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Effects of benzo[a]pyrene and CYP19a1b knockdown on zebrafish development

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EFFECTS OF BENZO[A]PYRENE AND CYP19A1B KNOCKDOWN ON ZEBRAFISH DEVELOPMENT

A dissertation presented in partial fulfillment of requirements for the degree of Doctor of Philosophy in the Department of BioMolecular Sciences Division of Pharmacology The University of Mississippi

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ABSTRACT

Benzo[a]pyrene (BaP) is a ubiquitous environmental contaminant that is both an endocrine disruptor and a carcinogen. Aromatase (CYP19) is a key enzyme in steroidogenesis playing a key role in the hypothalamus-pituitary-gonad feedback loop. We hypothesized that BaP would negatively impact cyp19a1b expression in zebrafish, in turn, adversely affecting development and physiology. Here, we consider whether the toxicities observed following BaP exposure are comparable to those following a transient morpholino (MO)-mediated CYP19a1b knockdown or exposure to an aromatase inhibitor (fadrozole) during early development. One-cell zebrafish embryos were injected with a CYP19a1b-MO or control-MO. Other non-injected embryos were exposed to nominal waterborne concentrations of BaP (0, 10 or 50 μg/L) and fadrozole (0, 10 or 50 μg/L) for 96 hours post-fertilization (hpf). Real-time PCR showed both BaP concentrations significantly decreased cyp19a1b expression in 96 hpf zebrafish larvae homogenates. Likewise, concentrations of E2 in 48 hpf whole body larval homogenates were significantly decreased by BaP, fadrozole and CYP19a1b-MO. Cumulative mortality of zebrafish larvae was significantly increased following BaP and fadrozole exposure and CYP19a1b knockdown compared to controls. Estradiol (E2, 10 nM) co-treatment rescued mortality mediated by 10 μg/L BaP, 10 μg/L fadrozole, and CYP19a1b-MO. In a treatment-blinded morphological assessment of larvae at 96 hpf, several phenotypes were negatively impacted by BaP, fadrozole, and CYP19a1b knockdown including body length, optic vesicle size, swim bladder inflation, pericardial and abdominal edema, and incidence of normal larval tail shape and these effects were reversed by exogenous E2-cotreatment. Decreased incidence of normal pectoral fins was only impacted by BaP exposure. In conclusion,
certain adverse developmental outcomes caused by BaP exposure are at least in part related to BaP-mediated CYP19a1b inhibition.
LIST OF ABBREVIATIONS

Aromatase inhibitors (AIs)

Aromatase knockout mice (ArKO mice)

Aromatase cytochrome P450 (P450arom)

Aryl hydrocarbon receptor (AHR)

Benzo[a]pyrene (BaP)

Benzo[a]pyrene diol epoxide (BPDE)

Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA)

Day post-fertilization (dpf)

Emission/energy units full scale (EUFS)

United States Environmental Protection Agency (EPA)

Follicle stimulating hormone (FSH)

Green florescent protein (GFP)

High molecular weight (HMW)

Hour post-fertilization (hpf)

High performance liquid chromatography (HPLC)
International Agency for Research on Cancer (IARC)

Low density lipoprotein (LDL)

Low molecular weight (LMW)

Luteinizing hormone (LH)

Maximum Contaminant Level (MCL)

National Institute for Occupational Safety and Health (NIOSH)

Occupational Safety and Health Administration (OSHA)

Permissible exposure limit (PEL)

Primordial germ cells (PGCs)

Polycyclic aromatic hydrocarbons (PAHs)

Tributyltin (TBT)

Triphenyltin (TPT)

1-hydroxypyrene (1-OHPyr)

5α-dihydropyosterone (DHT)

17β-estradiol (E2)
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CHAPTER 1. INTRODUCTION

1.1 Polycyclic aromatic hydrocarbons (PAHs)

1.1.1 PAHs and its abundance in the environment

PAHs are organic compounds that are only composed of carbon and hydrogen, and contain multiple fused aromatic (benzene) rings with no additional substituents or heteroatoms. Based on their molecular weight, PAHs are usually classified into two classes; low molecular weight (LMW) and high molecular weight (HMW) PAHs. Members that contain two to three benzene rings are known as LMW, and those members that have more than four benzene rings are known as HMW PAHs. Generally, characteristics of PAHs are high melting and boiling points, low vapor pressure, and very low aqueous solubility. The differences in their sizes and structures make HMW PAHs more lipophilic, less volatile, and more resistant to oxidation, reduction, and degradation by microorganisms (therefore, they can easily adhere to sediments for months). In comparison LMW PAHs can react with sunlight, ozone or NO$_2$ and break down in days or weeks (Perraudin et al., 2007, Bamforth and Singleton, 2005, Wang et al., 2005).

