS polyacrylamide gels (Invitrogen, Carlsbad, CA). Protein was transferred to a 0.45 µm PVDF Transfer Membrane (Thermo Fisher Scientific, Waltham, MA) for 1 h using 200 mA (constant) in 1× transfer buffer (25 mM Tris–base, 192 mM glycine, 10% methanol). Membranes were then incubated at 4 °C for 20 h in 1× TBST (200 mM NaCl, 50 mM Tris, 0.1% Tween–20) with vitellogenin (striped–bass) monoclonal antibody diluted

1:2500 (ND–1C8, Cayman Chemical, Ann Arbor, MI). Membranes were washed 3 times for 5 min each in 1× TBST and then incubated for 30 min at room temperature in1× TBST with 5% non–fat milk and goat anti–mouse IgG1–HRP secondary antibody sc–2969 diluted 1:5000 (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were finally washed 5 times for 5 min each in 1× TBST and once with deionized ultrafiltered water for 5 min. The Amersham ECL Advance Western Blotting Detection Kit (RPN2135, GE Healthcare, Buckinghamshire, UK) was used to detect immunoreactivity with CL–X Posure Film (Thermo Fisher Scientific, Waltham, MA). Bands were quantified by densitometry with ImageJ (Abramoff et al., 2004) and converted to relative intensity to a standard rainbow trout vitellogenin sample (Vtg–51, Biosense Laboratories, Bergen, Norway).

3.2.7. Statistical analyses

Statistical tests were performed using SigmaPlot™ (v. 11.0). Data is reported as mean ± standard deviation. Fold–changes were calculated using median values. A *p*–value $\leq$ 0.05 was regarded as significantly different. Unpaired t–tests were used to compare different treatments. When normality failed the Student–Neuman–Keuhls test was used. When equal variance failed the Mann–Whitney rank sum test was used. Discrete data was compared using Fisher's exact test.

3.3. Results

3.3.1. Study 1: endocrine responses of Naïve Tuckerton killifish caged at Newark Bay

Adult naïve killifish from Tuckerton were caged at Tuckerton and Newark Bay for up to 2 months to investigate the effects of contaminant exposure on expression of vitellogenin, ER α, ER βa and ER βb. Mortality was low for fish caged at both sites for 1 month, 59 of 60 fish survived at Tuckerton (98% survival) and 57 of 60 fish survived at Newark Bay (95% survival). Survival was decreased at Newark Bay after 2 months, although not significant. After two months, 23 of 30 (77%) fish survived at Tuckerton and 30 of 54 fish (56%) survived at Newark Bay. Animals were feeding at both sites and during both collection times (determined by presence of food in the gastrointestinal tract). No significant differences or changes in body morphometrics (liver and gonad organ weights, body lengths, and organ to body weight ratios) were observed between killifish caged at Tuckerton and Newark Bay for either gender after 1 and 2 months caging time, or from month to month (data not shown).

Vitellogenin expression (hepatic mRNA and circulating protein) was assessed in naïve Tuckerton male killifish caged at Tuckerton and Newark Bay, as a biomarker of exposure to estrogen mimics. Males caged at Newark Bay for 1 month exhibited significantly elevated levels of hepatic vitellogenin mRNA expression that were 53–fold higher than levels measured in killifish caged at Tuckerton (Fig. 3.1A). After 2 months, hepatic vitellogenin mRNA expression in males caged at Newark Bay returned to reference levels (Fig. 3.1A). Circulating vitellogenin protein was detected in 5 of 7 males caged at Newark Bay after 1month while no males in the Tuckerton group (N = 7)

expressed detectable levels (Fig. 3.1B). After 2 months, circulating vitellogenin protein was not detected in any males caged at either site (N = 7 per group). Hepatic mRNA expression of ER α, ER βa and ER βb was measured in killifish caged at both sites to investigate the potential for Newark Bay contaminants to alter expression of the estrogen receptors. ER α expression served as a second biomarker of xeno–estrogen exposure since ER α has been shown to be inducible by 17β–estradiol (Urushitani et al., 2003). Expression of ER α in males was significantly elevated 2.4–fold in males caged at Newark Bay for 1 month, relative to males caged at Tuckerton (Fig. 3.2A). The expression of ER α returned to basal levels after 2 months and was not different between males caged at Tuckerton and Newark Bay (Fig. 3.2A). Expression of ER βa and ER βb was not affected in killifish caged at Newark Bay for 1 month (Fig. 3.2B and C). Hepatic expression of ER α, ER βa and ER βb decreased significantly in killifish caged at Newark Bay by 3.2, 2.0, and 3.0–fold, respectively, from month 1 to month 2 (Fig. 3.2A–C). However, only expression of ER βb was significantly lower than levels measured in killifish caged at Tuckerton for 2 months (Fig. 3.2C). Hepatic vitellogenin mRNA expression and circulating protein levels were evaluated in females caged at Tuckerton and Newark Bay as a measure of contaminant impacts on vitellogenesis. Females caged at Newark Bay exhibited no significant change in hepatic vitellogenin mRNA expression or circulating protein levels after 1 and 2 months exposure time, relative to females caged at Tuckerton (Fig. 3.3A and B). Hepatic vitellogenin mRNA expression at each site decreased from month–to–month (Fig. 3.3A). Hepatic mRNA expression of ER α, ER βa and ER βb was not affected in female killifish caged at Newark Bay after 1 month and did not change from month–to–month in killifish caged at either site (Fig. 3.4A–C).

Expression of ER βa in females caged at Newark Bay for 2 months was significantly lower by 2.8–fold relative to levels measured in females caged at Tuckerton (Fig. 3.4B). There were no significant differences in expression of ER α and βb between females caged at Tuckerton and Newark Bay for 2 months.

3.3.2. Study 2A: population productivity: egg production, quality and embryonic survival

A fecundity study was performed using adult killifish collected from Tuckerton and Newark Bay to directly measure the reproductive capacity of each population and to assess the quality of eggs and embryos. All eggs that were evaluated were fertilized and proceeded through early embryonic stages. Newark Bay females produced significantly fewer mature eggs compared to Tuckerton females (Fig. 3.5A). In addition, significantly fewer embryos from Newark Bay successfully hatched by 24 days post fertilization (dpf), relative to Tuckerton embryos (Fig. 3.5A). Tuckerton killifish produced 140 ± 49 mature eggs per female and Newark Bay females produced 11 ± 13 mature eggs per female (92% fewer). Tuckerton females averaged 117 ± 48 embryos hatch per female by 24 dpf while Newark Bay females averaged 5 ± 3 embryos hatch per female by 24 dpf (95% fewer). Survival was not significantly affected in Tuckerton embryos, however Newark Bay embryos had a significant decrease in survival before 3 dpf (Fig. 3.5B). Mortality in Newark Bay embryos was primarily observed throughout the first 3 days of development (59% of total mature eggs were viable compared to Tuckerton's 93%) and had little change in survival by 24 dpf. Of the total eggs strip spawned, 82% at Tuckerton hatched by 24 dpf and 54% hatched from Newark Bay (Fig. 3.5B). Embryo morphometric

parameters (yolk volume and embryo mass) were measured at 24 hpf to investigate impacts on yolk development. Newark Bay embryos were visually smaller than Tuckerton embryos and appeared to have decreased yolk–volume (Fig. 3.6A). Quantitative measurements were made at 24 hpf so that only viable embryos were used. Embryo mass and yolk–volume were significantly decreased in Newark Bay embryos by 16 and 25%, respectively (Fig. 3.6B and C). The mean embryo mass at Newark Bay was 3.3 ± 0.1 mg per embryo, while at Tuckerton it was 3.9 ± 0.3 mg per embryo (Fig. 3.6B). The mean yolk–volume at Newark Bay was 2.2 ± 0.2 mm^3 per embryo compared to Tuckerton embryos which were 3.0 ± 0.2mm^3 per embryo (Fig. 3.6C).

3.3.3. Study 2B: circulating 17β–estradiol prior to spawning

Circulating 17β–estradiol levels were measured in females from Tuckerton and Newark Bay three days prior to the peak of spawning to investigate the role of 17β–estradiol levels in the inhibition of oocyte development and decreased vitellogenin levels in Newark Bay females. Circulating levels of 17β–estradiol are highest at this point in the killifish reproductive cycle (Cerdá et al., 1996). Concentrations of 17β–estradiol in blood plasma of Newark Bay females were 8–fold significantly lower than Tuckerton females (Fig. 3.7). Mean concentrations of circulating 17β–estradiol were 2.25 ± 1.78 ng/mL in Tuckerton females and 0.26 ± 0.26 ng/mL in Newark Bay females. Five of 10 composite samples analyzed from Newark Bay fell below the detection limit (0.02 ng/mL) and were recorded as the detection level.

3.3.4. Study 3: regulation of vitellogenin protein by a 17β–estradiol challenge

Adult male and female killifish from the Tuckerton and Newark Bay populations were given intra–peritoneal injection with graded doses of 17β–estradiol to assess the sensitivity and ability of the vitellogenin pathway to produce protein in response to an estrogen challenge. Vitellogenin was not detected in either control groups on day 0 (no injection) and day 4 (oil injection) for both males and females from both sites. Males and females from both sites were inducible with all doses tested (0.01, 0.1, 1, 10 and 100 ng/g bodyweight). However, killifish from Newark Bay were less responsive and had a dose–response shifted to the right, compared to Tuckerton killifish (Fig. 3.8A). Newark Bay males produced significantly less circulating vitellogenin protein in response to a 17β–estradiol challenge than males from Tuckerton at concentrations of 0.01–10 ng 17β–estradiol per gram body weight (Fig. 3.8B). At doses of 0.01, 0.1, 1 and 10 ng/g body weight, Newark Bay males exhibited induction levels of circulating vitellogenin protein that were lower by 32.6, 139.0, 53.9 and 1.8–fold, respectively, than induction levels in Tuckerton males. Induction of vitellogenin in Newark Bay males at doses of 0.01, 0.1, 1 and 10 ng/g bodyweight were therefore inhibited by 96.9, 99.3, 98.1 and 44.2%, respectively. At the highest dose tested (100 ng/g body weight), there were no differences in vitellogenin levels between Tuckerton and Newark Bay males. Female killifish from Newark Bay also produced significantly less circulating vitellogenin protein in response to 17β–estradiol at all doses tested, compared to Tuckerton females (Fig. 3.8C). At doses of 0.01, 0.1, 1, 10 and 100 ng/g body weight, females from Newark Bay exhibited induction levels of circulating vitellogenin protein that were lower by 9.0, 4.8, 2.5, 1.7 and 1.4–fold, respectively, than induction levels in Tuckerton females. Induction of

vitellogenin in Newark Bay females at doses of 0.01, 0.1, 1, 10 and 100 ng/g body weight were therefore inhibited by 88.9, 79.3, 60.5, 39.5 and 30.2%, respectively. As a result of the shifted–dose response for induction of circulating vitellogenin protein by 17β–estradiol, hepatic mRNA expression of ER α, ER βa and ER βb was assessed in Tuckerton and Newark Bay killifish. Expression prior to treatment (day 0 controls) acted as a surrogate measure of basal estrogen receptor levels. However, no significant differences were found between Tuckerton and Newark Bay killifish for either gender on day 0 for ER α, ER βa and ER βb (Fig. 3.9).

3.4. Discussion

Endocrine disruption at Newark Bay is a complex interaction of estrogenic and anti–estrogenic stressors that result in an overtly anti–estrogenic phenotype in Newark Bay killifish. Estrogenic responses (vitellogenin and ER α expression) in naïve males transplanted to Newark Bay were transiently induced before returning to basal levels over time. This demonstrated that Newark Bay is an acutely estrogenic environment with anti–estrogenic impacts resulting from prolonged exposure. In addition, we demonstrated that female killifish inhabiting Newark Bay exhibited decreased fecundity (number of mature eggs per female, viability, hatching success), as a result of (1) decreased 17β–estradiol signaling and (2) a decreased sensitivity of the vitellogenin pathway to a 17β–estradiol challenge. Impacts on oogenesis (yolk–development) in female killifish are proposed to result from contaminants that inhibit steroidogenesis and vitellogenesis, which cumulatively result in decreased vitellogenin expression.

3.4.1. Endocrine responses in naïve Tuckerton killifish caged at Newark Bay

Elevated levels of vitellogenin have not been detected in male killifish inhabiting Newark Bay or from naïve killifish injected intra–peritoneally with Newark Bay sediment extracts (McArdle et al., 2004; Bugel et al., 2010). These two separate studies speculated that the lack of an estrogenic response was due to either (1) insufficient levels of xeno–estrogens in Newark Bay to induce a vitellogenin response or (2) the presence of contaminants in Newark Bay that act antagonistically with xeno–estrogens (e.g., AhR agonists). These studies concluded that Newark Bay was not an overtly estrogenic environment, despite the presence of known xeno–estrogens (e.g., dioxin like compounds, PCBs, PAHs, synthetic estrogens, etc.). The caged study presented in this paper addressed the limitations of measuring vitellogenin expression in indigenous males that may have developed altered responses from chronic exposure to contaminants. We demonstrated that Newark Bay is an estrogenic environment that transiently induced hormonal responses, which were down–regulated over time. Elevated expression levels of vitellogenin and ER α in naïve male Tuckerton killifish caged at Newark Bay for 1 month were evidence of feminization (Figs. 3.1 and 3.2). However, these inductions were transient and expression of both vitellogenin and ER α returned to basal levels after 2 months (Figs. 3.1 and 3.2). It is unclear whether the down–regulation of ER α plays a role in the down–regulation of vitellogenin because ER α expression did not decrease below basal levels after 2 months (Fig. 3.2). In addition, the impacts on expression of the ER β isoforms and relevance to vitellogenin regulation are not clear but may indicate contaminant effects on these receptors. These results help to explain the lack of feminization of the indigenous male killifish population in Newark Bay and suggest that

the vitellogenin pathway has adapted to be non–responsive in this estrogenic environment. This is the first evidence to our knowledge to suggest that vitellogenin may not be an appropriate biomarker for xeno–estrogen exposure in a wild fish population chronically exposed to complex mixtures of contaminants. Therefore, we recommend that studies, such as transplant studies, should follow negative vitellogenin results (not up–regulated) to reduce the occurrence of false–negatives in environments known to contain xeno–estrogens. Our caged study was unable to determine the basis for the down–regulation of the elevated vitellogenin and ER α expression in males caged at Newark Bay. However, studies by Nash et al. (2004) reported that male zebrafish develop attenuated vitellogenic responses to 17α–ethynylestradiol after life–long exposure to environmentally relevant concentrations (5 ng/L). Desensitization of estrogenic responses may therefore be a normal outcome of chronic exposure to xeno–estrogens. Alternatively, reductions in hepatic expression of vitellogenin and ER α have been reported in female fathead minnows (*Pimephales promelas*) exposed to sediments impacted by pesticides, resulting in a similar down regulation of vitellogenin and ER α (Sellin et al., 2010). Further work is necessary to determine the chemical and biological reasons for down–regulation of estrogenic responses in Newark Bay killifish.

Our caged study is the first to demonstrate that killifish in Newark Bay are exposed to xeno–estrogens that can illicit estrogenic responses. We previously reported decreased spermatocytes and interstitial fibrosis in testis of male killifish from Newark Bay (Bugel et al., 2010). Exposure to estrogen mimics may help explain these reproductive impacts in the indigenous male population. Several studies have demonstrated that exposure to estrogenic compounds impairs gonadal development and

testis function in fish (Jobling et al., 1996; Nash et al., 2004; Kidd et al., 2007). Other studies investigating endocrine disruption within the NY–NJ Harbor Estuary have also reported feminization of nearby fish populations. Studies by Baldigo et al. (2006) reported elevated vitellogenin levels and altered 17β–estradiol/11–ketotestosterone ratios in common carp (*Cyprinus carpio*), bass (*Micropterus salmoides* and *Micropterus dolomieui*) and bullhead (*Ameiurus nebulosus*) in the nearby Hudson River (NY). Studies by McArdle et al. (2000) and Todorov et al. (2002) demonstrated that larval and juvenile sunshine bass (*Morone saxatilis* × *Morone chrysops*) exposed to effluent from several sewage treatment plants serving New York City exhibited significant elevations in plasma vitellogenin levels. Endocrine disruption resulting from xeno–estrogen exposure is likely to be widespread in fish populations throughout the NY–NJ Harbor Estuary.

The down–regulated vitellogenin response observed in naïve male killifish caged at Newark Bay could be related to the anti–estrogenic reproductive impacts (decreased vitellogenin mRNA and protein expression) that were previously reported in female killifish inhabiting Newark Bay (Bugel et al., 2010). However, the caged study was unable to replicate these effects in naïve female killifish caged at Newark Bay. Vitellogenin levels (mRNA and protein) in naïve female killifish caged at Newark Bay were not significantly different than those caged at Tuckerton after 1 and 2 months exposure (Fig. 3.3A and B). Hepatic expression of ER α, and ER βb in female killifish was unaffected by transplantation into Newark Bay while expression of ER βa was significantly lower after 2 months, but the biological significance of this change (2.8–fold) is not clear (Fig. 3.4A–C). We propose that vitellogenin and estrogen receptor regulation in females was relatively unaffected by caging at Newark Bay for two reasons.

First, two months exposure to contaminants within Newark Bay may not be enough to alter the reproductive status of female naïve killifish. Second, killifish were caged at Newark Bay after the reproductive cycle had initiated, and in 2 months contaminants did not interfere with hormonal signaling that were already in place. Events during ovarian recrudescence may be more sensitive to disruption by contaminants. We therefore recommended that future studies consider beginning caged–studies prior to the onset of oogenesis when investigating impacts on female fish.

3.4.2. Impacts on fecundity and yolk development in Newark Bay killifish

The fecundity study demonstrated that Newark Bay killifish have a significantly reduced capacity for reproduction which was due primarily to the reduction in egg production, rather than loss of embryos during development. The inhibition of follicular development in Newark Bay killifish reported by Bugel et al. (2010) correlated with a 92% reduction in egg production, relative to Tuckerton females (Fig. 3.5). In addition, many Newark Bay embryos did not survive to hatching due to high mortality in the first 3 days of development (Fig. 3.5). The high mortality in Newark Bay embryos may have been due to both elevated contaminant levels and poor embryo quality (reduced size and yolk supply). Elevated body burdens of contaminants found in the sediments and wildlife of Newark Bay (e.g., 2,3,7,8–tetrachlorodibenzo–*p*–dioxin, furans, PCBs) correlate with early life stage mortality in other fish populations (Casillas et al., 1991; Fitzsimons, 1995; Zabel et al., 1995). Despite dramatic impacts on egg production, the Newark Bay killifish population continues to sustain itself, as is evidenced by young–of–the–year. A small percentage of the population appears to perform the majority of the reproductive duties

and may be why this population persists. The three females with the highest fecundity at Newark Bay produced 47, 25 and 22 eggs with a population mean of 11 ± 13 eggs per female. The three females with the lowest fecundity in the reference population at Tuckerton produced 91, 94 and 95 eggs each with a population average of 140 ± 49 eggs per female. The few females at Newark Bay reproducing at a relatively higher capacity may represent non–responders that are relatively resistant to contaminant effects.

An inverse relationship between vitellogenin expression and fecundity in the fathead minnow has been established, suggesting that impacts on vitellogenesis directly translates to a reduction in egg production and population growth (Miller et al., 2007; Thorpe et al., 2007). Reduced vitellogenin expression in Newark Bay killifish has been previously associated with underdeveloped ovaries. The inhibition of development was demonstrated by many follicles present at early pre–vitellogenic stages and few that progressed through vitellogenin–dependent stages (Bugel et al., 2010). The relatively few eggs that are produced by Newark Bay females are less robust and lower quality, than those from Tuckerton (Fig. 3.6). Newark Bay embryo mass and yolk–volume was demonstrated to be reduced by 25% and 16%, respectively, relatively to Tuckerton embryos. Vitellogenin accumulation by developing oocytes largely determines size and quality of eggs and is estimated to account for 50% of the final egg size in killifish (Selman and Wallace, 1983). Therefore, the reduced vitellogenin levels in Newark Bay females are proposed to be the basis for the impacts on egg and yolk–development.

3.4.3. Role of altered 17β–estradiol signaling in reproductive impacts of Newark Bay killifish

Decreased vitellogenin expression in Newark Bay females is proposed to be the cumulative result of (1) deficiency of circulating 17β–estradiol and (2) desensitization of the vitellogenin pathway to induction by 17β–estradiol. Newark Bay females had an 8–fold decrease in circulating 17β–estradiol levels relative to Tuckerton females, indicating impacts on hormone regulation (Fig. 3.7). Studies with the fathead minnow by Ankley et al. (2008) established a relationship between circulating 17β–estradiol levels and both vitellogenin and fecundity. These studies demonstrate that impacts on circulating 17β–estradiol levels correlate with reductions in vitellogenin and fecundity. In addition, our challenge studies showed that vitellogenin was less inducible in Newark Bay killifish relative to Tuckerton killifish, which indicated a decrease in sensitivity of the vitellogenin pathway to 17β–estradiol (Fig. 3.8). This shifted dose–response confounds the use of vitellogenin as a biomarker for xeno–estrogen exposure in males and helps to explain why vitellogenin has not been previously detected in indigenous Newark Bay male killifish. In females, a decreased capacity to produce vitellogenin offers a unique explanation for the decreased vitellogenin expression levels and impaired egg development. Newark Bay females exhibited inhibited induction at levels of 17β–estradiol (0.1–100 ng/g body weight) that are relevant to those measured during reproduction in Tuckerton killifish (2.25 ng/mL blood plasma). A linear regression model formulated to predict fecundity from measured vitellogenin levels in fathead minnow estimated that a 50% decrease in vitellogenin concentration in females translates to an approximate 50% decrease in fecundity (Miller et al., 2007). The direct relationship

between vitellogenin and fecundity is important considering vitellogenin levels in Newark Bay females were 88.9, 79.3 and 60.5% the induction levels of Tuckerton females, at doses of 0.01, 0.1, 1 ng 17β–estradiol/g (Fig. 3.8). The decrease in vitellogenin inducibility of Newark Bay killifish is biologically important and will act in conjunction with the 17β–estradiol deficiency to result in decreased vitellogenin expression and reduced egg production.

The decreased 17β–estradiol levels in Newark Bay females were contrary to what was expected considering ovarian aromatase (cytochrome P450 19A) mRNA expression that was previously measured to be 210–fold higher than Tuckerton females (Bugel et al., 2010). Gonadal aromatase is stimulated by gonadotropin hormone I and was expressed at elevated levels, but failed to produce normal circulating levels. Circulating levels of 17β–estradiol are balanced by aromatase synthesis in granulosa cells of the ovary, and clearance by the liver and kidneys. Levels of 17β–estradiol in blood are an integrated measure of the ability of Newark Bay killifish to achieve normal circulating sex steroid levels. Our studies are unable to determine whether the decreased 17β–estradiol levels were due to a decrease in aromatase activity (synthesis), or an increase in 17β–estradiol metabolism (clearance). Future studies will need to examine the basis for this deficiency.