In the environment, PAHs exist ubiquitously as a complex mixture. At least thirty PAH compounds (including benzo[a]pyrene (BaP)) are listed by the United States Environmental Protection Agency (EPA) (http://www.epa.gov/region1/npdes/permits/generic/priority_pollutants.pdf) as priority pollutants. They are mainly formed by incomplete combustion of organic compounds including through natural processes like forest fires, oil seeps, volcanoes, microorganisms and anthropogenic processes including petroleum, electric power generation,
refuse incineration, home heating, production of coke, carbon black, coal tar, and asphalt, internal combustion engines and tobacco smoke. PAHs occur and are widely spread in air, water, soil, and sediment. PAHs emissions after combustion are able to suspend in the air, be transferred long distances (Ma et al., 2013, González-Gaya et al., 2014), be adsorbed to particulate matter such as diesel particulate matter (Wichmann 2007), and finally deposit in the soil. PAH pollution is not only restricted to the atmosphere (Zhao et al., 2015, Shen et al., 2013), but also affects the aquatic (Allan et al., 2012, Chen et al., 2015, Wu et al., 2011), urban (Pozo et al., 2012, Yu et al., 2014, Çabuk et al., 2014), and industrial (Martins et al., 2011, Abril et al., 2014, Dong et al., 2012) ecosystems.

1.1.2 Human exposure to PAHs

There are various routes through which humans can be exposed to PAHs including the respiratory and digestive systems and skin. Through the respiratory tract, exposure to PAHs occurs by inhalation of PAH-particulate matter in air such as first and second-stream cigarette smoke (Nelson 2001, Rubin 2001, DeMarini 2004), vehicle exhaust (Finlayson-Pitts and Pitts 1997), and smoke from residential cookstoves and heaters (Li et al., 2011b, Alkurdi et al., 2014, Shen et al., 2014). Exposure to PAHs through the digestive tract happens via consumption of PAHs-contaminated foodstuff including fried and charcoal grilled meat (Larsson et al., 1983, Sinha et al., 1994), and PAH-contaminated vegetables and fruits which grow in areas that are near to traffic and factories sources (Phillips 1999, Camargo and Toledo 2003). Several studies have shown that the exposure to PAHs through diet is much higher than exposure to PAHs through inhalation for non-smokers (Lioy et al., 1988, Vaessen et al. 1988, De Vos et al., 1990, Lodovici et al., 1995, Beckman et al., 1998). Finally, humans can be exposed to PAHs through the skin following
contact with petroleum substances (such as soot, tars, pitch) or with water and soil next to contaminated areas (Van Rooij et al., 1993, Moody et al. 2007). The total concentrations of PAHs on skin of roofing workers and road-paving crews can reach up to 1,400 ng/cm² (Jongeneelen et al., 1988).

Importantly, higher exposure to PAHs can occur in workers in occupational settings including aluminum production, coal gasification, coke production, iron and steel foundries, tar distillation, shale oil extraction, wood impregnation, roofing, road paving, carbon black production, carbon electrode production, chimney sweeping, and calcium carbide production and transport industry (Petry et al. 1996, Boffetta et al. 1997, Rota et al. 2014). Therefore, the Occupational Safety and Health Administration (OSHA) determined the permissible exposure limit (PEL) of 0.2 mg/m³ of PAH in work areas, measured as the benzene-soluble fraction of coal tar pitch volatiles. The OSHA standard for coke oven emissions is 0.15 mg/m³ of PAH. The National Institute for Occupational Safety and Health (NIOSH) has recommended that the workplace exposure limit for PAHs be set at 0.1 mg/m³ for coal tar pitch volatile agents.

Studies have shown that maternal PAH exposure during pregnancy could lead to prenatal PAH exposure, by testing maternal blood cord for PAH-DNA adducts (Jedrychowski et al., 2014, Jedrychowski et al., 2013). A recent study has used the urinary metabolite of pyrene, 1-hydroxypyrene (1-OHPyr) as a biomarker of total PAH exposure to detect the PAH levels in preschool children in Ohio. They found that the median urinary 1-OHPyr concentration was 0.33 ng/mL in these children (Morgan et al., 2015). This concentration was significantly higher compared to other US children. Moreover, as high as 0.67 µg/L of 1-OHPyr was detected in amniotic fluid, and 0.15 mmol/mol creatinine of 1-OHPyr was found in maternal urine following
smoking during pregnancy (de Barros Machado et al., 2014). These concentrations of 1-OHPyr are significantly higher compared to what are found in non-smoking mothers.

1.1.3 PAHs as a human health concern

Various harmful health effects can result follow either short or long-term PAHs exposure. Eye irritation, nausea, vomiting, diarrhea, and allergic skin response have been reported as short term effects after occupational exposures to PAHs (Unwin et al., 2006). In addition to above effects, headaches, dizziness, cough, respiratory diseases, and chest pain have been reported in individuals in Louisiana during early months of the Deep Water Horizon oil spill (Solomon and Janssen 2010). Also, impaired lung function in asthmatics and thrombotic effects in coronary heart disease patients are short term effects of PAH exposure (ACGIH, 2005). However, short term effects depend on length of exposure, concentrations of PAHs, route of exposure, age, and pre-existing health conditions.

The primary health concerns associated with long term PAHs exposure are their carcinogenic and mutagenic toxicities. Among PAHs, BaP, coal tars, coal-tar pitches and tobacco are listed in group 1 (carcinogenic to humans) in the World Health Organization International Agency for Research on Cancer (IARC) classification. Group 2A (probably carcinogenic to humans) includes dibenz(a,h)anthracene, dibenzo(a,l)pyrene and creosotes, while group 2B (possibly carcinogenic to humans) includes benz(a)anthracene, benzo(b)fluoranthene, benzo(c)phenanthrene, benzo(j)fluoranthene, benzo(k)fluoranthene, dibenzo(a,h)pyrene, dibenzo(a,i)pyrene, indeno(1,2,3-cd)pyrene, chrysene, and naphthalene (http://monographs.iarc.fr/ENG/Classification/index.php). Risk of developing lung cancer
following occupational (Armstrong et al., 1994) and environmental (Zhang et al., 2009) exposure to PAHs has been reported (Boffetta et al., 1997, Brüske-Hohlfeld, 2009). Also, different types of cancer are believed to be associated with PAHs exposure. These include colorectal cancer (Alexandrov et al., 1996), skin cancer (Mastrangelo et al., 1996), liver cancer (Chen et al., 2002), esophageal cancer (Gustavsson et al., 1998), laryngeal cancer (Elci et al., 2003), renal cancer (Karami et al., 2011), bladder cancer (Geller et al., 2008), prostate cancer (Rybicki et al., 2006), pancreas cancer (Alguacil et al., 2003), and breast cancer (Terry et al., 2004).

Negative impacts on reproduction associated with PAH exposure are another human health concern. The number of human primordial germ cells, which will differentiate and develop to form gonad, were reduced following PAH exposure (Kee et al., 2010). Increased risk of male idiopathic infertility was associated with increased urinary concentrations of the sum of PAH metabolites (Xia et al., 2009). Another study by Xia et al. (2009) found alterations in male semen quality, which was evaluated by semen volume, sperm concentration, sperm number per ejaculum, and sperm motility as a result of PAHs exposure (Xia et al., 2009). Severely damaged sperm DNA in infertile men was correlated with high PAH-adduct levels (Gaspari et al., 2003).

Increased infertility due to PAH exposure is not only restricted to men. Epidemiological studies have shown a correlation between cigarette smoking and menstrual abnormalities (Hornsby et al., 1998) and infertility in women (Laurent et al., 1992). Also, follicular fluid and serum of smoking women were found to have significantly higher concentrations of PAHs compared to non-smoking women. Ability of PAH to enter the placenta and negatively impact pregnancy outcomes, such as early pregnancy loss and preterm delivery, have been previously reported (Dejmek et al., 2000, Wu et al., 2010, Singh et al., 2008, Gladen et al., 2000).
Another human health concern is that developmental toxicities have been linked to PAH exposure. Fetal growth restriction and low birth weights were observed after prenatal PAH exposure from air pollution or maternal smoking (Ong et al., 2002, Choi et al., 2008). Other studies have shown that prenatal exposure to PAHs adversely impacts children’s cognitive development at 3 years of age (Perera et al., 2006), and children’s IQ at 5 years of age (Perera et al., 2009). High levels of PAH-DNA adducts in maternal and cord blood was associated with negative child behavior, at 6-7 years of age, characterized as anxiety and depression (Perera et al., 2012). Also, it has been recently reported that the development of left hemisphere white matter is disrupted due to prenatal exposure to PAH air pollutants that is, in turn, related to slower processing speed, attention-deficit/hyperactivity disorder symptoms, whereas postnatal PAH exposure lead to further disturbances in the development of white matter in dorsal prefrontal regions (Peterson et al., 2015). High PAH concentrations in maternal serum increases the risk of neural tube defects in offspring (Wang et al., 2015). Additionally, in a cohort from Krakow Poland, PAH-mediated birth-length deficit persisted, and children that had prenatal PAH exposure above 34.7 ng/m$^3$ was associated with decreased height by 1.1 cm at age nine (Jedrychowski et al., 2015).