The expression levels of ER α, ER βa and ER βb in killifish prior to treatment with 17β–estradiol in the challenge study are unlikely to explain differences in vitellogenin inducibility. No differences in expression levels for any of the isoforms measured were found between killifish from Newark Bay and Tuckerton, for males or females (Fig. 3.9). However, in the caged study, exposure to Newark Bay contaminants led to the induction and down–regulation of ER α expression to basal levels after 1 and 2

months, respectively (Fig. 3.1). Therefore, contaminants at Newark Bay have the ability to modulate ER α responses but with unclear consequences on the regulation of vitellogenin regulation.

Overall, we conclude that impacts on egg production and oocyte development in Newark Bay females are due to insufficient vitellogenin expression levels in the liver. The reduced vitellogenin expression is inadequate for yolk–development, and results from a combination of both altered regulation of vitellogenin and 17β–estradiol deficiency. Contaminants at Newark Bay alter various reproductive pathways that cumulatively result in an anti–estrogenic phenotype in females. The contaminants responsible for these effects are unknown. However, Newark Bay is heavily contaminated by AhR agonists, and reproductive processes in the liver and the ovary are known to be direct targets for a variety of AhR agonists (Hutz, 1999). Killifish within Newark Bay exhibit elevated basal levels of hepatic CYP1A expression (mRNA, protein, activity) indicative of exposure to AhR agonists (Prince and Cooper, 1995a,b; Bugel et al., 2010). Chronic exposure to AhR agonists at Newark Bay has also led to a resistance of CYP1A induction, although the mechanisms for this resistance are currently unknown (Prince and Cooper, 1995a,b; Elskus et al., 1999; Arzuaga and Elskus, 2002; Nacci et al., 2010). We have previously reported an inverse relationship between hepatic expression of CYP1A protein and vitellogenin mRNA demonstrating a potential relationship between the AhR and ER pathways in Newark Bay female killifish (Bugel et al., 2010). Exposure to AhR agonists may therefore play a role in the reproductive impacts of the Newark Bay population. For example, rodents exposed to 2,3,7,8–tetrachlorodibenzo–*p*–dioxin exhibited decreased serum levels of 17β–estradiol and early transition to

reproductive senescence (Petroff et al., 2000; Franczak et al., 2006; Shi et al., 2007). Zebrafish chronically exposed to 2,3,7,8–tetrachlorodibenzo–*p*–dioxin exhibited decreased sensitivity to gonadotropins, decreased 17β–estradiol and vitellogenin synthesis, and ultimately an inhibition of follicle development (King Heiden et al., 2006, 2008, 2009). There is mounting evidence from *in vitro* studies that AhR agonists can directly affect vitellogenin levels by inhibiting induction by 17β–estradiol in teleost hepatocytes (Anderson et al., 1996; Navas and Segner, 2000; Bemanian et al., 2004; Mortensen and Arukwe, 2007; Gräns et al., 2010). However, *in vivo* studies reporting direct effects of AhR agonists on liver regulation of vitellogenin is limited. For example, Vaccaro et al. (2005) demonstrated that acute exposure to PCB 126 can inhibit E2–induced vitellogenin synthesis in sea bass (*Dicentrarchus labrax*). The attenuation of vitellogenin in males caged at Newark Bay for 2 months may be explained by increased exposure to AhR agonists. The shifted dose–response in Newark Bay killifish may also be explained by inhibition from the highly active AhR pathway in this population. Reproductive impacts in the Newark Bay killifish population are phenotypic of dioxin toxicity and further studies are necessary to establish direct evidence for the role of AhR agonists in the endocrine disruption of this population.

A population of killifish from the heavily PCB–contaminated New Bedford Harbor (MA) has exhibited similar impacts on vitellogenesis and steroidogenesis as those from Newark Bay, although with several distinct differences. Male killifish from New Bedford Harbor are feminized, unlike those from Newark Bay, indicating contaminants at New Bedford Harbor result in a stable up–regulation of vitellogenin expression (Greytak et al., 2005). Our caged study demonstrated that Newark Bay is estrogenic, although

estrogenic responses (vitellogenin and ER α expression) are transient and down–regulate over time. Female killifish from New Bedford Harbor, similar to those from Newark Bay, exhibit decreased vitellogenin expression (mRNA and protein), decreased gonadal somatic index, and decreased circulating 17β–estradiol levels (Greytak et al., 2005). Challenge studies with 17β–estradiol demonstrated that larval killifish from New Bedford Harbor have a shifted dose–response for ER α expression, but exhibit normal vitellogenin inducibility (Greytak et al., 2010). The relevance of these impacts to the formation of eggs and reproductive success of females at New Bedford Harbor are not clear because fecundity has been shown to be normal and not impacted (Black et al., 1998). Naïve killifish caged at Newark Bay were shown to down regulate ER α expression to basal levels over time, and impacts on ER α regulation are not as clear as those in New Bedford Harbor killifish. Also strikingly different, is that killifish from New Bedford Harbor did not exhibit a shifted dose–response for induction of vitellogenin in larval fish or adult males. Decreased vitellogenin levels in adult female killifish from New Bedford Harbor are likely related to altered regulation of ER α and a deficiency in 17β–estradiol levels (Greytak and Callard, 2007). Impacts on oocyte development at Newark Bay are thought to be due to both altered regulation of vitellogenin and 17β–estradiol deficiency. Future studies in these two populations will generate new interest in site–specific comparisons of contaminant impacts on killifish reproduction.

4.5. Conclusion

We propose that the inhibition of ovarian follicle development in Newark Bay killifish has led to a reduction of egg production due to insufficient vitellogenin

expression. The decrease in hepatic vitellogenin synthesis is due to a combination of (1) deficiency of circulating 17β–estradiol levels, and (2) a decreased sensitivity of the vitellogenin pathway to 17β–estradiol. Contaminants at Newark Bay result in a multi–faceted disruption of reproductive events in the ovary and liver, which ultimately impact fecundity. Further studies are necessary to determine the mechanism for 17β–estradiol deficiency and the inhibition of vitellogenin induction by 17β–estradiol. Contaminant impacts on killifish reproduction are relevant to ecologically and commercially important species that inhabit or migrated through Newark Bay or for spawning. In addition, contaminants at Newark Bay interfere with the ability of vitellogenin to be a reliable biomarker of exposure to estrogen mimics in males due to the down–regulation of the vitellogenin pathway with chronic exposure. Killifish are a relatively insensitive species, and because teleost oogenesis is well conserved between species, contaminant impacts in other fish populations may be similar and widespread in estuaries with similar contaminant profiles.

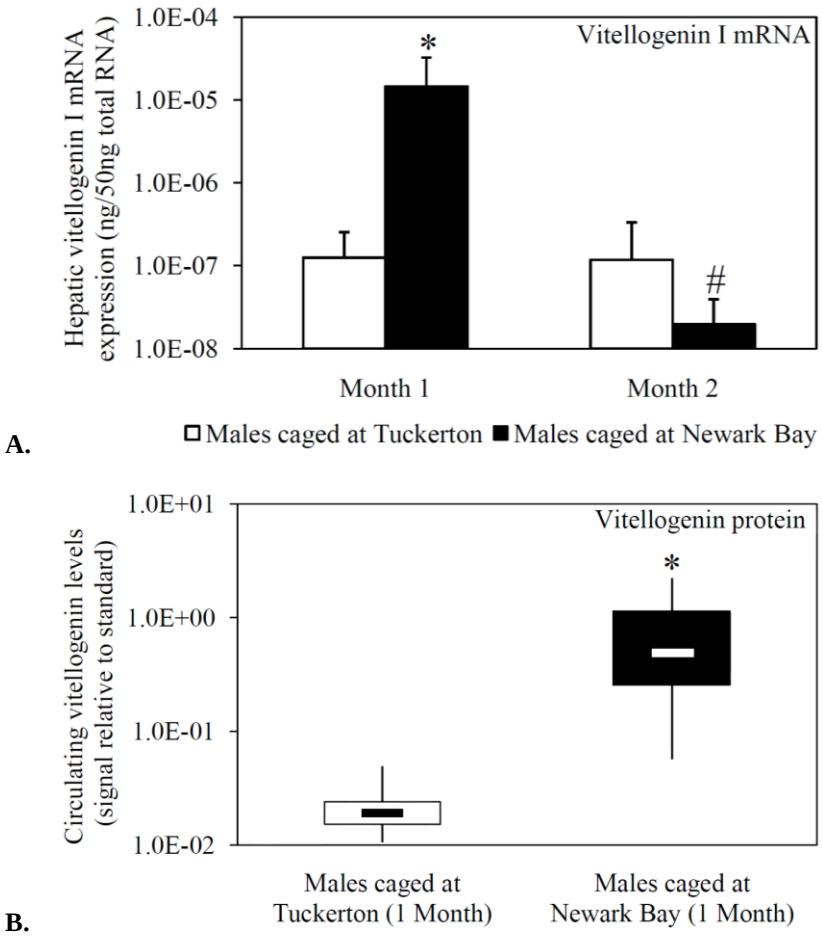


Fig. 3.1. (A) Hepatic vitellogenin mRNA expression for naïve males caged at Tuckerton and Newark Bay for 1 and 2 months. (B) Circulating levels of vitellogenin protein in naïve Tuckerton males after 1 month. No vitellogenin protein was detected in males after 2 months. Killifish were caged starting May 24, 2009, for 1 or 2 months duration with collections on June 22 and July, 21, 2009. Bar graphs are presented as mean ± standard deviation. The box–and–whisker plot represents the minimum and maximum values and the median, lower and upper quartiles. N = 6–7 individuals per group. *Significantly different from Tuckerton ($p \leq 0.05$). #Significantly different between months within the respective site and gender ($p \leq 0.05$).

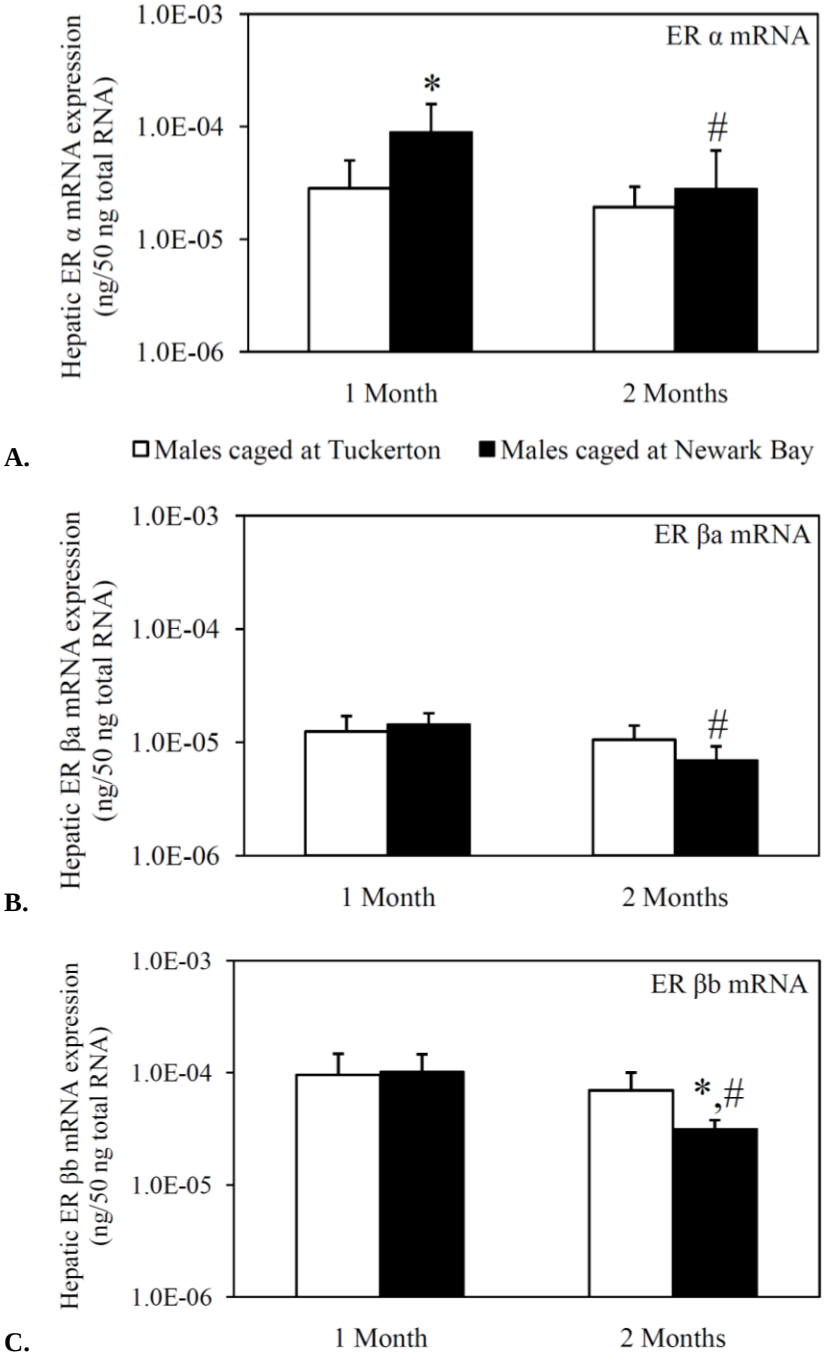


Fig. 3.2. Hepatic mRNA expression of (A) ER α, (B) ER βa and (C) ER βb in naïve males caged at Tuckerton and Newark Bay for 1 and 2 months. Killifish were caged starting May 24, 2009, for 1 or 2 months duration with collections on June 22 and July 21, 2009. Bar graphs are presented as mean ± standard deviation. N = 6–8 individuals per group. [*]Significantly different from Tuckerton ($p \leq 0.05$). [#]Significantly different between months within the respective site and gender ($p \leq 0.05$).

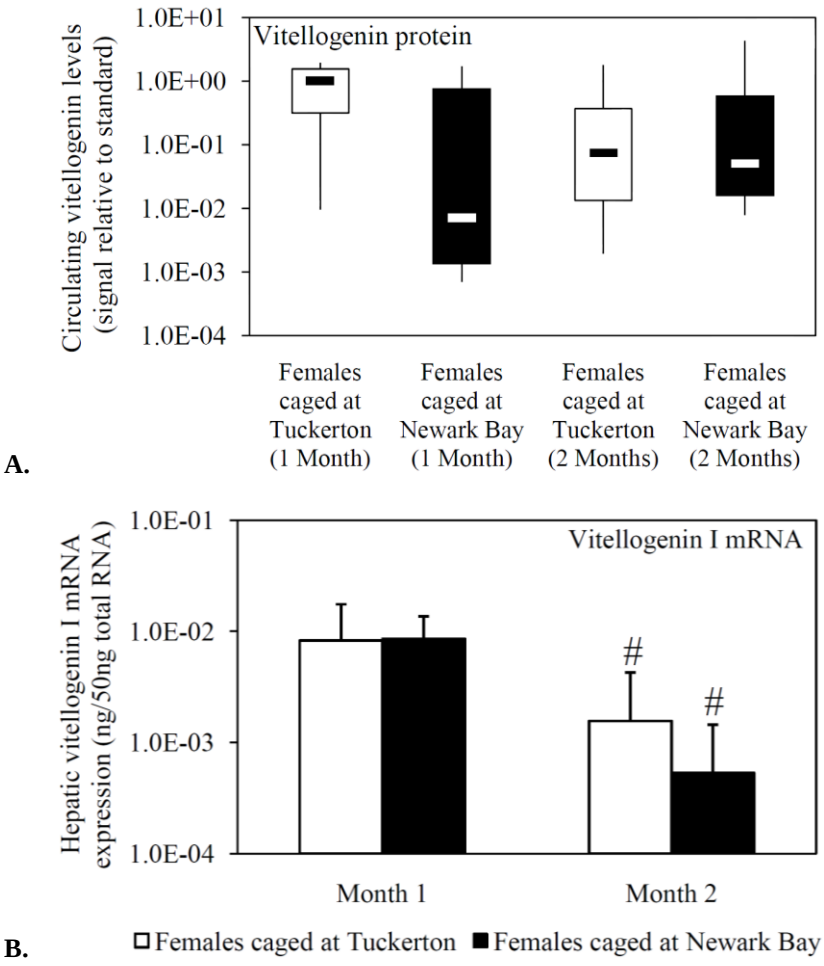
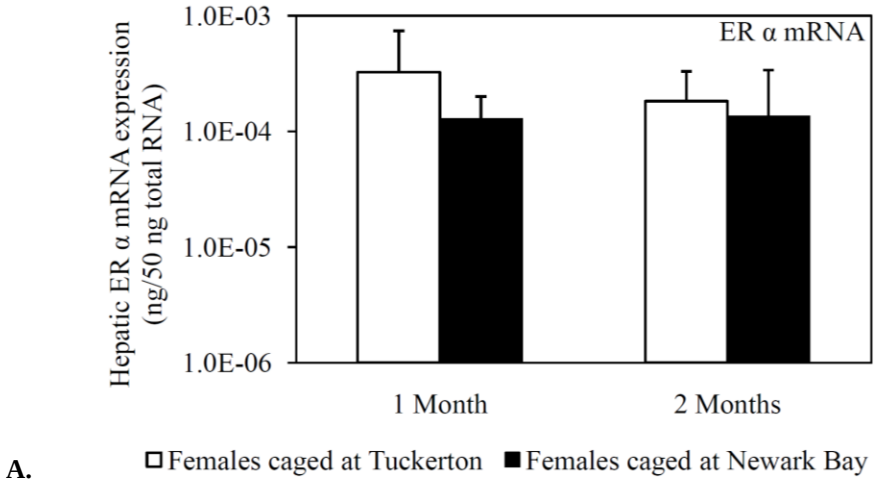
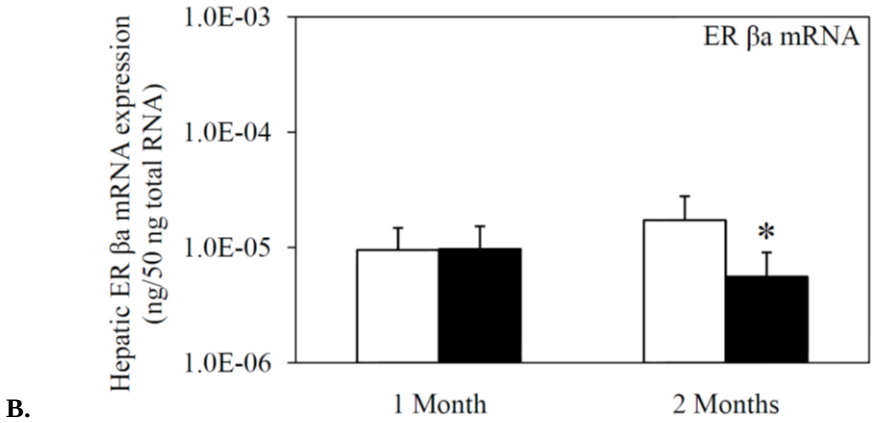


Fig. 3.3. (A) Hepatic vitellogenin mRNA expression for naïve females caged at Tuckerton and Newark Bay for 1 and 2 months. (B) Circulating levels of vitellogenin protein in naïve females after 1 and 2 months. Killifish were caged starting May 24, 2009, for 1 or 2 months duration with collections on June 22 and July 21, 2009. Bar graphs are presented as mean ± standard deviation. The box–and–whisker plot represents the minimum and maximum values and the median, lower and upper quartiles. N = 6–7 individuals per group. [#]Significantly different between months within the respective site and gender ($p \leq 0.05$).



A.



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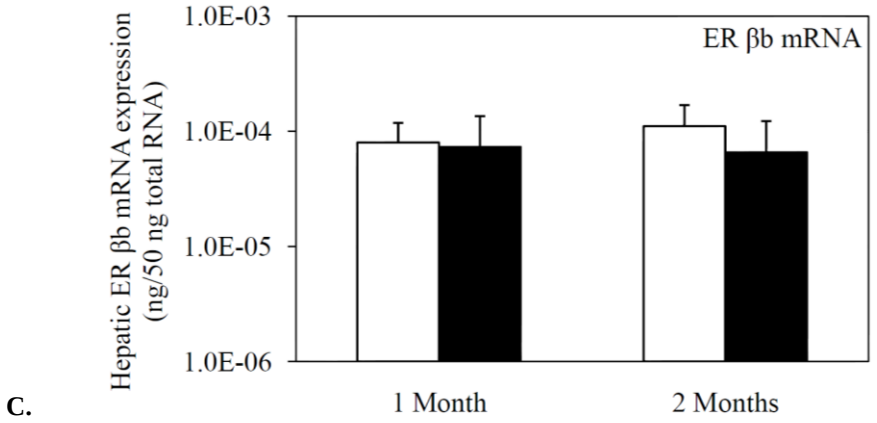
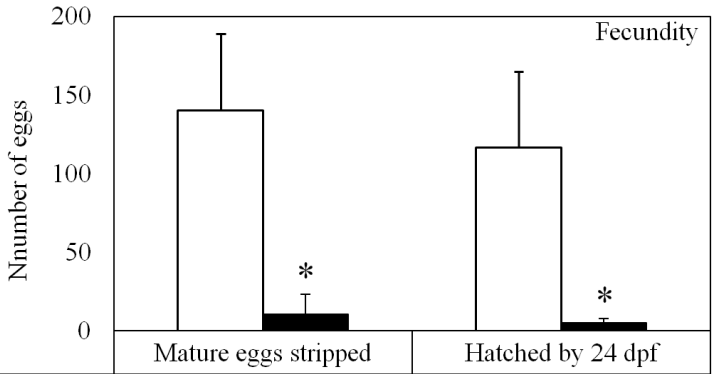


C.

Fig. 3.4. Hepatic mRNA expression of (A) ER α, (B) ER βa and (C) ER βb in naïve females caged at Tuckerton and Newark Bay for 1 and 2months. Killifish were caged starting May 24, 2009, for 1 or 2 months duration with collections on June 22 and July 21, 2009. Bar graphs are presented as mean ± standard deviation. N = 6–8 individuals per group. *Significantly different from Tuckerton ($p \leq 0.05$).

A.