1.1.4 Benzo[a]pyrene as a model PAH

BaP is a five ring polycyclic aromatic hydrocarbon (Fig 1), which is often found in PAH mixtures. On the recent ranking of CERCLA’s Priority List of Hazardous Substances, BaP was ranked as #8 (http://www.atsdr.cdc.gov/spl/). Like all PAHs, BaP is ubiquitously found in sediment, soil, and ambient air resulting from incomplete combustion of organic materials and processed food. Inhalation, ingestion of contaminated food, and drinking contaminated water are the major BaP exposure sources. The highest airborne BaP concentration reached 9.6 μg/m$^3$ in
traffic tunnels (De Fré et al., 1994). In Minnesota, a study of children’s exposure to PAHs has measured BaP concentrations in house dust, personal air, outdoor air, and food samples and found BaP in 43-58% of various types of air samples, 19% of household dust samples, and 22% of food samples (Clayton et al., 2003). A recent study of BaP concentrations in urban, industrial and semi-urban areas in Malaysia detected BaP concentrations as high as 0.61 ng/m$^3$ (Jamhari et al., 2014). Although, BaP is found in food, its concentrations vary based on food types. BaP concentrations in non-meat food such as greens and cereals were generally low ~0.5 ng/g. Meat-food including fried, grilled, and barbecued meat have higher BaP concentrations compared to non-meat food (Kazerouni et al., 2001). In a survey of nine Malaysian grilled meat meals, the highest BaP concentration was reported (up to 12.5 μg/kg) in barbecued beef satay (Farhadian et al., 2010). However, fat content (Chen and Chen, 2001, White et al., 2008), heat temperature, and heat source (Chung et al., 2011b, Reinik et al., 2007) are key factors that contribute to varied BaP concentrations in meat-food. Additionally, BaP can exist in drinking water, even though it has low water solubility (2.3 to 4 μg/L) (Mackay and Shiu, 1977). The EPA Maximum Contaminant Level (MCL) for BaP in concentrations drinking water is 0.2 μg/L (http://water.epa.gov/drink/contaminants/index.cfm#1).

![Figure 1. BaP and its metabolite structures (Bui et al., 2009)](image-url)
1.1.5 BaP and its reproductive and developmental toxicities

BaP is classified as an endocrine disruptor due to its adverse impacts on reproductive success. In humans, BaP exposure is related to alterations of sperm morphology and decreased sperm and eggs numbers (Cordier et al., 1997, Zenzes et al., 1998). Benzo(a)pyrene diol epoxide (BPDE) DNA adducts (Fig 1) have been detected at higher amounts in sperm cells of smokers compared to non-smokers (Zenzes et al., 1999a). Higher BaP concentrations were found in serum and follicular fluid of smoking women compared to non-smoking women. Those women, with up to $1.79 \pm 0.03$ ng/ml of BaP in their follicular fluid, did not conceive (Neal et al., 2008). In addition, BaP has also been found in maternal blood, placenta, cord blood and human breast milk (Madhavan and Naidu, 1995).