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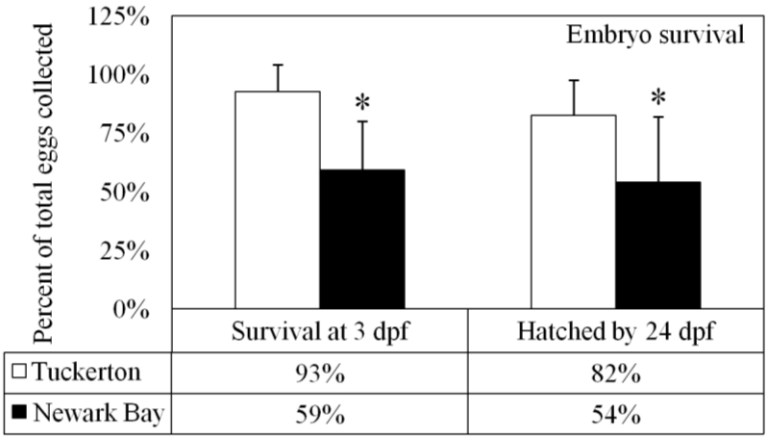


Fig. 3.5. (A) Total number of eggs stripped from each site on the peak of the lunar cycle and number of embryos to hatch by 24 dpf. (B) Percentage of total stripped–spawned eggs to survive 3 dpf and to hatch by 24 dpf. Data are presented as mean ± standard deviation. N = 20 females stripped per site. Eggs were fertilized with minced testis from 5 males from the respective site. *Significantly different from Tuckerton ($p \leq 0.05$).

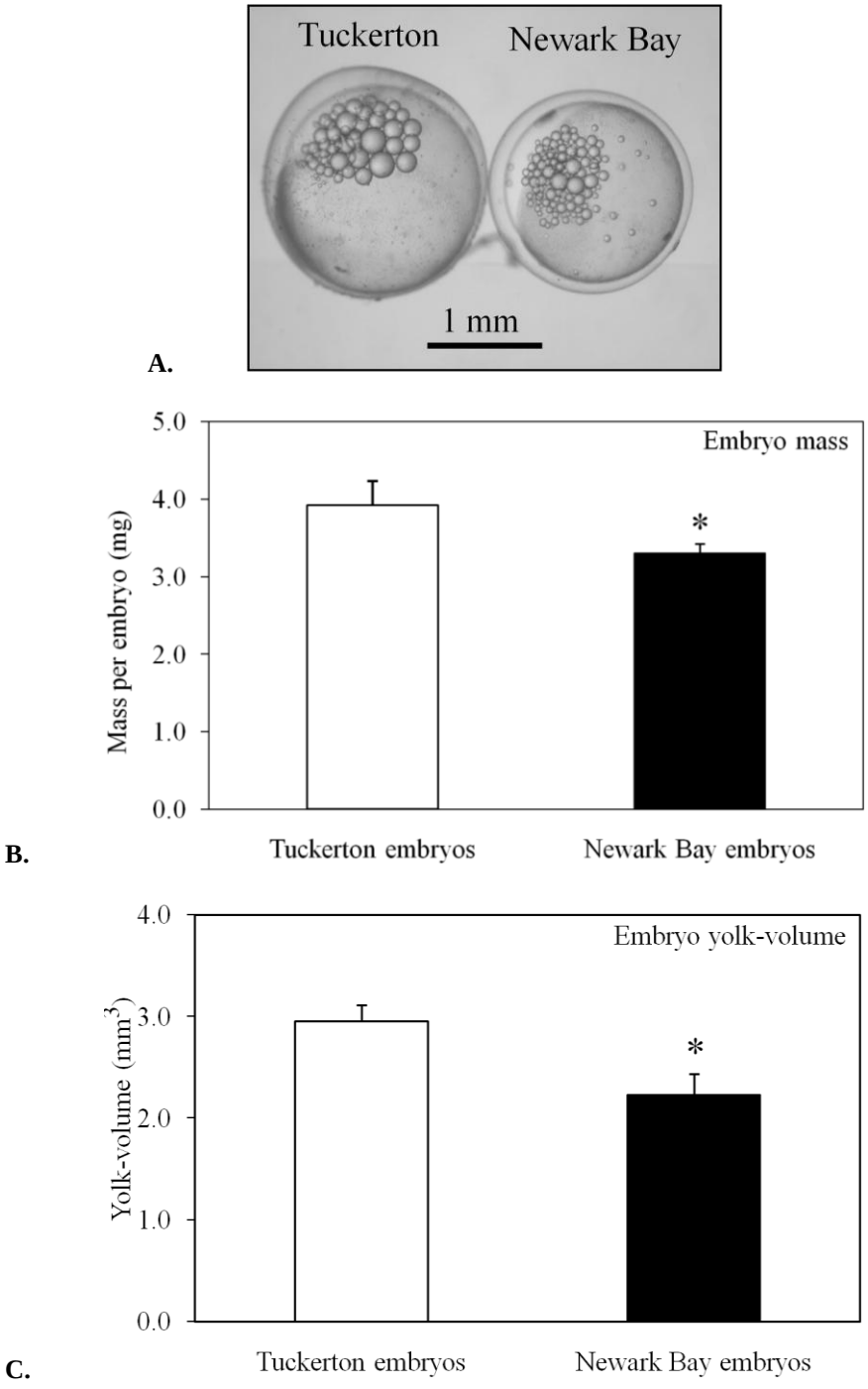


Fig. 3.6. (A) Representative photomicrograph of embryos taken side–by–side from each site at 24 hpf. (B) Embryo–mass and (C) yolk–volume of viable embryos at 24 hpf. Data are presented as mean ± standard deviation. N = 6 pools of 10 embryos for mass measurements (6 different females from each site). N = 21 for yolk–volume measurements (7 embryos from 3 different females from each site). *Significantly different from Tuckerton ($p \leq 0.05$).

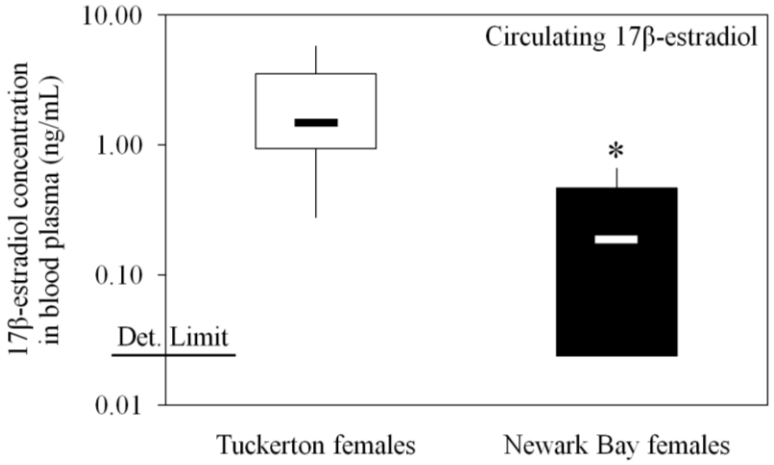
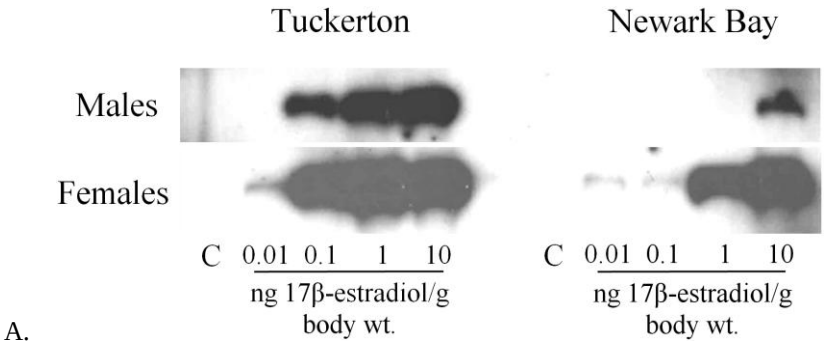
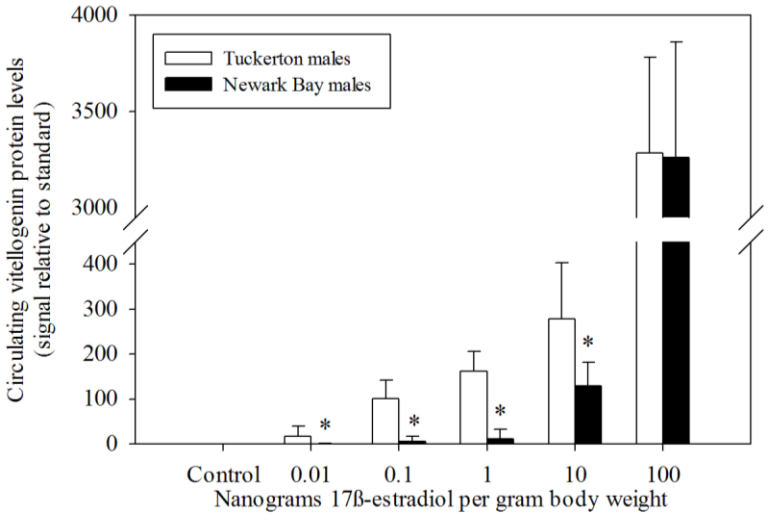


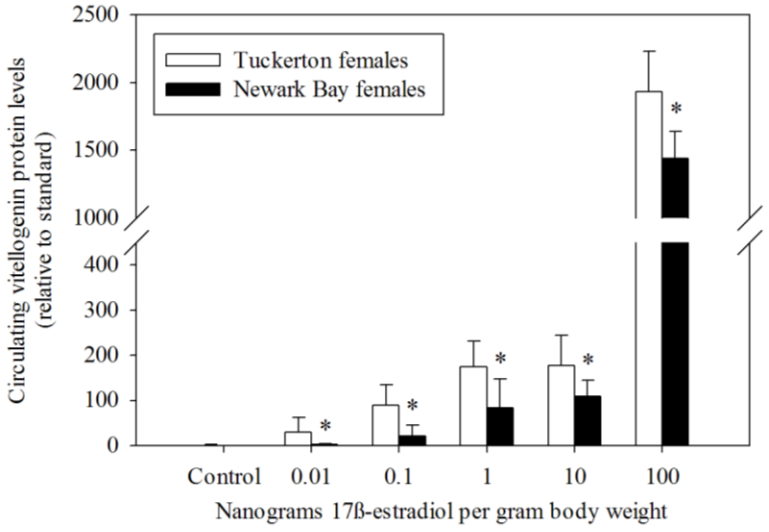
Fig. 3.7. Circulating 17β–estradiol levels in the blood plasma of female killifish 3 days prior to the peak of spawning. The box–and–whisker plot represents the minimum and maximum values and the median, lower and upper quartiles. The detection limit is shown on the axis (0.02 ng/mL). N = 10 pools of 5 individuals from each site. *Significantly different from Tuckerton ($p \leq 0.05$).



A.

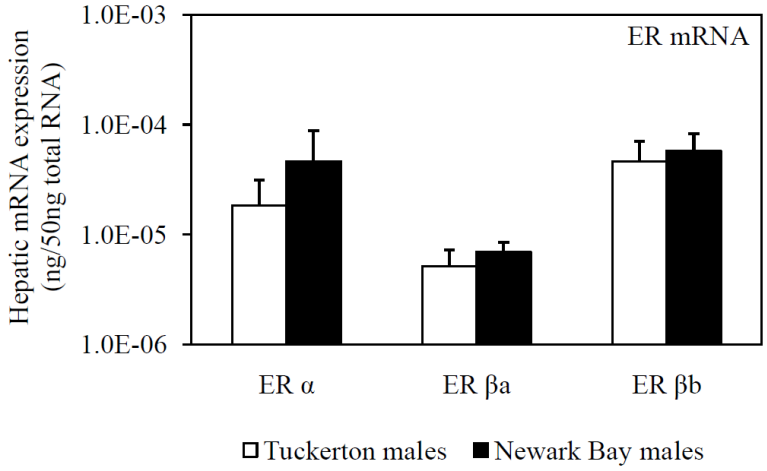


B.

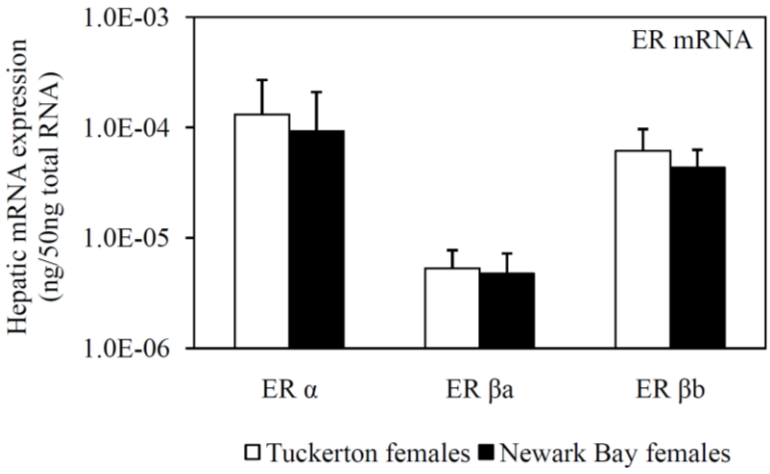


C.

Fig. 3.8. (A) Western blots for doses 0.01–10 ng 17β–estradiol/g body weight in males and females. Each sample is a composite of 3 individuals for each treatment group. Dose–response of vitellogenin protein induction by 17β–estradiol in reproductively inactive (B) males and (C) females from both Tuckerton and Newark Bay. N = 6 per treatment group. Data are reported as mean ± standard deviation. *Significantly different from the Tuckerton at $p \leq 0.05$ among each treatment group. All treatment groups were significantly higher than control groups (no vitellogenin detected).



A.



B.

Fig. 3.9. Day 0 (pre–injection) hepatic mRNA expression of three estrogen receptors (ER α, ER βa, ER βb) in reproductively inactive (A) male and (B) female killifish from both Tuckerton and Newark Bay. N = 6 per treatment group. Data are reported as mean ± standard deviation.

CHAPTER 4

Altered regulation of reproductive genes in a chemically impacted population of killifish (*Fundulus heteroclitus*) from Newark Bay, NJ

4.1. Introduction

Previous studies with the Atlantic killifish (*Fundulus heteroclitus*) from our laboratory have demonstrated that contaminants in Newark Bay (NJ, USA) down–regulate the vitellogenin (egg–yolk protein precursor) pathway in the liver and desensitize vitellogenin expression to induction by 17β–estradiol (Chapters 2 and 3). Impacts on vitellogenesis in Newark Bay killifish correlated with inhibition of yolk–development and reduced egg production. This was demonstrated to be caused by a deficiency of circulating 17β–estradiol and decreased expression of hepatic vitellogenin during spawning. Interestingly, male Newark Bay killifish do not express elevated levels of vitellogenin, a biomarker for exposure to xeno–estrogens, which are also known to be present within Newark Bay. Contrary to this, naïve Tuckerton male killifish transplanted into Newark Bay exhibited a high induction of vitellogenin after 1 month, which was transient in nature and down–regulated to basal levels after 2 months (Chapter 3). These studies demonstrated that prolonged exposure to contaminants in Newark Bay down–regulates the vitellogenin pathway. This is important considering a direct correlation between vitellogenin levels and fecundity (Miller et al., 2007; Thorpe et al., 2007). Therefore, contaminants that alter the gene regulation of critical reproductive genes (e.g. vitellogenin, choriogenin Hm, estrogen receptor α) will have consequences on a population's reproductive success and sustainability. The main purpose of the current

study was to determine contaminant effects on the gene regulation of estrogen–inducible genes (vitellogenin, choriogenin, estrogen receptor α).

The inhibition of vitellogenesis in fish exposed to AhR agonists can be caused by a variety of mechanisms (Fig. 1.7). Newark Bay is heavily contaminated with AhR agonists, which was shown to result in elevated levels of CYP1A mRNA and protein expression (Chapter 2). One possible way for AhR agonists to act as endocrine disruptors is by inducing hepatic cytochrome P450 enzymes that increase phase I and phase II metabolism of 17β–estradiol (Martucci et al., 1993; Zhu and Conney, 1998; Lee et al., 2001). Induction of cytochrome P450 enzymes can increase hydroxylation and/or conjugation of aromatic hydrocarbons and steroids such as 17β–estradiol. This can result in increased clearance of circulating 17β–estradiol through metabolism, and ultimately a decrease in the biological activity of estrogen. Vitellogenin is regulated by 17β–estradiol, therefore increased clearance rate of estrogen can have ramifications on vitellogenin induction and expression. The deficiency of circulating 17β–estradiol levels in Newark Bay females prior to spawning reported in Chapter 3 was hypothesized to be due to elevated hepatic clearance of 17β–estradiol.

Decreased vitellogenin expression and altered protein regulation explained the decreased embryo size and mass reported in Chapter 3. However, the relationship between effects on vitellogenin–dependent yolk–development and egg quality (i.e. embryo survival) is not clear. Yolk–proteins provide all the biomolecules necessary for growth and development of all embryonic fish from the single cell to eleutheroembryo (sac–fry) stages. Yolk–proteins are derived completely from vitellogenins 1 and 2 in killifish through a cathepsin mediated process (LaFleur et al., 2005). Therefore,

disruption of the cathepsin regulated derivation of yolk–proteins may impair the quality of eggs produced. I therefore hypothesized that eggs from Newark Bay females would have altered yolk–protein fingerprints due to impacts on the incorporation of vitellogenin and cathepsin processing.

Three studies presented here sought to investigate the altered regulation of vitellogenesis in the chemically impacted population of killifish in Newark Bay, relative to a reference population at Tuckerton, NJ. The first study evaluated the yolk–protein fingerprint in eggs from Tuckerton and Newark Bay killifish as a qualitative measurement of vitellogenin processing by cathepsins in mature eggs. The second study tested the hypothesis that estrogen responsive genes in killifish from Newark Bay have a decreased induction by 17β–estradiol. The genes of interest were vitellogenin 1 (VTG 1), vitellogenin 2 (VTG 2), choriogenin Hm (CHG Hm) and estrogen receptor α (ER α). Adult male killifish from Tuckerton and Newark Bay were challenged with 17β–estradiol to assess the sensitivity and responsiveness of VTG 1, VTG 2, CHG Hm and ER α in the liver. The third study tested the hypothesis that the down–regulation of estrogen–responsive genes was due to increased estrogen metabolism (clearance). Hepatic elimination of 17β–estradiol was evaluated in adult male and female killifish from Tuckerton and Newark Bay *in vitro* as a surrogate measure of the clearance ability of each population. Overall, these studies demonstrated that vitellogenin 1, vitellogenin 2 and choriogenin Hm are refractory (i.e. resistant) to induction at the mRNA level by 17β–estradiol in Newark Bay killifish. This was not explained by elevated 17β–estradiol clearance. Furthermore, the yolk–protein analysis does not indicate a functional deficit or impairment of vitellogenin processing by oocyte cathepsins.

4.2. Materials and methods

4.2.1. Site selection and necropsy and husbandry protocols

All animal husbandry and methods for collection of animals and tissue samples were approved by the Rutgers University Animal Rights Committee in accordance with AALAC accreditation and NIH guidelines (Protocol #08–025). Adult killifish were collected from two estuaries in New Jersey. The reference population was collected from the relatively pristine Tuckerton, NJ (Rutgers Marine Station, 39°30´32.52″N, 74°19´26.50″W). Killifish from the chemically impacted Newark Bay were collected from Richard Rutkowski Park (Bayonne, 40°41´17.0″N, 74°06´42.0″W). Fish were collected using stainless still minnow traps and immediately transported back to the lab in aerated water from the site. Killifish used for laboratory controlled studies were acclimated and maintained in laboratory conditions previously described (Bugel et al., 2011; Chapter 3). Fish to be sacrificed were euthanized with an overdose of MS–222 (tricaine methanesulphonate), weighed and measured for body length. The liver was removed, weighed, snap frozen in liquid nitrogen and stored at −80 °C until analysis.

4.2.2. Study 1: Yolk–protein (YP) assessment

To investigate site–specific differences in yolk–protein (YP) processing, mature eggs were collected from Tuckerton and Newark Bay killifish and the yolk–protein content in eggs of each population were compared using protein gel electrophoresis. Mature eggs were strip spawned and collected from six killifish from Tuckerton and Newark Bay (2–8 g, 5–9 cm). The collection was conducted on May 14, 2011, which was

3 days prior to the full moon (peak of spawning). For each female, twelve to fifteen eggs were homogenized and centrifuged at 14,000 × g for 10 minutes at 4 °C. The supernatant was diluted 1:100 in RIPA buffer and electrophoresed on a 4–12% bis–tris gel, transferred to a PVDF membrane, and stained using coomassie blue using methods previously described using equal volume of yolk (0.1 µL equivalent) per lane (Bugel et al., 2011; Chapter 2–3). The major yolk–proteins reported by LaFleur et al. (2005) were identified my size in eggs from both populations and qualitatively evaluated for presence and absence.

4.2.3. Study 2: Hepatic mRNA gene regulation of vitellogenin, choriogenin Hm and estrogen receptor α

To assess site–specific differences in hepatic mRNA regulation of various reproductive genes, killifish from Tuckerton and Newark Bay were injected intra–peritoneally with 17β–estradiol (≥ 98%, Sigma–Aldrich, St. Louis, MO) to induce gene expression. 17β–estradiol was dissolved and injected in corn oil (10 µL/g body weight). Adult male killifish (3–10 g) were collected from Tuckerton and Newark Bay outside of the breeding season (October, 2010) and acclimated to laboratory conditions for 1–2 weeks. Doses of 17β–estradiol used were 10 and 100 ng per gram body weight, which are doses that have been shown to result in significantly different induction levels of vitellogenin protein in Tuckerton and Newark Bay killifish (Bugel et al., 2011; Chapter 3). Treated killifish were sacrificed 4 days post–injection because circulating vitellogenin levels are maximally induced 4–8 days post–injection with 17β–estradiol (Pait and Nelson, 2003). The control group was a group of non–injected killifish sacrificed on Day

0. Hepatic mRNA expression of four reproductive genes were evaluated by quantitative polymerase chain reaction (qPCR): VTG 1, VTG 2, CHG Hm and ER α.

4.2.4. Analysis of mRNA expression by qPCR

Expression of hepatic mRNA was analyzed using qPCR. First, total RNA was isolated from liver tissue using TRIzol® (Invitrogen, San Diego, CA), treated with DNA–free (DNA–free, Ambion, Austin, TX), and reverse transcribed using a High Capacity cDNA Reverse Transcript Kit (Applied Biosystems, Foster City, CA). qPCR was performed on 50 ng of cDNA using Bio–Rad iQ SYBR Green Supermix with a Bio–Rad iCycler and iCycler iQ Detection System (Bio–Rad, Hercules, CA). Samples were analyzed in triplicate for each gene and normalized to the population's median β–actin value. After normalization, expression was quantified using a standard curve to calculate the nanogram amount of gene template per 50 ng RNA. Primers used to quantitate β–actin mRNA expression are the same as those used previously by Bugel et al. (2010) and Chapter 2. Primers used for qPCR analysis of vitellogenin 1 (VTG 1) and estrogen receptor α (ER α) are the same as those developed by Bugel et al. (2011) and Chapter 3. New primer sets were designed for vitellogenin 2 and choriogenin Hm using criteria described previously (Bugel et al., 2010; Chapter 2–3). Vitellogenin 2 (VTG 2, GenBank U70826) primers were F: 5´–CAA GCA GTA CAA CAC CAC–3´ and R: 5´–GAT GTA AGT AGG GAG TCT GG–3´ (100 bp product). Choriogenin Hm (CHG Hm, GenBank AB533329) primers were: F: 5´–ATA CAC TGT GAT GCT GCT GTG TGC–3´ and R: 5´–CCT TGC TGC TAA CAA TGG TGG CTT–3´ (136 bp product).

4.2.5. Study 3: Hepatic 17β–estradiol metabolism analysis

Metabolism of 17β–estradiol was evaluated *in vitro* as a surrogate measure of clearance in killifish collected from Tuckerton and Newark Bay. Liver was collected from adult male and female killifish (2–8 g, 5–9 cm) collected from Tuckerton and Newark Bay on May 14, 2011 (N = 8 per group). This collection was 3 days prior to the peak of a full moon, which is the peak level of circulating 17β–estradiol during the reproductive cycle of killifish (Cerdá et al., 1996).

Livers were thawed and homogenized on ice in Assay Buffer (100 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, 250 mM sucrose, pH 7.6). Homogenates were centrifuged at 9,000 × g for 20 minutes at 4 ºC to obtain the S9 fraction (supernatant). The protein concentration of the S9 fraction was quantified by the modified Lowry protein assay (Pierce Biotechnology) and stored at –80 ºC until analysis. The 17β–estradiol reduction activity was assessed in the S9 fraction by measuring the quantity of 17β–estradiol lost during the *in vitro* assay. Assay Buffer was used to prepare all reagents. Each assay contained 100 µg of protein, 5 µM 17β–estradiol, and approximately 100 mM NADPH (Promega NADPH Regeneration System) in a 0.2 mL volume. Prior to initiating the reaction with NADPH, 1 µL of the assay was diluted and stored on ice for analysis to obtain the initial concentration of 17β–estradiol. The reaction was initiated by the addition of NADPH and incubated at 30 ºC for 1.5 hours. At the end of the incubation, the assays were chilled on ice (to slow reaction) and 1 µL of the assay was diluted for analysis to obtain the final concentration of 17β–estradiol). Samples to be analyzed were diluted 1:1500 and the concentration of 17β–estradiol was measured using Coat–A–Count Estradiol Radioimmunoassay (Siemens Medical Solutions Diagnostics, Los

Angeles, CA). Hepatic enzyme activity was calculated as pmol 17β–estradiol lost per microgram of protein per minute.

4.2.6. Statistical analyses

Statistical tests were performed using SigmaPlot™ (v. 11.0). Data was compared for each treatment between sites, and between control and treatment within each site. Data is reported as mean ± standard deviation. Fold–changes were calculated using median values. Unpaired t–tests were used to compare different treatments. When normality failed the Student–Neuman–Keuhls test was used. When equal variance failed the Mann–Whitney rank sum test we used. A p–value ≤ 0.05 was regarded as significantly different.

4.3. Results

4.3.1. Study 1: Yolk–protein analysis

Yolk–proteins from mature eggs from Tuckerton and Newark Bay females were separated and identified using protein electrophoresis (Fig. 4.1). The major yolk–proteins, which are derived from vitellogenin 1 and 2, were identified in mature eggs from females of both populations. No overt differences in yolk–protein processing were identified. All the major yolk–proteins were present in both populations and with no qualitative differences in band density for any of the yolk–proteins.

4.3.2. Study 2: mRNA inducibility of vitellogenin 1, vitellogenin 2, choriogenin Hm and ER α

To explore population differences in the regulation of various reproductively relevant genes (VTG 1, VTG 2, CHG Hm and ER α), the mRNA inducibility of each gene by 17β–estradiol was evaluated using adult males from Tuckerton and Newark Bay. Males were used because of their low levels of endogenous estrogen, and the low basal expression associated with these genes. There were no significant differences between populations for basal mRNA expression levels measured in the control group for any of the four genes tested (Fig. 4.2–4.4). VTG 1, VTG 2 and CHG Hm were significantly inducible in both populations by 17β–estradiol, although ER α was not (Fig. 4.2–4.4). For the genes that were inducible by 17β–estradiol, there were statistical differences between induced mRNA expression levels measured in Tuckerton and Newark Bay killifish. For vitellogenin 1, at doses of 10 ng/g and 100 ng/g 17β–estradiol Newark Bay killifish had expression levels that were 11–fold and 5–fold lower than those in Tuckerton killifish (Fig 4.2). For vitellogenin 2, at doses of 10 ng/g and 100 ng/g 17β–estradiol expression levels in Newark Bay killifish were 8–fold and 4–fold significantly lower than levels in Tuckerton killifish (Fig. 4.2). In general, vitellogenin 1 was more sensitive to induction by 17β–estradiol than vitellogenin 2, in the Tuckerton population. For Choriogenin Hm, Newark Bay killifish had a 5–fold lower expression level than those in Tuckerton when dosed with 10 ng/g 17β–estradiol, although no statistical differences were found between sites for the 100 ng/g dose (Fig. 4.3). Overall, relative to each population's respective control levels, the fold–induction of vitellogenin 1, vitellogenin 2 and choriogenin Hm

over control levels was much higher than the fold–induction measured in Newark Bay killifish (Table 4.2).

4.3.3. Study 3: In vitro clearance of 17β–estradiol by S9 liver homogenates

To determine if hepatic 17β–estradiol metabolism contributes to the down–regulation of estrogen–responsive genes in Newark Bay killifish, the 17β–estradiol elimination activity was evaluated in liver homogenates *in vitro*. There were no significant differences between Tuckerton and Newark Bay killifish for either gender, and between genders (Fig. 4.5).

4.4. Discussion

Exposure to complex mixtures of contaminants in Newark Bay has previously been shown to result in the down–regulation of the vitellogenin pathway in killifish (Chapters 2 and 3). Current studies demonstrated that chronic exposure to complex mixtures of contaminants in Newark Bay results in the down–regulation (i.e. inhibition) of vitellogenin (VTG 1 and 2) and choriogenin (CHG Hm). These refractory genes were less inducible by 17β–estradiol in Newark Bay killifish at the mRNA level, indicating contaminant disruption of the estrogen receptor signaling pathway. *In vitro* hepatic metabolism studies demonstrated that Newark Bay killifish do not exhibit increased clearance of 17β–estradiol. Therefore, the attenuated mRNA induction of vitellogenin and choriogenin cannot be explained by differences in 17β–estradiol metabolism. In addition, I demonstrated that the yolk–protein fingerprints in Newark Bay eggs were qualitatively identical to those in Tuckerton eggs, which indicated normal cathepsin

mediated vitellogenin processing in developing oocytes from the Newark Bay population. Decreased vitellogenin expression and subsequent effects on egg development in Newark Bay killifish were demonstrated by these studies to be the result of a refractive phenotype, in which mRNA induction of vitellogenin genes was lower than normal due to a decreased sensitivity to 17β–estradiol. Our studies presented here further established a link between chemical impacts on vitellogenin gene regulation and the inhibition of egg development which reduced the reproductive success of this population.

4.4.1. Evaluation of cathepsin mediated yolk–protein formation

Decreased embryo–mass and yolk–volume in Newark Bay embryos were two previous observations that correlated with decreased survival during the first 3 days of embryonic development (Chapter 3). I had previously proposed that the decreased egg quality in the Newark Bay population was the result of contaminant effects on yolk–development caused by reduced vitellogenin availability during oocyte growth. However, this did not fully explain the decreased survival during early embryo development in this population. Alternatively, I hypothesized that decreased egg quality was due to disruption of yolk–protein formation, and that inhibition of cathepsin mediated yolk formation would be evident in the yolk–protein fingerprint. Vitellogenin processing in oocytes and the genesis of yolk–proteins are mediated by oocyte cathepsin activity (LaFleur et al., 2005). Cathepsins have been proposed to be a biomarker for embryonic toxicity because of the potential for chemical disruption of cathepsin regulation and function (Gündel et al., 2007; Maradonna et al., 2007). However, the data shows that the yolk–protein fingerprints of Tuckerton and Newark Bay eggs were qualitatively identical (Fig. 4.1).

Each yolk–protein in killifish eggs that was identified by LaFleur et al. (2005) was present in Bay oocytes indicating no major impacts on the cathepsin mediated process of yolk–protein formation from vitellogenins 1 and 2. Due to this, inhibition on yolk–protein formation and cathepsin activity are not suspected to contribute to the mortality observed in the Newark Bay population. Decreased survival is therefore most likely due to chemical burdens disrupting embryonic development. For example, studies have demonstrated that 2,3,7,8–TCDD can be maternally transferred in zebrafish through association with vitellogenin to result in embryonic developmental toxicity and decreased survival (Monteverdi and Di Giulio, 2000a,b; King Heiden et al., 2005). 2,3,7,8–TCDD and many other potent dioxin congeners (1,2,3,7,8–PeCDD) are present in Newark Bay (Rappe et al., 1991; Fernandez et al., 2004; Dimou and Pecchioli, 2006; Muñoz et al., 2006) and maternal transfer was likely to be the basis for decreased embryo survival in the Newark Bay population.

The presence of YP 122 indicates that the developing oocyte underwent the final steps of maturation (meiosis II) and hydration, which is stimulated by the maturation inducing steroid (LaFleur 2005). All samples from Newark Bay had YP 122 present indicating that the eggs analyzed from both sites were fully mature oocytes (Fig. 4.1). Overall, the yolk–protein content (YP–density) of Tuckerton and Newark Bay oocytes looks indistinguishable with no major differences between populations (Fig. 4.1). This is important to determining why eggs are smaller at Newark Bay (Chapter 3). I had previously argued that decreased vitellogenin uptake was the etiology for the decreased embryo–mass and yolk–volume. Alternatively, differences in embryo size and mass could have been equally due to decreased oocyte hydration (water content) during the

final stages of maturation of the eggs. The YP–content per unit volume was identical for all observed yolk proteins in eggs from both populations. Therefore, the differences in embryo–size and yolk–volume between the populations were truly due to differences in vitellogenin–dependent growth (vitellogenin uptake) and not hydration.

4.4.2. Chemical impacts on the gene regulation of vitellogenin, choriogenin and ER α mRNA in the liver

Studies have previously shown that female Newark Bay killifish have reduced expression levels of vitellogenin mRNA and protein during reproduction (Chapter 2). In addition, males from Newark Bay are exposed to xeno–estrogens and do not express vitellogenin (Chapter 2). Newark Bay is both an estrogenic and anti–estrogenic environment because naïve male killifish transplanted into Newark Bay exhibited transient induction of vitellogenin which are attenuated over time (Chapter 3). In addition, adult male and female killifish from Newark Bay exhibited a refractory induction of circulating vitellogenin protein levels by a 17β–estradiol challenge relative to killifish from the reference site at Tuckerton (Chapter 3). The current studies show that this refractory activation of the vitellogenin pathway is due to the altered gene regulation of vitellogenins 1 and 2 in the liver (Fig. 4.2). Relative to Tuckerton killifish, male killifish from Newark Bay had a shifted dose–response for vitellogenin 1 and 2 mRNA induction (less inducible) over their respective controls at reproductively relevant doses of 17β–estradiol (Table 4.1). The inhibition of vitellogenin protein induction is due to altered mRNA induction of both vitellogenin isoforms in Newark Bay killifish. This is further support for the development of a suppressed vitellogenin pathway in the Newark

Bay killifish population due to chronic exposure to complex mixtures of contaminants. This refractory vitellogenin pathway is correlated with decreased egg production and reproductive success of the population. I propose that the reduction of fecundity and inhibition of oocyte development were due to reduced vitellogenin expression levels caused by the inhibition of mRNA induction of vitellogenins 1 and 2 by 17β–estradiol. These studies illustrate how impacts on the hepatic gene regulation of reproductively vital genes can affect egg development and reproductive success of a population.

In addition to vitellogenin, other reproductively relevant genes known to be sensitive to 17β–estradiol were evaluated (choriogenin Hm, ER α). The purpose of this evaluation was to investigate whether the refractory induction of vitellogenin is gene–specific or is representative of a global down–regulation of estrogen–responsive genes. Hepatic choriogenin Hm is one of the choriogenin genes involved in chorion development in oocytes and is inducible by 17β–estradiol (Urushitani et al., 2003). Like vitellogenins 1 and 2, hepatic Choriogenin Hm mRNA was also refractory to induction with physiologically relevant doses of 17β–estradiol (Fig. 4.3, Table 4.1). However, the sensitivity of hepatic ER α mRNA to 17β–estradiol was indeterminate due to the lack of induction using our doses of 17β–estradiol (Fig. 4.4). Studies by Greytak et al., (2010) and Urushitani et al. (2003) have shown that ER α is inducible by 17β–estradiol although this has only been tested at concentrations (5–10 µg/g body weight) that would result in a body burden much greater than what would be estimated from reproductively and environmentally relevant (nanogram levels per mL plasma). In our studies, ER α was not inducible by the concentrations used (10 and 100 ng/g). ER α expression levels measured in the control group support results reported previously in Chapter 3 that Newark Bay

killifish do not have altered ER α levels. This reiterates the idea that the refractory induction of vitellogenin and choriogenin Hm mRNA in the liver is not due to altered estrogen receptor levels. Overall, these studies demonstrate that multiple estrogen–responsive genes are refractory to induction, and indicate a global down–regulation of estrogen receptor regulated genes. In addition, it is difficult to determine the importance of a refractory choriogenin response in Newark Bay killifish because previous studies had not investigated impacts on chorion formation or choriogenin gene expression. However, the dose–response studies reported suggest that choriogenin expression levels may also be down–regulated in Newark Bay killifish and may correlate with effects on chorion development (i.e. thinner chorionic membrane). Future studies are needed to explore this possibility.

Hepatic activity of 17β–estradiol metabolism (elimination) was evaluated in Tuckerton and Newark Bay killifish to explore the possibility that increased metabolism is the etiology for reduced 17β–estradiol levels during spawning and the refractory vitellogenin and choriogenin responses. Induction of genes involved in 17β–estradiol metabolism is one of the six possible mechanisms for AhR–ER cross–talk (Fig. 1.7). Previously, studies conducted in our laboratory have demonstrated that Newark Bay killifish have elevated levels of CYP1A mRNA, protein and activity (Chapter 2). The CYP1A biomarker indicates exposure to AhR agonists and elevated AhR activity. Therefore, induction of cytochrome P450 enzymes in the liver that are involved in 17β–estradiol metabolism was hypothesized to contribute to the decreased sensitivity of vitellogenin and choriogenin gene–induction. However, I demonstrated that male and female killifish from Newark Bay exhibit normal 17β–estradiol metabolism activity in

the liver, relative to levels measured in Tuckerton killifish (Fig. 4.5). Further studies are necessary to investigate the other possible AhR–ER interactions that may be resulting in the down–regulation of estrogen–responsive gene induction (vitellogenin and choriogenin).

4.5. Conclusions

I propose that the down–regulation of vitellogenin pathway in Newark Bay killifish is due to altered gene regulation at the mRNA level. The refractory hepatic induction of vitellogenins 1 and 2 by 17β–estradiol in Newark Bay killifish is believed to be the etiology for a variety of effects in this population reported in Chapters 2 and 3, including (1) the inhibition of oocyte development and reduced egg production, (2) decreased expression levels of vitellogenin during spawning, (3) the decreased inducibility of vitellogenin protein and (4) the transient induction and down–regulation of vitellogenin over time in naïve Tuckerton male killifish transplanted into Newark Bay. These studies demonstrate that chemical effects on the gene regulation of hepatic proteins involved in oocyte production correlated with impacts on the reproductive success of a population, which therefore threatens population sustainability.

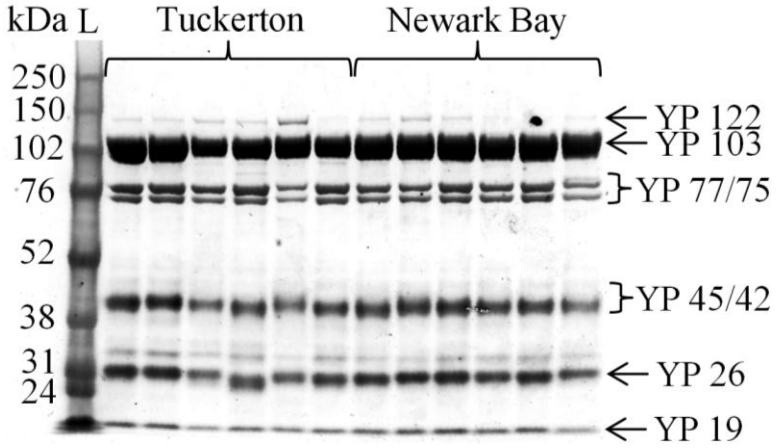
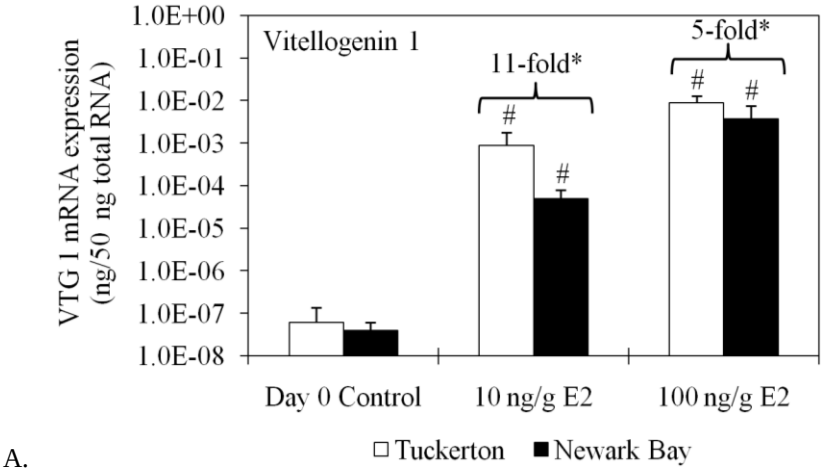
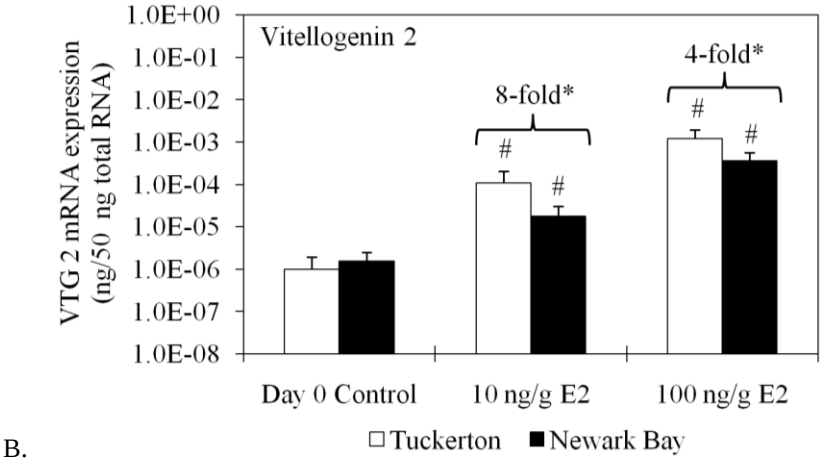


Fig. 4.1. Yolk–protein (YP) analysis in mature eggs collected from Tuckerton and Newark Bay females directly prior to a peak in spawning (full moon, May 17, 2011). Twelve to fifteen embryos were homogenized together and analyzed per female, N = 6 per site females per site. The ladder (L) is shown and was used to identify the major killifish YPs identified by LaFleur et al. (2005). Each yolk–protein is named according to its previously estimated molecular weight.



A.



B.

Fig. 4.2. Induction of hepatic (A) vitellogenin 1 and (B) vitellogenin 2 mRNA expression by 17β–estradiol in adult male killifish collected from Tuckerton and Newark Bay. Killifish were collected in October, 2010. N = 7 per treatment group. Data are reported as mean ± standard deviation. *Significantly different between Tuckerton and Newark Bay at $p \leq 0.05$ for respective treatment group. Fold–difference between mRNA expression in Tuckerton and Newark Bay are shown for treatment groups with significant differences. Fold–differences were calculated using median values. #Significantly different from respective Day 0 control group. Fold–inductions for each treatment over the population's respective control are shown in Table 4.1.

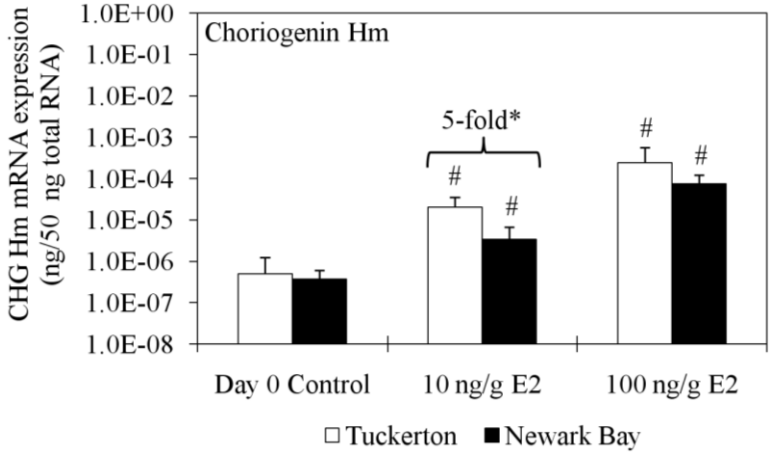


Fig. 4.3. Response of choriogenin Hm mRNA expression to 17β–estradiol in adult male killifish collected from Tuckerton and Newark Bay. Killifish were collected in October, 2010. N = 7 per treatment group. Data are reported as mean ± standard deviation. *Significantly different between Tuckerton and Newark Bay at $p \leq 0.05$ for respective treatment group. Fold–difference between mRNA expression in Tuckerton and Newark Bay are shown for treatment groups with significant differences. Fold–differences were calculated using median values. #Significantly different from respective Day 0 control group. Fold–inductions for each treatment over the population's respective control are shown in Table 4.1.

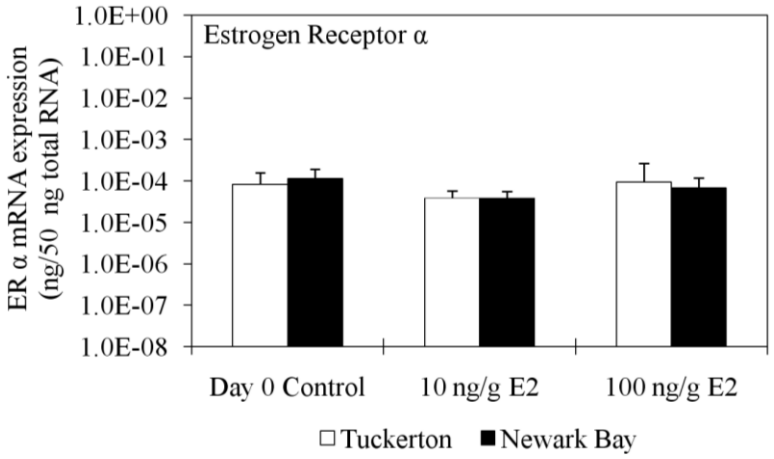


Fig. 4.4. Response of estrogen receptor α mRNA expression to 17β–estradiol in adult male killifish collected from Tuckerton and Newark Bay. Killifish were collected in October, 2010. N = 7 per treatment group. Data are reported as mean ± standard deviation. *Significantly different between Tuckerton and Newark Bay at $p \leq 0.05$ for respective treatment group. Fold–difference between mRNA expression in Tuckerton and Newark Bay are shown for treatment groups with significant differences. Fold–differences were calculated using median values. #Significantly different from respective Day 0 control group. Fold–inductions for each treatment over the population's respective control are shown in Table 4.1.

	17β-Estradiol Dose	
	10 ng/g	100 ng/g
Vitellogenin 1		
Tuckerton	26641[#]	379623[#]
Newark Bay	1593[*,#]	51782[*,#]
Fold difference:	17	7
Vitellogenin 2		
Tuckerton	173[#]	1963[#]
Newark Bay	8[*,#]	185[*,#]
Fold difference:	21	11
Choriogenin Hm		
Tuckerton	159[#]	1459[#]
Newark Bay	12[*,#]	184[#]
Fold difference:	14	8
ER α		
Tuckerton	0.2	0.3
Newark Bay	0.2	0.7
Fold difference:	1.2	0.4

Table 4.1. Fold–inductions levels for hepatic mRNA expression of each gene over the population's respective control group for experiments in Figs. 4.1–4.3. Fold–induction was calculated using median values. Fold–differences between fold–induction levels over control are also shown. [*]Significantly different mRNA expression level between Tuckerton and Newark Bay at $p \leq 0.05$ for respective treatment group. [#]Significantly different from the respective population's Day 0 control group.

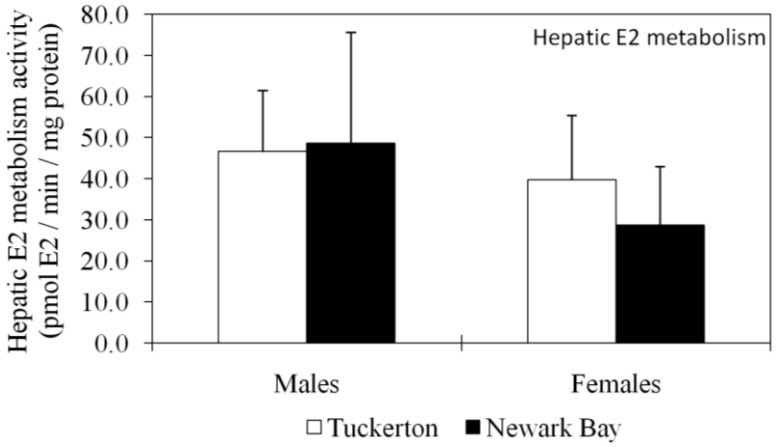


Fig. 4.5. Hepatic activity of 17β–estradiol (E2) clearance *in vitro* in liver S9 fractions from Tuckerton and Newark Bay killifish. Killifish were collected May 15, 2011, 3 days prior to the peak of a full moon and the peak of circulating 17β–estradiol during the reproductive cycle of killifish (Cerdá et al., 1996). N = 8 per treatment group. Data are reported as mean ± standard deviation. No significant differences were found ($p \leq 0.05$).

CHAPTER 5

Role of the aryl hydrocarbon receptor 2 (AhR2) in the inhibition of vitellogenesis by dioxin in zebrafish (*Danio rerio*)

5.1. Introduction

Previous studies demonstrated that killifish from the heavily contaminated Newark Bay (NJ, USA) exhibited decreased vitellogenin production, reduced egg production and decreased growth of oocytes at vitellogenin–dependent stages (Chapters 2 and 3). Newark Bay killifish had a decreased inducibility of vitellogenin at the protein and mRNA level with physiologically relevant doses of 17β–estradiol, which indicated a disruption of gene regulation (Chapters 3 and 4). Newark Bay is heavily contaminated by AhR agonists (e.g. PCDDs, PCDFs, PAHs, PCBs) and Newark Bay killifish have elevated CYP1A levels, which are biomarkers for AhR activation (Chapter 2). I have also reported an inverse correlation between CYP1A and vitellogenin expression, implicating a role of AhR activation in the down–regulation of the vitellogenin pathway through estrogen receptor (ER) cross–talk (Chapter 3). The hypothesis tested by the current study was that activation of the AhR2 by PCDDs inhibits the up–regulation of vitellogenesis in zebrafish (*Danio rerio*).

Zebrafish chronically exposed to 2,3,7,8–tetrachlorodibenzo–*p*–dioxin (2,3,7,8–TCDD) exhibit an inhibition of follicular development, and decreased levels of gonadotropins, 17β–estradiol and vitellogenin (King Heiden et al., 2006; King Heiden et al., 2008; King Heiden, 2009). This was thought to be due primarily to toxicity in the ovary, although changes in liver morphology suggested possible effects on hepatic vitellogenin production. Studies with teleost hepatocytes *in vitro* have also shown that AhR agonists can result in the inhibition of vitellogenin induction by 17β–estradiol

(Anderson et al., 1996a; Navas and Segner, 2000; Bemanian et al., 2004; Mortensen and Arukwe, 2007; Gräns et al., 2010). Bemanian et al. (2004) demonstrated that an AhR–inhibitor can protect against the inhibition effect of 2,3,7,8–TCDD on vitellogenesis *in vitro*. These studies suggest that impacts on the down–regulation of vitellogenin can occur locally in the liver through AhR activation, regardless of toxicity in the ovary.

Zebrafish can be used to study AhR–ER interactions *in vivo* to gain mechanistic insight into AhR impacts on vitellogenin gene–regulation. In vertebrates, the function of the AhR is considered highly conserved (Hahn, 2002). Unlike higher vertebrates, teleosts have multiple isoforms of the aryl hydrocarbon receptor. For example, zebrafish have 3 isoforms: AhR 1a, 1b and 2. In zebrafish, AhR2 and ARNT 1 have been shown to mediate CYP1A induction and developmental toxicity of PCDDs, although the mechanism by which the AhR2 induces developmental toxicity is not clear (Prasch et al., 2003; Prasch et al., 2004; Dong et al., 2004; Prasch et al., 2006). I used zebrafish to study the interaction of the AhR2 pathway with the estrogen receptor mediated regulation of vitellogenin. The AhR and ER pathways are both active in developing embryonic zebrafish, demonstrated by CYP1A and vitellogenin induction, respectively. Zebrafish have seven vitellogenin genes that are expressed virtually exclusively in the liver, and three of these genes were chosen for analysis to represent the three structural classes of vitellogenin genes (Wang et al., 2000; Tong, 2004; Wang et al., 2005). Vitellogenin 1 (and vitellogenins 4–7) contains all three domains (lipovitellin I, phosvitin, and lipovitellin II) but lacks the C–terminal half of lipovitellin II. Vitellogenin 2 contains all three domains intact and vitellogenin 3 lacks phosvitin and the C–terminal half of lipovitellin II. Conducting studies in zebrafish embryos also allows for the transient gene

knock–down of the AhR2 to investigate AhR–ER cross–talk interactions and effects on the regulation of all three major classes of vitellogenins. Because of this, morpholino anti–sense technology was used to investigate the role of the AhR 2 in cross–talk inhibition with the estrogen receptor mediated induction of vitellogenins 1, 2 and 3 (Fig. 5.1). In zebrafish, anti–sense technologies such as morpholino oligonucleotides allow for the transient knock–down of specific gene targets *in vivo* (Nasevicius and Ekker, 2000). This molecular tool is useful in probing the function of specific genes by knocking down a pathway in developmental and toxicological studies. Morpholino oligonucleotides are similar in structure to DNA, but differ by having a morpholine ring instead of the ribose in the backbone. This structural difference allows morpholino oligonucleotides to remain resilient to intracellular degradation while interacting with homologous DNA sequences. Morpholino oligonucleotides can decrease expression of functional proteins by blocking translation, transcription, or splicing of specific genes, depending on the type of morpholino used. In the current studies, a morpholino was designed specifically to block normal splicing of the AhR2 gene (Fig. 5.1).

The studies presented within this paper investigated cross–talk between the AhR and ER pathways in zebrafish *in vivo*, as it relates to the regulation of vitellogenin 1, 2 and 3. I demonstrated that exposure to 2,3,7,8–tetrachlorodibenzo–*p*–dioxin and 1,2,3,7,8–pentachlorodibenzo–*p*–dioxin (1,2,3,7,8–PeCDD), but not dibenzo–*p*–dioxin (DD), inhibits induction of vitellogenin 1, 2 and 3 by 17α–ethynylestradiol (EE2). A morpholino gene knockdown approach was used to investigate the role of the AhR2 in the 2,3,7,8–TCDD inhibition of vitellogenin induction by EE2. These studies demonstrate

that AhR cross–talk inhibition of estrogen receptor vitellogenin regulation is mediated by the AhR2.

5.2. Materials and methods

5.2.1. Chemicals

Dimethyl sulfoxide (>99.9%, DMSO) was obtained from Fisher Scientific (Hampton, NH). 2,3,7,8–tetrachlorodibenzo–*p*–dioxin (>99.9%) and dibenzo–*p*–dioxin (> 99%) were obtained from ULTRA Scientific (N. Kingstown, RI). 1,2,3,7,8–pentachlorodibenzo–*p*–dioxin (>99.9%) was obtained from Accustandard, Inc. (New Haven, CT). 17α–ethynylestradiol (>98%) was obtained from Sigma–Aldrich Corporation (St. Louis, MO).

5.2.2. Zebrafish husbandry

All animal husbandry and methods for collection of animals and tissue samples were approved by the Rutgers University Animal Rights Committee in accordance with AALAC accreditation and NIH guidelines (Protocol #08–025). Zebrafish were maintained in a re–circulating Aquatic Habitats (Apopka, FL) Stand–Alone System (80 gallon total, 10% water change daily, 25–27 °C) and maintained with a 14:10 light:dark photoperiod. Breeding adults were fed brine shrimp (*Artemia salina*) and a mixture of Aquatox Protein Flakes and TetraMin Rich Mix Flakes (4:1, respectively). Tap water fed into the system was filtered through sand and activated carbon filters, and was

continually re–circulated through coarse–particle, UV, activated carbon and bio–filters. Zebrafish embryos were raised in egg water (60 µg/mL Instant Ocean) at 28.5 °C.

5.2.3. Dioxin and 17α–ethynylestradiol exposure protocol for induction and inhibition studies

To investigate the potential for dioxins to inhibit vitellogenesis, zebrafish embryos (AB strain) were treated with either (1) 0.1% DMSO as a solvent control, (2) dioxin, (3) 17α–ethynylestradiol, or (4) a combination of dioxin and 17α–ethynylestradiol treatments. Embryos treated with 17α–ethynylestradiol were exposed from 6 hpf to 4 days post fertilization to 1000 pptr EE2 (pg/mL, 1 embryo/mL, 3.37 nM) with daily renewal. This exposure protocol has been previously demonstrated to result in a significant induction of vitellogenin (Muncke and Eggen, 2006). Embryos treated with dioxin were exposed as a group for 1 hour starting at 4 hours post fertilization to 50 or 400 pptr dioxin (parts–per–trillion, pg/mL, 10 embryos per mL). Dioxins used included 2,3,7,8–TCDD, 1,2,3,7,8–PCDD and dibenzo–*p*–dioxin. Doses of each dioxin were: 50 and 400 pptr 2,3,7,8–TCDD (0.16 and 1.24 nM), 50 and 400 pptr 1,2,3,7,8–PCDD (0.14 and 1.40 nM), and 400 pptr dibenzo–*p*–dioxin (2.17 nM). The dioxin exposure protocol was adapted from studies by Prasch et al. (2003), which previously demonstrated that a 1 hour exposure to 400 pptr 2,3,7,8–TCDD resulted in pericardial edema in >95% of animals treated and a dose–dependent induction of cytochrome P450 1A. Dibenzo–*p*–dioxin is known to not be an agonist for the AhR, and thus served as a negative control for AhR activation. Quantitative PCR was used to measure mRNA expression of cytochrome P4501A (CYP1A) and vitellogenins 1, 2 and 3 (VTG 1, VTG 2, and VTG 3)

in 4 day old embryos. For each experiment, embryos were also observed daily for mortality, and developmental toxicity (pericardial edema, yolk–sac edema and hemorrhages).

5.2.4. Microinjection and morpholino rescue

To determine the role of the AhR2 in dioxin inhibition of vitellogenesis, a splice–blocking morpholino was designed against the zebrafish AhR2 Exon 2–Intron 2 (E2I2) junction to knock–down expression of the AhR2 gene. Morpholino (MO) oligonucleotides were designed and obtained from Gene Tools, LLC (Philomath, OR). This morpholino interferes with normal splicing to result in an mRNA variant missing Exon 2, which also frame–shifts the sequence downstream of Exon 1. The standard Gene Tools control morpholino (C–MO) used was: 5′–CCT CTT ACC TCA GTT ACA ATT TAT A–3′. The AhR2 morpholino sequence was: 5′–AAT AAT CTC ACC ATT GAA GAA GCT C–3′. PCR primers were also designed to flank Exon 2 in AhR2 (Exon 1 and Exon 4) and were used to verify the presence of the splice variant in morpholino injected embryos (an amplicon missing the 188 bp Exon 2). Morpholinos were tagged with fluoroscein to visually confirm incorporation of morpholinos into the developing embryo.

Morpholinos were reconstituted and diluted to 250 µM for injection in 1X Danieau's solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5mM N–2–hydroxyethylpiperazine–N^1–2–ethane sulfonic acid, pH 7.6) (Nasevicius and Ekker, 2000). Prior to injection, morpholinos were heated to 55 °C for 10 minutes. Microinjection was performed using a General Valve Corporation Picospritzer II. Embryos (1–2 cell stage) were injected with 4.2 nL (1.1 pmol, 9.4 ng) of morpholino into

the yolk near the cell mass as previously described (Nasevicius and Ekker, 2000). Damaged or dead embryos were discarded. At 3 hours post fertilization viable embryos were screened for homogenous morpholino uptake into the cell mass using an Olympus IX51 inverted microscope equipped with an X–Cite 120 Fluorescence Illumination System.

5.2.5. RNA isolation and expression analysis by quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from groups of embryos using TRIzol® (Invitrogen, San Diego, CA). RNA was quantified using the absorbance at 260 nm. Ten micrograms of total RNA were treated with DNA–free (DNA–free, Ambion, Austin, TX), and reverse transcription was performed on 1 µg of DNA–free treated RNA using a High Capacity cDNA Reverse Transcript Kit (Applied Biosystems, Foster City, CA). qPCR was performed on 50 ng of cDNA using Bio–Rad iQ SYBR Green Supermix with a Bio–Rad iCycler and iCycler iQ Detection System (Bio–Rad, Hercules, CA). Cycle parameters were: Step 1: 95 °C for 3 minutes, Step 2 (35×): 95 °C for 10 sec, 60 °C for 1 min, Step 3: 60 °C for 1 min. Each sample was quantified in triplicate for each gene and normalized to the median quantity of β–actin in all samples. Expression was quantified using a standard curve to calculate the nanogram amount of each gene amplicon per 50 ng RNA. β–actin (GenBank BC063950) primers were F: 5´– CGA GCA GGA GAT GGG AAC C–3´ and R: 5´–CAA CGG AAA CGC TCA TTG C–3´ (101 bp product). Cytochrome P450 1A (CYP1A, GenBank AF210727) primers were F: 5´–TGC CGA TTT CAT CCC TTT CC–3´ and R: 5´–AGA GCC GTG CTG ATA GTG TC–3´ (279 bp product). Aryl

hydrocarbon receptor 2 (AhR2, GenBank AF063446) primers were F: 5´–GAC TGT ACA TAC TCG ACT CCT G–3´ and R: 5´–GCC CAA GTA GTC CTG TAT AG–3´ (597 bp product). Vitellogenin 1 (VTG 1, GenBank BC094995) primers were F: 5´–GCC AAA AAG CTG GGT AAA CA–3´ and R: 5´–AGT TCC GTC TGG ATT GAT GG–3´ (210 bp product). Vitellogenin 2 (VTG 2, GenBank BC154732) primers were F: 5´–GGT GAC TGG AAG ATC CAA G–3´ and R: 5´–TCA TGC GGC ATT GGC TGG–3´ (190 bp product). Vitellogenin 3 (VTG 3, GenBank AF254638.1) primers were F: 5´–TTC ATA CAG TTT AGA ACC AGC AAA GGA TGC–3´ and R: 5´–AGG TGT TCT CAT CTC TTT TCT CCT TAA ATA C–3´ (149 bp product).

5.2.6. Statistical analyses

Statistical tests were performed using SigmaPlot™ (v. 11.0). Data is reported as mean ± standard deviation. Fold–changes were calculated using mean values. Unpaired t–tests were used to compare different treatments. When normality failed the Student–Neuman–Keuhls test was used. When equal variance failed the Mann–Whitney Rank Sum test was used. For multiple comparisons, one–way ANOVA was used. When normality failed, a Ranked Student–Neuman–Keuhls ANOVA was used. A p–value $\leq$ 0.05 was regarded as significantly different.

5.3. Results

5.3.1. Dioxin developmental toxicity and induction of CYP1A

Cytochrome P450 1A (CYP1A) mRNA expression was measured to demonstrate the activation of the AhR2 pathway in 4 dpf embryos that were exposed to dioxin for 1 hour (4–5 hpf). Developmental toxicity (e.g. pericardial edema, yolk–sac edema) was also evaluated to demonstrate the effect of these exposures on normal development.

Exposure to 2,3,7,8–TCDD and 1,2,3,7,8–PeCDD, but not dibenzo–*p*–dioxin, resulted in a dose–dependent increase of CYP1A mRNA expression (Fig. 5.2). Expression of CYP1A in embryos exposed to 50 pptr and 400 pptr 2,3,7,8–TCDD were 52–fold and 149–fold higher than control, respectively. Embryos treated with 50 pptr and 400 pptr 1,2,3,7,8–PeCDD expressed CYP1A levels that were 89–fold and 175–fold higher, respectively. Expression of CYP1A remained unchanged in embryos treated with 400 pptr dibenzo–*p*–dioxin, while 400 pptr 2,3,7,8–TCDD (positive control) exhibited a 426–fold induction, relative to control.

Exposure to 2,3,7,8–TCDD and 1,2,3,7,8–PeCDD elicited developmental toxicity that is phenotypic of dioxin exposure (Fig. 5.3). When exposed to 50 pptr and 400 pptr 2,3,7,8–TCDD for one hour, 25% and 100% of embryos developed pericardial edema, respectively, compared to 0% in the control. Treatment with 50 pptr and 400 pptr 1,2,3,7,8–PeCDD for one hour also resulted in pericardial edema in 25% and 100% of embryos, respectively, relative to the 0% control. Embryos exposed to 50 pptr 2,3,7,8–TCDD and 1,2,3,7,8–TCDD exhibited pericardial edema that were generally less severe than observed in the 400 pptr treatment group. Exposure to 400 pptr dibenzo–*p*–dioxin

for one hour did not result in any lesions associated with dioxin exposure. Survival was not affected in any treatment group.

5.3.2. Verification of morpholino effectiveness

Effective morpholino knock–down of the AhR2 gene was verified by the presence of an AhR2 splice–variant in AhR2–MO injected embryos, which was absent in non–injected control (NIC) or C–MO injected embryos (Fig. 5.4). This splice variant was missing Exon 2, which therefore shifts the sequence down–stream of Exon 1 out of frame, resulting in an improperly translated AhR protein. The functional ability of this knock–down to block AhR2–mediated developmental toxicity was verified by the rescue of the developmental toxicity caused by 2,3,7,8–TCDD. When treated with 400 pptr 2,3,7,8–TCDD, 100% of embryos (4 dpf) exhibited severe pericardial edema in NIC or C–MO injected embryos, versus approximately 25% in those injected with AhR2–MO. In addition to decreased prevalence of pericardial edema in AhR2–MO injected embryos, lesions were also generally less severe than observed in NIC or C–MO injected embryos (Fig. 5.3).

5.3.3. Dioxin inhibition of vitellogenin induction by 17α–ethynylestradiol (EE2)

Vitellogenin mRNA expression serves as a sensitive and reproductively relevant biomarker for estrogen receptor pathway activity in teleosts. To demonstrate the ability for dioxins to inhibit the induction of vitellogenesis in zebrafish, mRNA expression of three vitellogenin genes (VTG 1, 2 and 3), was measured. Embryos were exposed to

either 17α–ethynylestradiol (EE2), or pre–treated with a dioxin congener and then exposed to EE2.

Exposure to 1000 pptr EE2 significantly induced mRNA levels of VTG 1, 2 and 3 in all experiments, relative to control. The order of sensitivity of the three vitellogenin genes to EE2 were consistently: VTG 2 > VTG 1 >> VTG 3 (determined by fold–induction). Embryos that were pre–treated with 2,3,7,8–TCDD and 1,2,3,7,8–PeCDD prior to EE2 exposure exhibited a significant dose–dependent inhibition of VTG 1, 2 and 3 (Fig. 5.5). Exposure to 400 pptr 2,3,7,8–TCDD and 1,2,3,7,8–PCDD inhibited vitellogenin induction by > 98% for all vitellogenin genes measured (Table 5.1). Exposure to 50 pptr 2,3,7,8–TCDD and 1,2,3,7,8–PeCDD inhibited vitellogenin induction by 73–79% and 94–96%, respectively (Table 5.2). Dibenzo–*p*–dioxin did not have an inhibitory effect on the induction of VTG 1, 2 and 3 by EE2, whereas exposure to the positive control (2,3,7,8–TCDD) inhibited induction by > 96% (Fig. 5.5, Table 5.1).

5.3.4. AhR2 knock–down rescue of the 2,3,7,8–TCDD inhibition of vitellogenesis

To investigate the role of AhR2 activation by dioxin congeners in the inhibition of vitellogenesis, a splice blocking morpholino (AhR2–MO) was used to transiently knock–down the AhR2 gene. In these experiments, knock–down of the AhR2 gene reduces (protects) against the 2,3,7,8–TCDD inhibition of VTG 1, 2 and 3 (Fig. 5.6, Table 5.2). C–MO injected embryos did not have a significant decrease in the capacity for induction of VTG 1–3 by EE2, compared to the NIC treatment group. Knock–down of AhR2 did not have a significant effect on the capacity for induction of VTG 1–3 by EE2, compared

to the C–MO injection groups. As previously demonstrated, treatment with dioxin significantly inhibited the induction of VTG 1–3 by > 95% in NIC and C–MO injection groups. Embryos injected with AhR2–MO had significantly less inhibition for VTG 1–3 than the NIC and C–MO injection groups, and ranged from 69–75% inhibition. Statistically, mRNA expression of VTG 1–3 in the AhR2–MO group treated with 2,3,7,8–TCDD and EE2 were the same induction levels as those treated with EE2 alone (Table 5.2). This corresponded to a 20–27% significant rescue from the 2,3,7,8–TCDD inhibition (Table 5.2).

5.4. Discussion

In teleosts, the regulation of vitellogenesis in the liver and the function of the AhR is highly conserved. Therefore, the use of embryonic zebrafish to develop an *in vivo* model to study the effect of AhR activation on the gene regulation of vitellogenin and AhR–ER interactions is justified. These studies demonstrate that exposure to AhR agonists (2,3,7,8–TCDD and 1,2,3,7,8,–PeCDD) inhibit the induction of vitellogenins 1, 2 and 3 by 17α–ethynylestradiol. In addition, it was demonstrated using morpholino gene knock–down that the inhibition of vitellogenesis by 2,3,7,8–TCDD is mediated by aryl hydrocarbon receptor 2 activity. Overall, these studies demonstrate that AhR2 activation can result in toxicity specifically in the liver that results in the down–regulation of vitellogenin gene expression. These studies are the first to demonstrate the role of AhR2 for cross–talk with estrogen receptor regulated genes in fish *in vivo* and provides insight into reproductive effects observed in oviparous species exposed to AhR agonist contaminants.

5.4.1. Dioxin toxicity and AhR2 morpholino effectiveness

Developmental malformations and CYP1A induction were used to verify effectiveness of the 1 hour dioxin exposure protocol to induce AhR2 mediated toxicity. Exposure to both 2,3,7,8–TCDD and 1,2,3,7,8–PeCDD, but not dibenzo–*p*–dioxin, resulted in severe developmental toxicity and a high induction of CYP1A mRNA (Figs. 5.2 and 5.3). As a result of these preliminary studies, 400 pptr was the dose chosen to be used in the subsequent inhibition studies due to their effectiveness in activating the AhR2. In addition, dibenzo–*p*–dioxin did not result in developmental lesions or induce CYP1A mRNA confirming that this compound did not activate the AhR and can therefore serve as a negative control for AhR activation in the inhibition studies. In my studies, 1,2,3,7,8–PeCDD consistently induced CYP1A more than 2,3,7,8–TCDD for all doses tested and developmental toxicity (pericardial edema) was also more severe in embryos treated with 1,2,3,7,8–PeCDD, relative to those treated with 2,3,7,8–TCDD. This is consistent with previous work showing that the potency of 1,2,3,7,8–PeCDD to induce CYP1A is slightly higher than that of 2,3,7,8–TCDD in zebrafish (Abnet et al., 1999; Henry et al., 2001).

To transiently knock–down gene expression of the AhR2, I used a morpholino oligonucleotide to block splicing at the exon 2–intron 2 junction. My studies demonstrated that embryos injected with the AhR2–MO were protected against the developmental toxicity of 2,3,7,8–TCDD. Developmental toxicity and CYP1A induction of 2,3,7,8–TCDD has been previously shown to be mediated by the AhR2, and my data also demonstrate this (Prasch et al., 2003; Prasch et al., 2004; Dong et al., 2004; Prasch et al., 2006). The morphological rescue is further supported by the effectiveness of the

morpholino to block AhR2 splicing (Fig. 5.4). Taken together, my studies demonstrate effective morpholino knock–down of AhR2 using morphological and molecular endpoints.

5.4.2. In vivo zebrafish embryo model for studying dioxin inhibition of vitellogenesis

The zebrafish embryo model developed by the current study to examine AhR–ER interactions *in vivo* relates to vitellogenesis in the liver. Vitellogenin is synthesized exclusively in the hepatic tissue of teleosts, including zebrafish (Tong, 2004). The zebrafish embryo model allows for the use of gene knock–down tools to study the mechanism of AhR–ER interaction of hepatic vitellogenesis. However, some studies suggest that the zebrafish intestine can account for 5% of expression while the liver accounts for 95% (Wang et al., 2000; Wang et al., 2005). These studies conflict with those by Tong et al. (2004), which showed vitellogenin is expressed solely in the zebrafish liver. Studies by Wang et al. (2000, 2005) did not standardize vitellogenin expression to β–actin, which was greatly reduced in their liver relative to other tissues. β–actin expression has been shown to be uniform across tissues including liver (Råbergh et al., 2000; Tong et al., 2004), and many studies commonly use β–actin to normalize mRNA expression between tissues (Tanguay et al., 2000; Sawyer et al., 2006). Considering this, studies by Wang et al. (2005) clearly demonstrate after normalization to β–actin that nearly 100% of vitellogenins 1–7 expression is synthesized in the liver, with relatively little to no expression in extra–hepatic tissues. Based on this, vitellogenesis is considered to occur exclusively in the hepatic tissue of zebrafish, which allows my results to be applied to AhR–ER cross–talk in the liver as it relates to vitellogenesis.

The inhibition studies clearly demonstrate that 2,3,7,8–TCDD and 1,2,3,7,8–PeCDD inhibit the mRNA induction of vitellogenins 1, 2 and 3 (Fig. 5.5, Table 5.1). This inhibition was not observed for dibenzo–*p*–dioxin, the negative control for AhR activation. 1,2,3,7,8–PeCDD was consistently more potent in inhibiting VTG 1–3 at 50 pptr than 2,3,7,8–TCDD and these results concur with my earlier evaluation that 1,2,3,7,8–PeCDD is more potent based on CYP1A induction. For example, at 50 pptr, 1,2,3,7,8–PeCDD inhibited inductions of VTG 1, 2 and 3, by 94.9%, 96% and 96.1%, respectively, compared to 73.5%, 76.7% and 78.9% with 2,3,7,8–TCDD (Table 5.1). These results suggest that the potency for AhR agonists to down–regulate vitellogenesis follows the relative potencies based on CYP1A induction (Henry et al., 2001). Studies by Anderson et al. (1996a) also noted that inhibition of vitellogenesis was directly related to the compounds potency for inducing CYP1A.

Overall, both AhR agonists tested by the current studies inhibited vitellogenin 1, 2 and 3 equally, based on % inhibition with 50 pptr doses. This indicates that the structurally different vitellogenins are down–regulated by AhR agonists similarly. In addition, the data show that VTG 2 is more sensitive to induction than VTG 1 and VTG 3. These findings are contrary to findings by Wang et al. (2005), which showed that VTG 1 is 100 times more sensitive to induction than VTG 2. In my studies, VTG 2 induction was usually 1 to 3 times that of induction levels for VTG 1. Based on these results, vitellogenin 2 may therefore play a more dominant role in oocyte and yolk–development than previously thought.

Reproductive toxicity of dioxins and other AhR agonists have traditionally been primarily thought of as the result of toxicity localized in the ovary. For example,

reproductive dysfunction in zebrafish exposed to 2,3,7,8–TCDD is caused by changes in ovarian gene–expression, which indirectly down–regulated vitellogenesis and inhibited follicle development (Wannenmacher et al., 1992; King Heiden et al., 2006; King Heiden et al., 2008; King Heiden et al., 2009). This was further correlated with effects on expression of genes involved in estrogen biosynthesis and gonadotropin sensitivity. My studies demonstrated that dioxins inhibit the gene regulation of vitellogenins 1–3 directly in the liver of zebrafish without toxicity in the ovary. *In vitro* studies with teleost hepatocyte cultures have also showed that a wide variety of AhR agonist contaminants can inhibit the induction of vitellogenin directly, such as PCDDs/PCDFs (Anderson et al., 1996a; Bemanian et al., 2004), PAHs (Navas and Segner, 2000; Gräns et al., 2010), and PCBs (Mortensen and Arukwe, 2007). Relatively fewer studies have studied the inhibition of hepatic vitellogenin gene regulation *in vivo*. Studies by Vaccarro et al. (2005) and Anderson et al. (1996b) have shown that PCBs and PAHs can depress vitellogenin induction *in vivo* using sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*). The down–regulation of vitellogenin gene–expression can clearly occur without ovarian toxicity, although effects on hepatic vitellogenesis will be exacerbated by disruption of ovarian estrogen biosynthesis. Based on my results, I propose that reproductive toxicity in teleosts can occur through combined effects directly in both the ovary and liver. Decreased vitellogenin production has been inversely correlated with reduced egg production in the fathead minnow (*Pimephales promelas*), and consequently results in reproductive failure (Miller et al., 2007; Thorpe et al., 2007). Therefore, the inhibition of vitellogenin gene–induction by AhR agonists can have a

direct role in reproductive effects and result in ramifications on a population's sustainability.

5.4.3. Direct role of AhR2 activation in the inhibition of hepatic vitellogenesis

My studies are the first to clearly demonstrate in a teleost model that AhR2 activation by 2,3,7,8–TCDD mediates inhibition of hepatic vitellogenins 1, 2 and 3 (Fig. 5.6, Table 5.2). Morpholino knock–down of the AhR2 effectively reduced the dioxin inhibition (> 95%) of VTG 1–3 by 21.6–27.4%, demonstrating a necessary role of the AhR2 (Table 5.2). The AhR2 is known to regulate developmental toxicity and CYP1A induction in zebrafish and my studies expand the role of the AhR2 to include mediating cross–talk with the estrogen receptor pathway. Studies by Ohtake et al. (2008) suggested that the AhR functions to co–regulate ER processes. My studies show that knock–down of the AhR2 does not significantly alter EE2 induction levels of VTG 1–3, relative to control morpholino injected embryos (Fig. 5.6, Table 5.2). This suggests that basal AhR2 activity does not play an intrinsic role in the co–regulation of vitellogenins with the ER pathway. Instead, the AhR2 modulates vitellogenin expression only when activated by the potent ligand 2,3,7,8–TCDD, which can result in greater than 95% inhibition. Overall, these studies demonstrate that the vitellogenin pathway is a target for contaminants that activate the AhR2.

The studies presented do not explain the mechanism by which AhR2–ER cross–talk occurs, although AhR2 mediated inhibition of vitellogenesis is likely a combination of cross–talk mechanisms. Cross–talk can occur through direct inhibition of transcription via inhibitory elements, co–factor squelching, AhR–mediated synthesis of an inhibitory

protein, ER degradation, and increasing E2 metabolism (Matthews and Gustafsson, 2006). It has been shown that AhR agonists (2,3,7,8–TCDD) can inhibit ER induction of vitellogenin, and that 17β–estradiol can inhibit AhR induction of CYP1A (Anderson, et al., 1996a). However, studies by Gräns et al. (2010) suggest that the AhR activation inhibits ER–mediated gene induction, but not vice versa. These two studies are conflicting, but the mechanism of cross–talk likely depends on the concentrations and ratios of AhR and ER agonists. Future studies should be directed to understand how the AhR2 modulates ER–signaling in the teleost liver, which can ultimately impair gonadal development.

5.5. Conclusions

In summary, these studies demonstrate that dioxins 2,3,7,8–TCDD and 1,2,3,7,8–PeCDD inhibit the mRNA induction of vitellogenins 1, 2 and 3 by 17α–ethynylestradiol in zebrafish. I demonstrated using morpholino knock–down of the AhR2 that activation of the AhR2 mediates the inhibition of vitellogenin by the prototypical dioxin 2,3,7,8–TCDD. These are the first studies to report a direct role of AhR2 activation in the inhibition of the ER–regulation of vitellogenesis although the mechanism by which AhR2 activation results in inhibition is not clear. These studies were instrumental in developing a model for studying AhR–ER cross–talk *in vivo* and future studies should focus on the mechanism by which AhR–ER cross–talk inhibition occurs. Demonstrating that AhR2 activation inhibits vitellogenesis offers insight into reproductive impacts observed in oviparous species inhabiting aquatic systems heavily contaminated by AhR agonists.

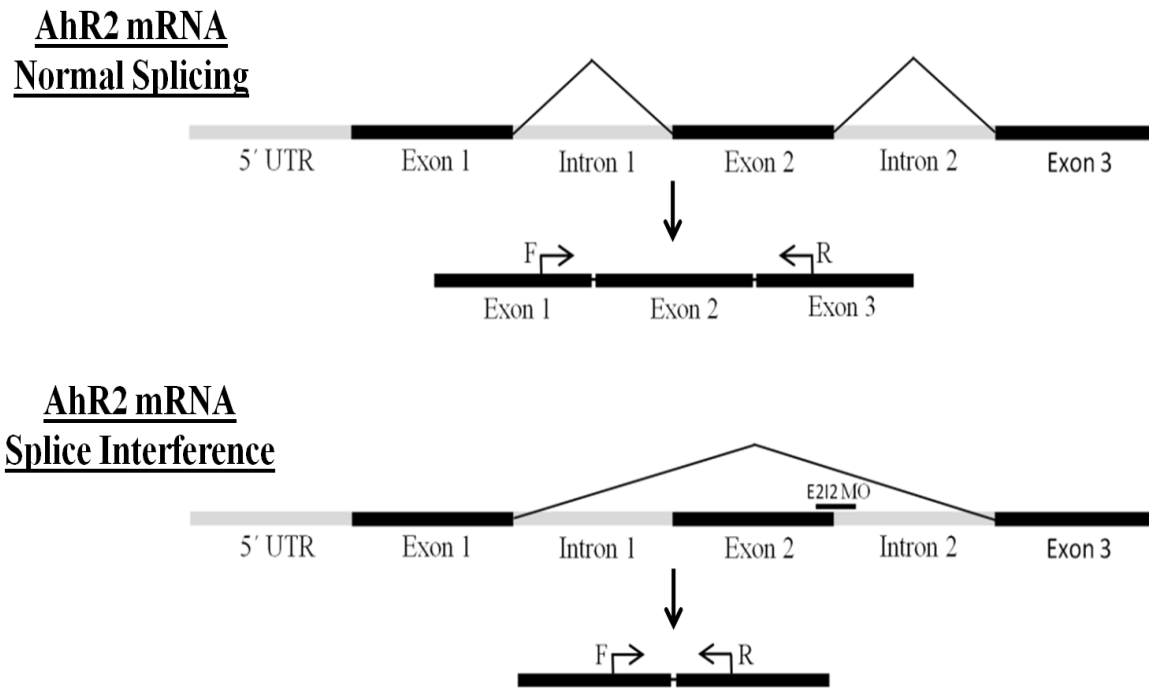
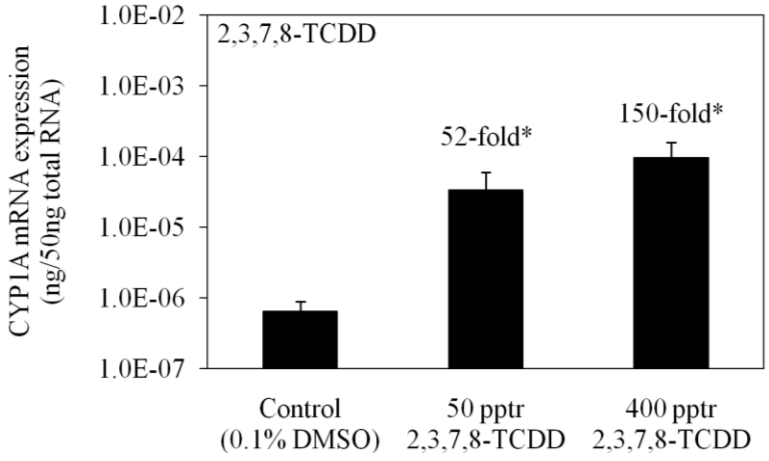


Fig. 5.1. Morpholino down–regulation of AhR2 transcription by interference with E2I2 splicing. The morpholino used in the current studies blocks the E2I2 junction forcing Exon 2 to be spliced out, resulting in a splice variant missing Exon 2 and also shifted out of frame down–stream of Exon 1. The splice variant is verified using PCR primers flanking Exon 1. Abbreviations: E2I2, Exon 2–Intron 2; F, forward primer; MO, morpholino, R, reverse primer; UTR, untranslated regions.



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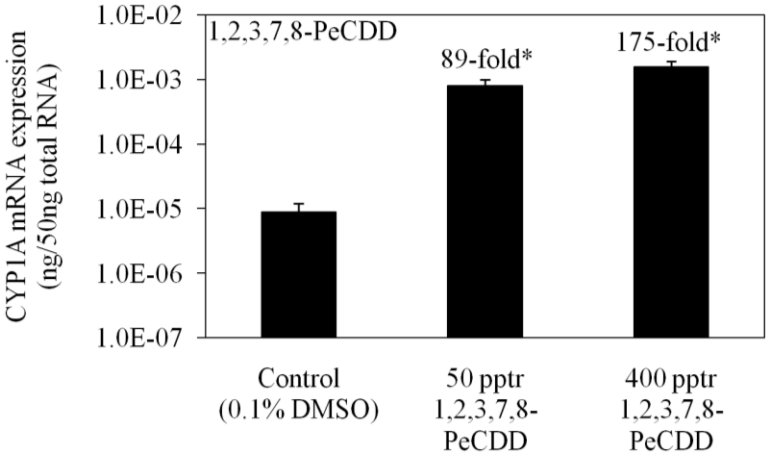

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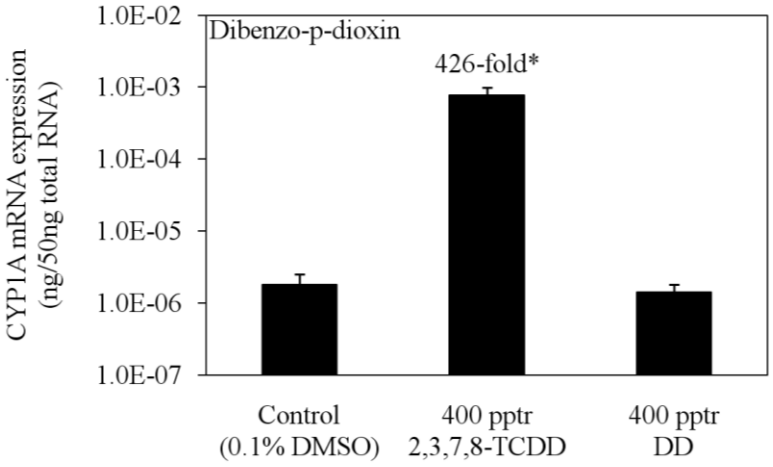


C.

Fig. 5.2. Expression and induction of CYP1A mRNA in embryos at 4 dpf by (A) 2,3,7,8–TCDD, (B) 1,2,3,7,8–PeCDD, and (C) dibenzo–*p*–dioxin (DD). Data are reported as mean ± standard deviation. *Significantly higher than control levels at $p \leq 0.05$ (Student's t–test). Fold–induction levels over control are shown. N = 6 biological replicates per treatment group, each with 10–12 embryos. Embryos were treated with dioxin for 1 hour from 4–5 hpf. 2,3,7,8–TCDD was used as a positive control for the dibenzo–*p*–dioxin experiment.

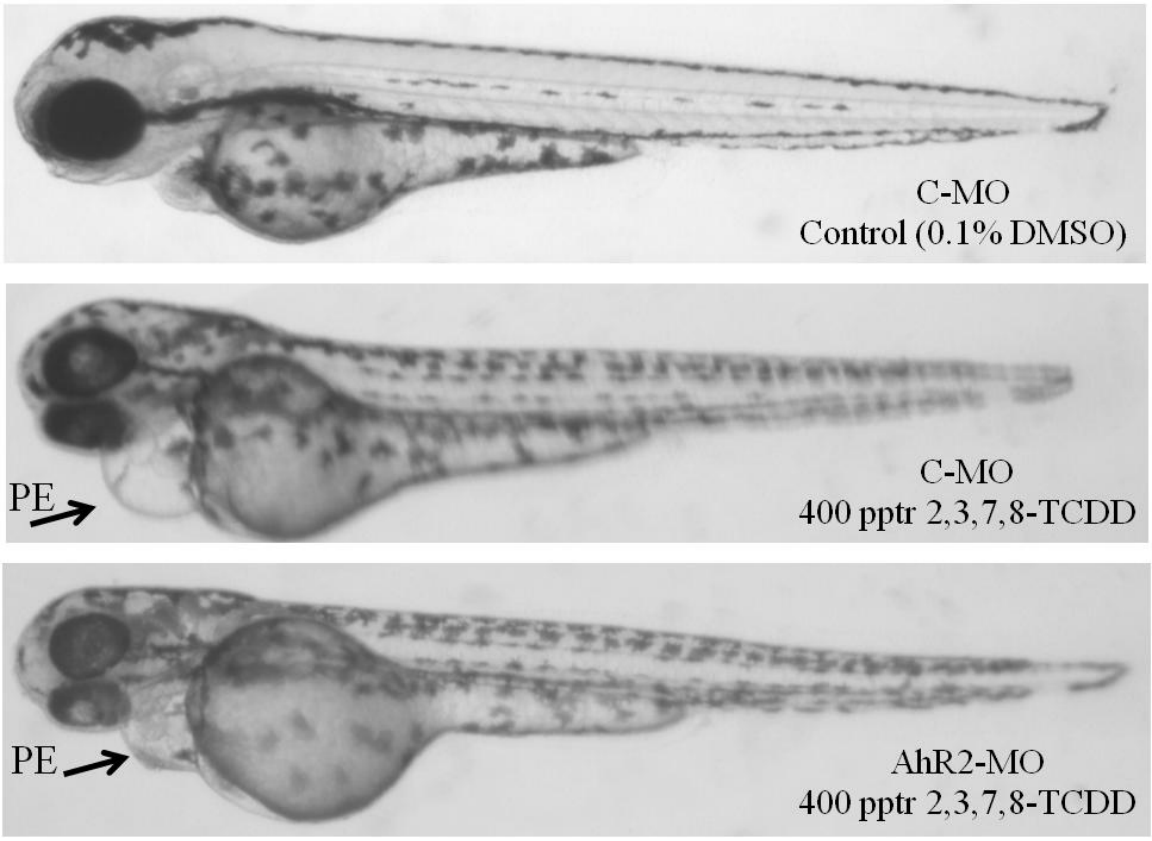


Fig. 5.3. Representative photomicrographs of 3 day old embryos in control (0.1% DMSO) exposed, 2,3,7,8–TCDD exposed (400 pptr) exposed embryos injected with the control morpholino (C–MO), and 2,3,7,8–TCDD exposed (400 pptr) embryos injected with AhR2–MO. Pericardial edema (PE) is a classic sign of dioxin developmental toxicity and was present in embryos treated with 2,3,7,8–TCDD and 1,2,3,7,8–PeCDD, but not dibenzo–*p*–dioxin. 100% of embryos treated with 400 pptr 2,3,7,8–TCDD injected with C–MO exhibited severe pericardial edema while approximately 25% of embryos injected with AhR2–MO exhibit pericardial edema, that was generally less severe (0% in controls). Embryos were treated with dioxin for 1 hour from 4–5 hpf.

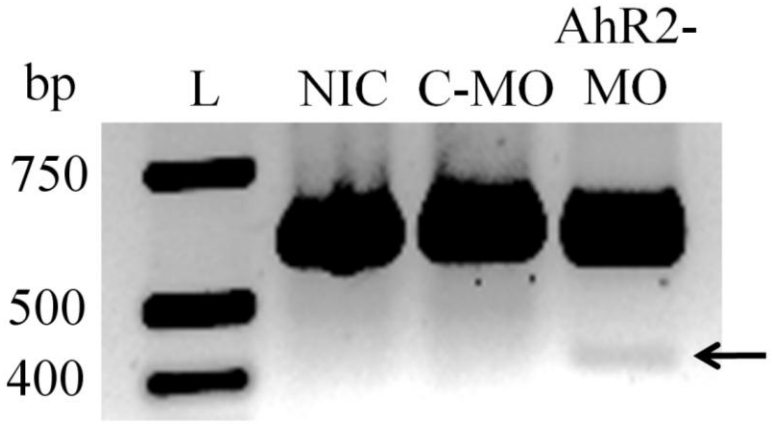


Fig. 5.4. PCR products (596 bp) of the AhR2 gene from cDNA isolated from non–injected control (NIC) embryos and embryos injected with control (C–MO) and AhR2 morpholinos (AhR2–MO) at 24 hpf. Primers flanked Exon 2 to detect the presence of a splice–variant (arrow) in AhR2–MO injected embryos. This splice variant (408 bp) was detected in 1 dpf and 3 dpf embryos, and is shifted out of frame because it is missing Exon 2 (188 bp).

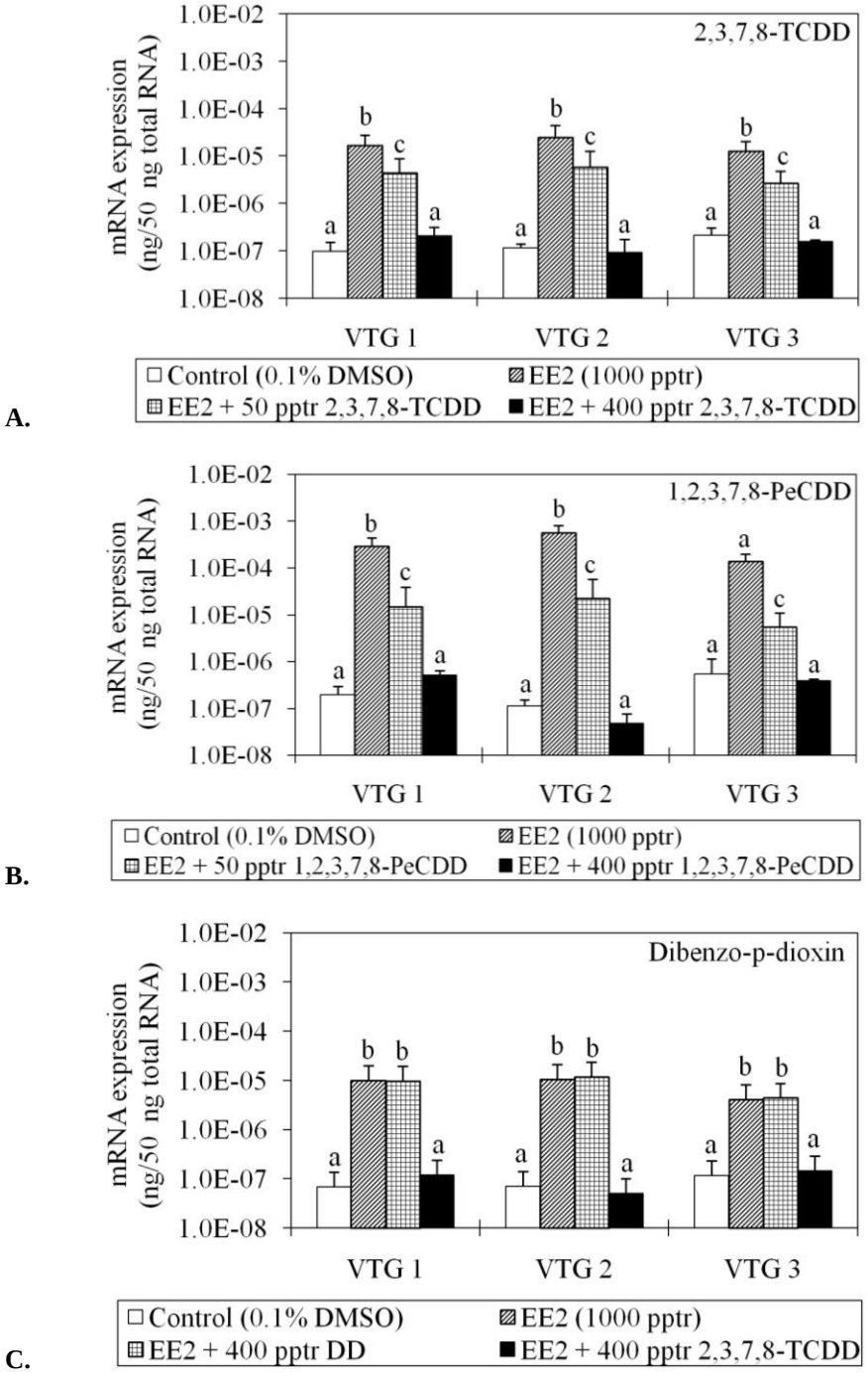
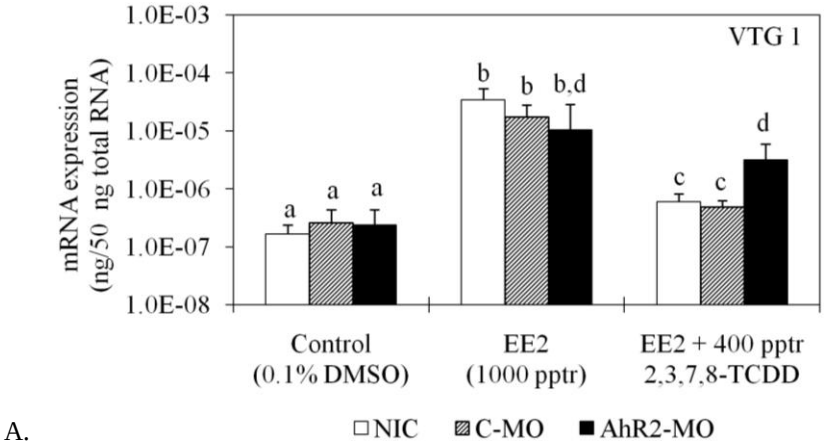


Fig. 5.5. Effect of (A) 2,3,7,8–TCDD, (B) 1,2,3,7,8–PeCDD, and (C) dibenzo–*p*–dioxin (DD) on the mRNA induction of VTG 1, 2 and 3 by EE2 in 4 dpf embryos. Embryos were treated with EE2 alone or pre–treated with dioxin for 1 hour (4–5 hpf) before EE2 exposure. 2,3,7,8–TCDD and 1,2,3,7,8–PeCDD inhibited induction of VTG 1–3, while dibenzo–*p*–dioxin had no effect. 2,3,7,8–TCDD served as a positive control for dibenzo–*p*–dioxin. Data are reported as mean ± standard deviation. Bars not labeled with the same letter are significantly different (ANOVA, Student–Newman–Keuls, $p \leq 0.05$). N = 6 biological replicates (10–12 embryos each).

Treatment	Treatment	VTG 1	VTG 2	VTG 3
EE2	1000 pptr	169-fold	212-fold	59-fold
EE2 + 2,3,7,8-TCDD	+ 50 pptr	45-fold (73.5%)	50-fold (76.7%)	12-fold (78.9%)
	+ 400 pptr	2.1-fold (98.9%)	0.8-fold (100%)	0.7-fold (100%)
EE2	1000 pptr	1500-fold	4923-fold	262-fold
EE2 + 1,2,3,7,8-PeCDD	+ 50 pptr	77-fold (94.9%)	197-fold (96.0%)	10-fold (96.1%)
	+ 400 pptr	2.6-fold (99.8%)	0.4-fold (100%)	0.7-fold (100%)
EE2	1000 pptr	146-fold	147-fold	35-fold
EE2 + Dibenzo-p-dioxin	+ 400 pptr	141-fold (3.3%)	164.6-fold (0%)	37.5-fold (0%)
EE2 + 2,3,7,8-TCDD	+ 400 pptr	1.7-fold (98.8%)	0.7-fold (100%)	(1.2-fold) 96.4%

Table 5.1. Fold–inductions of VTG 1, 2 and 3 by EE2 (1000 pptr) and EE2 + dioxin treatments, relative to control, for the experiments shown in Fig. 5.5. Percent inhibition of EE2 induction levels by pre–treatment with dioxin are shown in parenthesis. Induction of all vitellogenins tested were inhibited in a dose–dependent manner by 2,3,7,8–TCDD and 1,2,3,7,8–PeCDD, relative to expression levels in EE2 induced embryos. Dibenzo–*p*–dioxin had no inhibitory effect. Fold induction was calculated by dividing the average expression values in treatment by control expression levels. Percent inhibition was calculated using the average fold–inductions of EE2 treated and dioxin/EE2 co–treated embryos.



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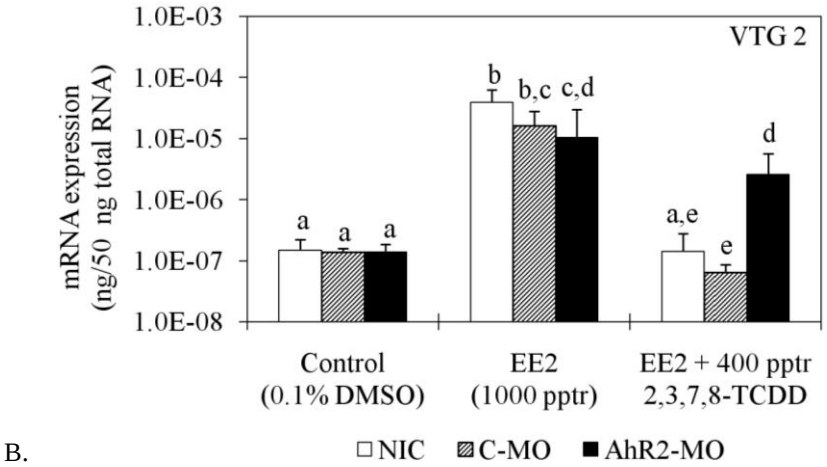

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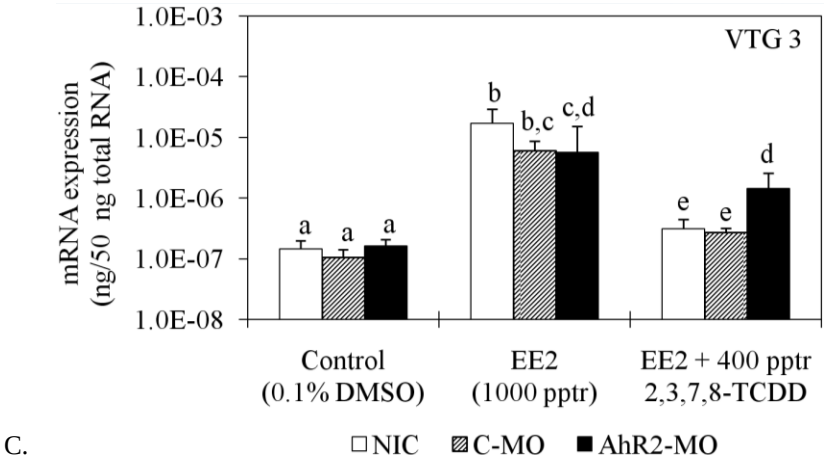

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Fig. 5.6. Effect of AhR2 knock–down on the 2,3,7,8–TCDD inhibition of (A) vitellogenin 1, (B) vitellogenin 2 and (C) vitellogenin 3 mRNA expression induction. Injection groups included a non–injection control (NIC), control morpholino (C–MO) and AhR2 morpholino (AhR2–MO) injected groups. Data are reported as mean ± standard deviation. Bars not labeled with the same letter are significantly different (ANOVA, Student–Newman–Keuls, $p \leq 0.05$). N = 6 biological replicates (10–12 embryos each).

	Group	VTG 1	VTG 2	VTG 3
Fold-Induction	NIC	205-fold	262-fold	119-fold
EE2 (1000 pptr)	C-MO	67-fold	118-fold	58-fold
	AhR2-MO	44-fold	74-fold	35-fold
% Inhibition	NIC	3.6-fold (98.2%)	1.0-fold (99.6%)	2.1-fold (98.2%)
EE2 + 2,3,7,8-TCDD	C-MO	1.9-fold (97.2%)	0.5-fold (100%)	2.6-fold (95.5%)
	AhR2-MO	13.3-fold (69.8%)	18.6-fold (74.8%)	8.8-fold (74.8%)
% Rescue		11.4-fold (27.4%)	18.2-fold (24.8%)	6.2-fold (20.6%)

Table 5.2. Fold–induction of VTG 1, 2 and 3 by EE2 (1000 pptr) and EE2 + dioxin treatments, relative to control, for the experiments shown in Fig. 5.6. Percent inhibition of EE2 induction levels by pre–treatment with dioxin are shown in parenthesis for all injection treatment groups. Fold induction was calculated using the average fold–inductions of EE2 treated and dioxin/EE2 co–treated embryos. Percent inhibition was calculated by dividing the average fold–induction of dioxin/EE2 co–treated embryos by the fold–induction in EE2 treated embryos. Percent rescue was determined by the difference between % inhibition in the C–MO group and the AhR2–MO group.

CHAPTER 6

General Discussion

The studies presented in this Dissertation demonstrated that reproductive deficits (reduced egg production) in killifish from Newark Bay, NJ was due to altered steroid–signaling and attenuation of the vitellogenin pathway (Fig. 6.1). Contaminants in Newark Bay were demonstrated to down–regulate vitellogenin expression and inducibility. Based on these findings it is being proposed that this down–regulation was a phenotypic response to PCDDs/PCDFs, which are the predominant toxicants in Newark Bay. Newark Bay killifish had elevated CYP1A levels, a biomarker for AhR2 activation, and decreased expression levels of vitellogenin. An inverse correlation between hepatic CYP1A and vitellogenin expression in this population strengthened this argument, indicating AhR–ER cross–talk in this population decreased sensitivity of estrogenic responses to 17β–estradiol. However, not being able to collect and conduct knock–down studies in Fundulus year–round required the use of an alternative model. Using the zebrafish model, it was demonstrated that AhR2 activation by 2,3,7,8–TCDD mediates the estrogen receptor cross–talk inhibition of vitellogenesis. The results from this model teleost offers insight into the altered vitellogenin gene regulation in Newark Bay killifish. These studies taken together with the field studies demonstrated that the vitellogenin pathway is down–regulated following disruption by AhR2–inducing contaminants, and that altered gene regulation in the liver can result in effects on oocyte development in the ovary. The fact that many bodies of water are contaminated with AhR2 inducing

contaminants and that oviparous organisms have conserved vitellogenin pathways raises concerns for population sustainability due to decreased fecundity.

6.1. Down–regulation of vitellogenin gene–expression and reproductive dysfunction in killifish from Newark Bay, NJ

Findings from the studies in chapters 2, 3 and 4 collectively demonstrated that reproductive dysfunction of a population of killifish from Newark Bay, NJ, was due to the inhibition of oocyte development resulting in decreased egg production, decreased embryo mass and reduced yolk–volume. A clear relationship was established between contaminant effects on hormonal signaling that led to the down–regulation of the vitellogenin pathway, and the inhibition of egg production due to reduced vitellogenin–dependent growth (Fig. 6.1). Exposure to AhR agonists, the predominant type of contaminant in Newark Bay resulted in AhR–ER cross–talk inhibition of the vitellogenin pathway.

It was demonstrated using four different approaches that oocyte growth and development was inhibited in Newark Bay killifish, and that this was caused by contaminant effects on the vitellogenin–dependent growth of the oocytes. First, females collected from Newark Bay in the biomarker studies of Chapter 1 had decreased gonadal weights and gonad–to–body weight ratios, which were general indicators of abnormal gonad development and underdeveloped ovaries (Table 2.1). Second, histological examination of the ovaries demonstrated that Newark Bay females had a significant increase in the percent of pre–vitellogenic follicles over that of Tuckerton females (43% at Tuckerton, 64% at Newark Bay) and a significantly decreased percent of follicles at

the mid–vitellogenic and mature stages (25% mature at Tuckerton and 3% at Newark Bay) (Table 2.4). The pre–vitellogenic stage is the earliest stage at which vitellogenin accumulation begins, and this was an early indicator that contaminants in Newark Bay down–regulate the vitellogenin pathway in the liver to result in inhibition of oocyte developmental progression. Third, the ramifications of reduced oocyte development on egg production were demonstrated by a fecundity study, which showed that Newark Bay killifish produce greatly reduced numbers of mature eggs during spawning (Fig. 3.5). These data show that on average, Tuckerton females produced 140 eggs per female and Newark Bay females produced 11 eggs per female, during peak spawning. Embryos from Newark Bay had 34% greater mortality during the first 3 days of development and 28% fewer embryos hatch compared to the reference population. Newark Bay killifish had a significantly reduced capacity for reproduction due primarily to the reduction in egg production, rather than loss of embryos during development. Fourth, histomorphometric measurements of mature eggs from each population demonstrated that eggs from Newark Bay killifish were generally smaller due to reduced yolk–development (Fig. 3.6). Newark Bay embryo mass and yolk–volume was demonstrated to be reduced by 25% and 16%, respectively, relative to Tuckerton embryos. Taken together, these four studies clearly demonstrated that living in a contaminated estuary inhibited the vitellogenin–dependent process of yolk–development, which resulted in decreased fecundity and poor egg quality in Newark Bay female killifish. Despite these reproductive effects, the Newark Bay killifish population sustains itself to present day. While many animals collected in the fecundity study produced no mature oocytes, some individuals produced several dozen oocytes and these animals are likely responsible for maintaining and carrying the

population. Alternatively, the Newark Bay population may have an acyclical reproductive cycle, which could allow them to continually reproduce at a reduced capacity, but with greater frequency than the Tuckerton population. However, our studies focused on the peak of spawning (full or new moons) and did not investigate the periodicity of the Tuckerton and Newark Bay populations. In addition, a semi–lunar spawning cycle is energetically costly due to the great amount of resources required for egg production and turnover within the ovary every two weeks. The down–regulation of egg production in the Newark Bay population may be an adaptive advantage that allows the animals to survive in a contaminated habitat through resource competition and by balancing appropriated resources for reproduction and those required for detoxification and cell repair (Fig. 1.6).

Further studies demonstrated on the molecular and biochemical level that the contaminant induced down–regulation of vitellogenesis was the root cause for the inhibition of oocyte development in Newark Bay killifish. In the Newark Bay population, vitellogenin protein expression levels (and mRNA levels) during spawning were 27–fold lower than levels measured in the reference Tuckerton population (Fig. 2.5). Vitellogenin accumulation drives the growth of oocyte development through the pre–, early– and mid–vitellogenic stages. A direct correlation between vitellogenin levels and fecundity has been established in the fathead minnow, demonstrating its importance for oocyte development (Miller et al., 2007; Thorpe et al., 2007). Due to vitellogenin's role in yolk development, reduced vitellogenin expression was clearly the reason why fewer oocytes progress beyond the pre–vitellogenic stage of development to reach maturity in Newark Bay killifish. Reduced vitellogenin expression was shown to be caused by a combination

of toxic effects in the ovary and liver that resulted in (1) altered steroid signaling and (2) altered gene regulation of the hepatic vitellogenin pathway.

Vitellogenesis in the liver is regulated by circulating levels of 17β–estradiol, which are regulated by the ovaries. Several studies demonstrated that exposure to complex mixtures of contaminants at Newark Bay had altered ovarian 17β–estradiol steroidogenesis and hormone signaling (Chapters 3 and 4). One direct contributing factor for the reduced vitellogenin levels measured during spawning was a deficiency of circulating 17β–estradiol levels, which were 8–fold lower in Newark Bay killifish (0.26 ng/mL) relative to Tuckerton (2.25. ng/mL) (Fig. 3.7). Interestingly, gonadal aromatase expression was shown to be elevated in Newark Bay females, relative to Tuckerton (Fig. 2.6). Gonadal aromatase (CYP19A1) is the enzyme involved in the conversion of androgens to 17β–estradiol. Elevated expression levels in Newark Bay females were likely due to gonadotropin signaling to increase circulating levels of 17β–estradiol and reach levels required for vitellogenesis. Tuckerton expression levels of CYP19A were lower, likely due to feedback mechanisms that down–regulate CYP19A1 after sufficient circulating 17β–estradiol levels were reached. However, normal levels were never achieved in Newark Bay killifish and the feedback mechanism never turned the gonadotropin/aromatase pathway off. It was hypothesized that the reduced circulating hormone levels were due to contaminants that induce hepatic cytochrome P450 enzymes involved in estrogen metabolism. Contrary to what we expected, hepatic metabolism (elimination) of 17β–estradiol was not shown to be up–regulated in Newark Bay killifish, and metabolism activity was not–significantly different than activity measured in Tuckerton killifish livers (Fig. 4.5). The studies carried out to date were unable to

determine the reason for reduced 17β–estradiol levels, but likely involve an inhibition of aromatase activity and translation, or AhR–impacts on other steroidogenesis pathways (e.g. StAR). This is an area of further studies since my studies were primarily focused to investigate contaminant effects on vitellogenin gene regulation in the liver.

A series of studies on the gene regulation of vitellogenin in the liver demonstrated that although circulating vitellogenin protein is inducible with physiologically relevant levels of 17β–estradiol in Newark Bay killifish, Newark Bay fish are significantly less sensitive to induction than Tuckerton killifish (Chapters 3 and 4). Dose responses were carried out for protein induction (males and females) and for mRNA induction (males only). For mRNA studies, males were used instead of females because of their low background levels of 17β–estradiol, which can interfere with mRNA dose–response studies in the female. Adult killifish from both sites were dosed with 17β–estradiol at doses of 0.01, 0.1, 1, 10 and 100 ng/g body weight and induction levels of circulating vitellogenin in Newark Bay females were inhibited by 89, 79, 61, 40 and 30%, respectively, compared to Tuckerton females (Fig. 3.8). At doses of 0.01, 0.1, 1 and 10 ng/g body weight, induction levels of circulating vitellogenin in Newark Bay males were significantly inhibited by 97, 99, 98 and 44%, respectively, compared to Tuckerton males. Using male killifish to further study the regulation of vitellogenin demonstrated a refractory induction at the mRNA level for vitellogenin 1 and vitellogenin 2 in the liver of Newark Bay killifish (Fig. 4.2, Table 4.1). These studies collectively show that the hepatic vitellogenin pathway in Newark Bay killifish is less inducible and less sensitive to a 17β–estradiol challenge due to impacts on the mRNA regulation of vitellogenin 1 and 2. In addition to vitellogenin, the dose–response of choriogenin Hm, which is another

ER–regulated gene that is inducible by 17β–estradiol, was also examined. Choriogenin Hm was also less inducible and this suggested that decreased responsiveness to estrogen is not gene–specific to vitellogenin but may be due to a global down–regulation of estrogen receptor signaling (Fig. 4.3). Contrary to what was expected, hepatic expression of ER α, βa and βb levels were normal in Newark Bay killifish, relative to Tuckerton (Fig. 3.9). It was expected that the refractive induction of ER–regulation genes would be correlated with the down–regulation of the various estrogen receptor isoforms. Overall, these studies demonstrate a direct role for the contaminant effects on the gene regulation of vitellogenin and the reproductive effects characterized in the Newark Bay population. The decreased responsiveness of the vitellogenin pathway in the liver can result in decreased circulating levels of vitellogenin. However, taking into consideration the reduced levels of circulating 17β–estradiol during spawning, it is proposed that contaminant impacts in the ovary and liver worked in combination to down–regulate vitellogenin expression during spawning in this population. Consequently, this resulted in the inhibition of vitellogenin–dependent growth of oocytes.

6.2. Potential Role of AhR2–ER cross–talk in inhibition of vitellogenesis in Newark Bay killifish and vitellogenin biomarker responses in males

The predominant classes of toxicants in Newark Bay are polychlorinated dioxins/furans, and were suspected to be the cause for the inhibition of vitellogenesis through AhR–ER cross–talk. In addition to PCDDs and PCDFs, other AhR agonists are abundant in the Newark Bay, including PAHs and PCBs (Table 1.1). A series of studies performed for this Dissertation correlated contaminant exposure with down–regulation of

the vitellogenin pathway, which brings into question whether the ability to detect vitellogenin in males should be used as a valid biomarker of xeno–estrogen exposure in heavily contaminated locations with known AhR agonists. The biomarker studies provide correlative evidence for a relationship between AhR activity and the ER–mediated expression of vitellogenin, suggesting that AhR–ER cross–talk is the mechanism for the disruption of oogenesis in the Newark Bay population.

In Chapter 2, it was reported that there was a lack of a biomarker response for vitellogenin in Newark Bay males, despite the presence of many known xeno–estrogens in this ecosystem. Hepatic vitellogenin mRNA expression was normal and no detectable protein levels were measured in Newark Bay males (Fig. 2.5). Studies by McArdle et al. (2004) also reported the lack of vitellogenin responses in killifish injected with Newark Bay sediment extracts and speculated that contaminants may antagonize the ER–induction of vitellogenin. The caged study demonstrated that these vitellogenin biomarker responses were "false negatives" due to contaminant down–regulation of the vitellogenin pathway. Naïve Tuckerton males were transplanted into Newark Bay as an alternative approach and were sensitive to estrogenic contaminants, which resulted in the induction of hepatic vitellogenin. This approach should be used when the indigenous population has been chronically exposed or is suspected to have adapted altered responses to contaminants (i.e. altered AhR signaling). Results from the caged study demonstrated that vitellogenin (mRNA and protein) and ER α (mRNA) are transiently induced by xeno–estrogens in naïve Tuckerton males transplanted to Newark Bay after 1 month, but are down–regulated after 2 months (Figs. 3.1–3.2). Vitellogenin expression is not known to attenuate over time, and is one of the reasons why vitellogenin is a

commonly used biomarker. Therefore, this was direct evidence that contaminants in Newark Bay antagonize estrogenic responses and down–regulate ER–mediated pathways. Considering the caged study, and the down–regulation of vitellogenin induction (Chapters 3 and 4), it was proposed that complex mixtures of contaminants in Newark Bay may interfere with the utility of vitellogenin to be used as a biomarker for exposure to xeno–estrogens. This may also occur in other aquatic environments that are similarly contaminated, although this is a novel finding. The caged study offers an alternative method for measuring vitellogenin biomarker responses in populations where vitellogenin responses are "negative" when xeno–estrogens are known to be present.

Using biomarker responses (CYP1A) and chemical exposure analyses (bile PAHs), it was shown that killifish from Newark Bay had elevated exposure to AhR agonists. Bile PAHs are inexpensive and rapid endpoint to demonstrate PAH exposure. We demonstrated in Chapter 2 that Newark Bay killifish had elevated levels of naphthalene, pyrene, and benzo[a]pyrene in the bile (Table 2.2). A more general biomarker of exposure to AhR agonists is hepatic CYP1A expression levels, which is regulated by activation of the AhR2 pathway (Clark et al., 2010). Therefore, CYP1A expression can be thought of as a measure of AhR2 activity. In our current study, it was demonstrated that CYP1A was significantly elevated in males and females in Newark Bay (Fig. 2.2). Prince and Cooper (1995a,b) had previously demonstrated elevated basal levels of CYP1A activity, and taken together with the current studies demonstrated that killifish in Newark Bay are chronically exposed to AhR contaminants with long–term induction of the AhR2 pathway. Using hepatic expression levels for vitellogenin and CYP1A, an inverse correlation between CYP1A and vitellogenin was found in female

killifish (Fig. 2.8). Although this relationship is only correlative, this relationship indicates the potential for AhR2–ER cross–talk inhibition of the vitellogenesis pathway. There have been many studies using teleost hepatocytes *in vitro* to demonstrate that AhR agonists antagonize and inhibit the ER–mediated vitellogenin induction by estrogen (Anderson et al., 1996; Navas and Segner, 2000; Bemanian et al., 2004; Mortensen and Arukwe, 2007; Gräns et al., 2010). Considering the findings from these *in vitro* studies and the *in vivo* studies in the Newark Bay killifish population, it was proposed that the down–regulation of vitellogenin is a phenotypic effect of AhR–ER cross–talk. In order to better characterize this interaction an alternative teleosts model (zebrafish) was used to examine the role of AhR2 activation on the down–regulation of vitellogenin.

6.3. Role of AhR2 activation in the inhibition of vitellogenesis in zebrafish

The studies with the zebrafish demonstrate a clear role for AhR2 activation in the 2,3,7,8–TCDD inhibition of vitellogenin (VTG 1, 2 and 3) induction (Fig. 6.2). The AhR and vitellogenin (ER) pathways are highly conserved in teleosts, therefore this model was used as a comparative tool to help explain the reproductive effects in the Newark Bay killifish population and other populations exposed to similar contaminant profiles.

Our inhibition studies in the zebrafish clearly demonstrated that 2,3,7,8–TCDD and 1,2,3,7,8–PeCDD inhibit the mRNA induction of vitellogenins 1, 2 and 3 in a dose–dependent manner (Fig. 5.5, Table 5.1). In the 400 pptr exposure group (2,3,7,8–TCDD and 1,2,3,7,8–PeCDD), vitellogenin induction (VTG 1–3) was inhibited by greater than 95% (Table 5.1). These two contaminants were tested because they are found at elevated levels in Newark Bay sediments. From the results it is clear that AhR2 activation is

responsible for the cross–talk inhibition of vitellogenin (Fig. 5.6, Table 5.2). Morpholino knock–down of the AhR2 effectively reduced 2,3,7,8–TCDD inhibition of VTG 1–3 by 21.6 to 27.4% compared to the control morpholino injected group. These studies do not explain the exact mechanism by which AhR2 mediates cross–talk inhibition and future studies should examine in more detail the possibilities discussed below. It is likely to be a combination of mechanisms, including: direct inhibition of transcription via inhibitory elements, co–factor squelching, AhR–mediated synthesis of an inhibitory protein, ER degradation, and increasing E2 metabolism (Matthews and Gustafsson, 2006). The studies with the killifish demonstrated high AhR2 activity, and a correlation between AhR2 activity (CYP1A) and vitellogenin expression. The findings from the zebrafish study suggest that the AhR2 agonists in Newark Bay contributed to the down–regulation of the vitellogenin pathway through AhR2–ER cross–talk. However, studies in the killifish will need to explore and demonstrate the role of AhR2 activation in cross–talk and investigate contaminant effects in extra–hepatic tissues involved in the HPGL regulation of reproduction (Fig. 1.2). Future studies will also need to investigate the exact mechanism by which the AhR2 inhibits the ER pathway in teleosts (Fig. 6.2).

6.4. General Conclusions

These studies have shown an important relationship *in vivo* in teleosts between the highly conserved AhR and vitellogenin pathways in the liver. Killifish inhabiting Newark Bay have reduced fecundity that is due in part to AhR2 inducing contaminants, were shown to inhibit vitellogenesis through AhR2–ER cross–talk in the zebrafish model. From the literature it has been shown that decreased vitellogenin in oviparous species can

cause the reduced follicular development that was observed in the Newark Bay population. The deficiency of 17β–estradiol levels could explain the decreased vitellogenin levels but the mechanism behind the reduced 17β–estradiol levels is not known. In addition, the Newark Bay fish required higher 17β–estradiol to induce vitellogenin indicating the vitellogenin pathway in the liver of Newark Bay killifish is down–regulated (refractive). The attenuated vitellogenin response was not due to decreased expression of estrogen receptors in the liver or increased hepatic clearance of 17β–estradiol. The inhibition of vitellogenesis in Newark Bay killifish is a phenotypic effect of AhR2–ER cross–talk, which this work suggested to result in the inhibition of vitellogenin–dependent growth of oocytes.

Reproductive failure poses a serious threat to the sustainability of finfish populations. This Dissertation demonstrated that reproductive dysfunction of the Newark Bay killifish population was due to the inhibition of vitellogenin–dependent growth of oocyte (yolk–development) through vitellogenin down–regulation and reduced steroidogenesis (Fig. 6.1). The down–regulation of vitellogenesis was demonstrated to be mediated by the AhR2 in the zebrafish (Fig. 6.2). Taken together, these findings also help to explain why many other oviparous species (finfish and shellfish) have failed to sustain healthy populations within the NY–NJ Harbor Estuary. Cross–talk between the AhR– and ER–pathways may also explain why a number of reproductive endocrine disruptors cause reproductive effects in multi–generational studies even if they do not bind directly to the estrogen receptors. Other aquatic systems polluted with contaminants that induce AhR2 to elevated levels may also exhibit impacts on the vitellogenin pathway in native populations, which poses a threat to population sustainability. In addition, due to the

conserved nature of AhR– and ER–pathways amongst vertebrates, contaminants within Newark Bay resulting in the reproductive dysfunction of the killifish population may also pose a risk to the reproductive health of humans that consume fin and shellfish harvested from this system.

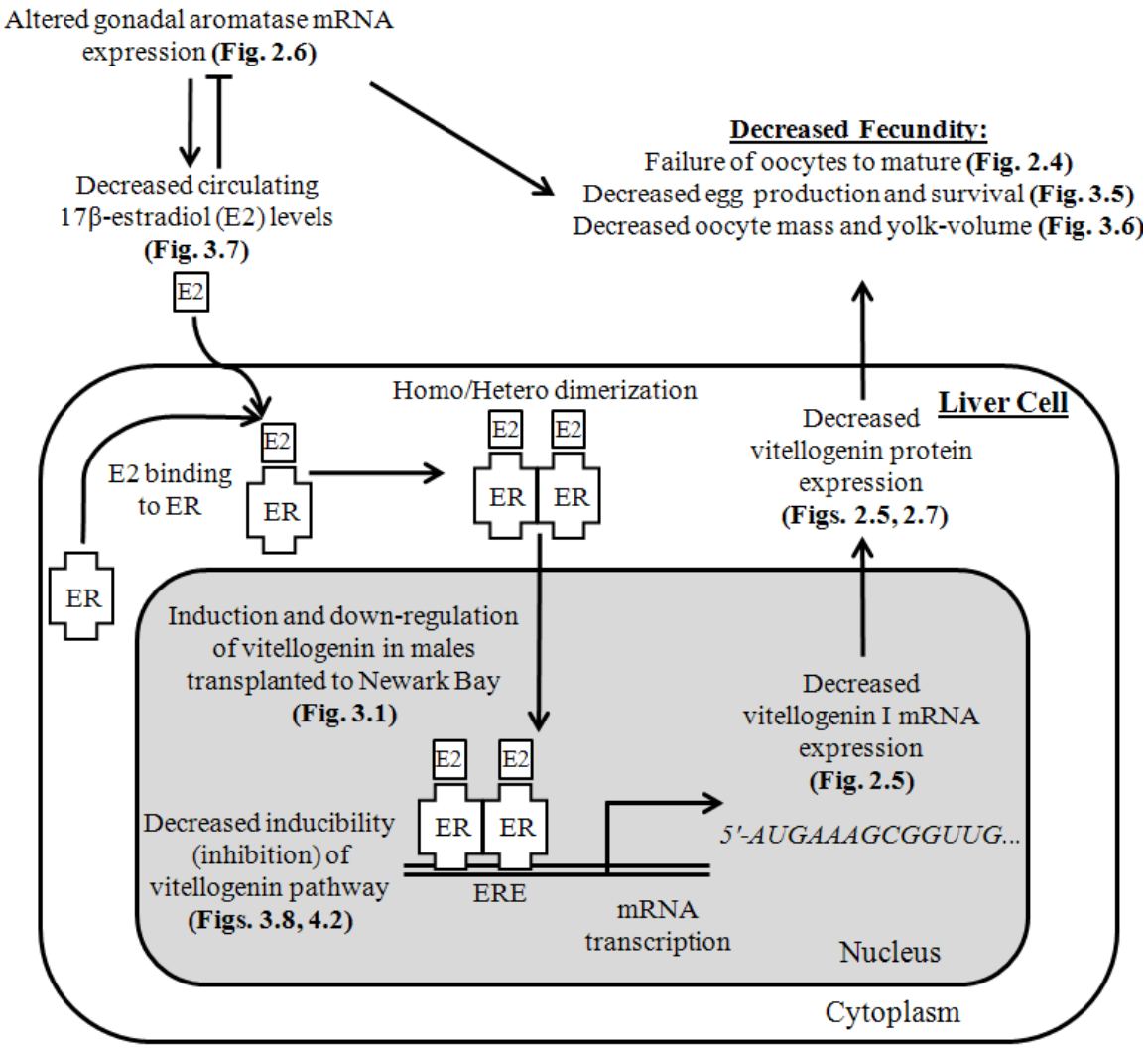

Fig. 6.1. Summary diagram of impacts on the gene regulation of vitellogenin in Newark Bay killifish population resulting in reproductive dysfunction (decreased egg production). Studies also showed that hepatic 17β–estradiol metabolism was normal in Newark Bay killifish (Fig. 4.5). Abbreviations: E2, 17β–estradiol; ER, estrogen receptor; ERE, estrogen responsive element.

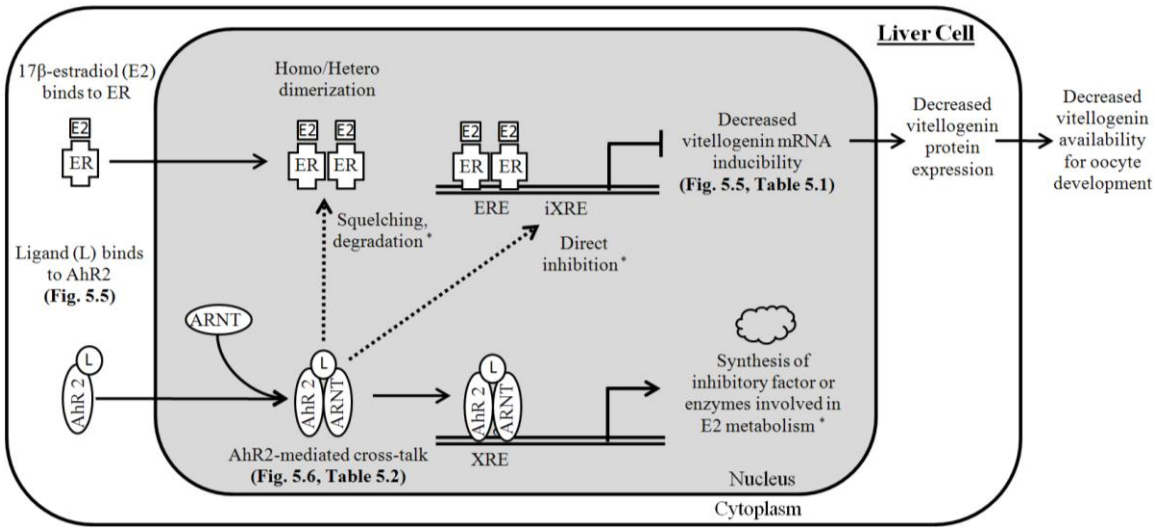

Fig. 6.2. Summary diagram of proposed AhR–ER cross–talk in zebrafish (Chapter 5). Studies with the zebrafish demonstrated that AhR2 activation mediates cross–talk. *Cross–talk can occur through a variety of mechanisms, including direct inhibition of transcription via inhibitory elements, co–factor squelching, AhR–mediated synthesis of an inhibitory protein, ER degradation, and increasing E2 metabolism (Matthews and Gustafsson, 2006). These findings provide insight into the reproductive impacts observed in killifish. Abbreviations: E2, 17β–estradiol; ER, estrogen receptor; ERE, estrogen responsive element; L, ligand, AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; XRE, xenobiotic response element; iXRE, inhibitory xenobiotic response element.

FUTURE AREAS OF RESEARCH

Based on the studies carried out for my Master's Thesis and Doctoral Dissertation, I recommend several future studies and areas of research requiring further attention. These are areas of teleost reproductive biology and toxicology that are fundamental to understanding contaminant impacts on reproduction, and had presented challenges when interpreting the data from my studies. They are listed in no particular order.

Teleost reproduction in general:

On the regulation of vitellogenin genes:

It is not clear how the different estrogen receptor isoforms interact to regulate each specific vitellogenin isoform. Each vitellogenin isoform should also be considered a uniquely regulated gene, which can be impacted by contaminants differently. Furthermore, in most oviparous species we also do not know which vitellogenin each yolk–protein is derived from, making it difficult to know how biologically important each vitellogenin isoform is and which isoform studies should focus on. Consider the vitellogenins in killifish: Studies by LaFleur et al. (2005) have characterized the yolk–proteins to demonstrate that the majority of yolk–proteins are derived from vitellogenin 1. Therefore, should studies focus on vitellogenin 1 because more yolk–proteins are derived from that specific isoforms? Is vitellogenin 2 less biologically important? Unlike the killifish, we do not know the derivation of yolk–proteins in the zebrafish (which have

7 known vitellogenin isoforms). Therefore, which vitellogenin should toxicological studies focus on without knowing how each contributes to yolk–protein synthesis? We also have nearly no understanding on the biological role of each yolk–protein in oocyte formation and embryonic development. How do impacts on yolk–protein formation by the cathepsins affect egg–quality and embryonic development? Do specific yolk–proteins perform specific roles in embryonic development? By expanding our knowledge of the biological importance of each vitellogenin isoform and yolk–protein, future studies can focus on specific parent vitellogenin isoforms.

On AhR–ER cross–talk:

Considerably less is known regarding the AhR–ER interaction in fish and impacts on reproduction in comparison to that of higher vertebrates. Considering vitellogenesis occurs in an organ with potentially high AhR activity, the AhR–ER interaction becomes extremely important to understand for chemical impacts on fish reproduction. Our studies were the first to demonstrate that the AhR2 in zebrafish mediates the inhibition of vitellogenesis by 2,3,7,8–TCDD. However, the roles of other AhR components such as the AhR1 and ARNT in cross–talk with the estrogen receptor are not clear. Future studies with fish models also need to investigate the proposed mechanisms of AhR–ER cross–talk (Fig. 1.7). The mechanism by which the specific estrogen receptor isoforms directly cross–talk with the AhR also needs to be explored. This emphasizes the need for studies that determine the mechanisms of estrogen receptor vitellogenin regulation. It is difficult to directly detect AhR–ER cross–talk in a field population of fish, as no biomarkers exist to indicate the different AhR–ER interactions. Therefore, development of biomarkers

specific for AhR–ER interactions would be a useful tool in directly evaluating reproductive impacts in a population from AhR agonist contamination.

On neuroendocrine disruption of the HPGL:

Considerably less is known regarding chemical impacts on neuroendocrine control of the hypothalamus and pituitary than chemical impacts on the ovary. The earliest events of oogenesis begin in the hypothalamus–pituitary which begin a cascade of events that turns on reproduction in the gonad and liver. Therefore, these earliest events may be susceptible to chemical disruption that may disrupt oogenesis and vitellogenesis that may result in reproductive failure and decrease the sustainability of populations. Therefore, studies are needed to correlate effects on the hypothalamus and pituitary regulation of reproduction with reproductive success and fecundity.

In Newark Bay killifish and other field studies:

Impacts on neuroendocrine and aromatase systems:

Future studies of the Newark Bay killifish population should focus efforts on steroidogenesis. Our studies have showed Newark Bay killifish have a high mRNA expression of aromatase and decreased circulating levels of 17β–estradiol (Chapters 2 and 3). The decreased circulating levels of estrogen were shown to be not due to elevated clearance in the liver (Chapter 5). Chemicals at Newark Bay may act as antagonists with aromatase and this effect could result in decreased circulating levels of 17β–estradiol.

Vitellogenin gene methylation:

The studies in this Dissertation demonstrated that the vitellogenin pathway is a target for contaminants found at Newark Bay, resulting in an attenuated pathway that is less inducible by 17β–estradiol in killifish. Studies by Strömqvist et al (2010) demonstrated that DNA methylation of CpG sites upstream of vitellogenin 1 in zebrafish were correlated with expression levels. Therefore, epigenetic modifications such as methylation are one likely regulatory factor in determining the activity of this pathway. The regulation of vitellogenins 1 and 2 by methylation is likely to offer insight into how these genes are controlled in the killifish. For example, the vitellogenin genes in Newark Bay may be hyper–methylated, which may explain the down–regulation of these genes. In addition, future studies should explore how chronic low–dose exposure to xeno–estrogens modifies the methylation state of estrogen–inducible genes.

Impacts on the male:

In Chapter 2 we demonstrated that males at Newark Bay have altered testis morphology, which may be due to chronic exposure to xeno–estrogens (Chapter 3). Future studies should investigate the etiology of the altered testis morphology and determine whether contaminants at the NY–NJ Harbor Estuary reduce the reproductive success of males.

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