In various in vitro and in vivo animal studies, the reproductive toxicity of BaP is well established. BaP exposure inhibited follicle growth in isolated rat follicle culture assay (Neal et al., 2007) and reduced fertility and primordial oocyte number in mice in a dose-dependent manner (Mattison et al., 1979). Fertility parameters such as testis histology, sperm count, and sperm motility of male mice were significantly altered by BaP exposure, and these negative effects were observed in three subsequent generations (Mohamed et al., 2010). Also, plasma progesterone, estrogen, and prolactin concentrations of female rats were reduced following BaP exposure (Archibong et al., 2002). In male rats, BaP exposure reduced testis weight, plasma testosterone concentrations, and increased luteinizing hormone (LH) concentrations (Ramesh et al., 2008). Similarly, waterborne BaP exposures of Fundulus caused significantly decreased testosterone concentrations and testes weights in males and decreased estradiol concentrations in females. Also, BaP significantly altered egg fertilization (Booc et al., 2014).
Besides the reproductive success, developmental success is significantly compromised by BaP. In humans, BaP-DNA adducts have been detected in preimplantation embryos of smoking parents (Zenzes et al., 1999b). Dietary BaP intake during pregnancy was associated with low birth weight (Duarte-Salles et al., 2013). In animals, maternal BaP exposure can lead to distribution of BaP into placenta. This was reported in mice (McCabe and Flynn, 1990), rats (Withey et al., 1993), guinea pigs (Kihlström, 1986), and primates (Lu et al., 1993). Prenatal BaP exposure lead to decreased fetal survival (Archibong et al., 2002), low birth weight, and developmental abnormalities (Barbieri et al., 1986, LeGraverend et al., 1984).

1.2 Aromatase

1.2.1 Mammalian aromatase

Aromatase, which is encoded by CYP19 gene, is a complex enzyme that is formed of two components: aromatase cytochrome P450 (P450arom) and, coupled to it, a ubiquitous flavoprotein, NADPH-cytochrome P450 reductase (reductase). This enzyme is responsible for the conversion of C19 androgen (typically testosterone and androstenedione) to C18 estrogen (Nelson et al., 1996). Aromatase in all mammals, with the exception of pigs, have a single form of the CYP19 gene (Sebastian and Bulun, 2001). The CYP19 gene in humans is a single copy gene located on chromosome 15. This entire gene spans over 123 kb of DNA, but only 30 kb represents the coding region (exons II to X) (Sebastian and Bulun, 2001). Human tissue specificity in aromatase gene regulation is due to alternative promoter splicing (Simpson et al., 1993). For example, untranslated first exons notated I.1, 2a, I.4, I.5, I.f, I.2, I.6, I.3, and PII are spliced for expression in placenta (major), placenta (minor 2), skin/adipose, fetal tissues, brain, placenta
(minor 1), bone, adipose/breast cancer, ovary/breast cancer/endometriosis, respectively (Fig. 2) (Meinhardt and Mullis, 2002, Sebastian and Bulun, 2001).

**Figure 2.** Human aromatase (CYP19) gene. In humans, expression of the aromatase gene is regulated by the tissue-specific activation of a number of promoters via alternative splicing reprinted with permission (Bulun et al., 2005).

However, aromatization of testosterone mainly occurs in the endoplasmic reticulum of estrogen-producing cells (Simpson et al., 2002). Aromatase is expressed in different cells, including the ovarian granulosa cell, the placental syncytiotrophoblast, the testicular Leydig cell, and various extraglandular tissues, including the brain, adipose stromal cells, osteoblasts in bone, skin fibroblasts and fetal tissues (Conley and Hinshelwood, 2001, Simpson et al., 1994). In premenopausal women, the ovarian granulosa tissues have the highest levels of aromatase
expression and produce estradiol as a primary product during the follicular phase. This ovarian aromatase expression is induced by follicle stimulating hormone (FSH) through activation FSH receptors that mediate the cAMP production and activation of promotor II (Simpson et al., 1994). In contrast, extragonadal tissues, such as adipose tissue and skin fibroblasts, are the major aromatase-expressing tissues in men and women after menopausal period (Grodin et al., 1973). Aromatized estrogen by these extragonadal tissues is very critical for many physiological processes such as closure of bone plates and bone mineralization (Bulun, 1999). In adipose and skin fibroblast tissues, the aromatase expression is exerted by a distal promoter (I.4) located 70 kb upstream of the coding region. A dual action of glucocorticoids and cytokines [e.g., interleukin (IL)-6, IL-11, leukemia inhibitory factor, and oncostatin-M] regulates promoter (I.4) (Zhao et al., 1995). However, the primary aromatization product of adipose tissues is the estrone, which is biologically weaker than estrogen. Because a relatively large amount of estrone is produced by adipose tissues, at least half of this peripherally produced estrone eventually could be converted to estradiol in tissues outside of the ovary (Perel and Killinger, 1979). In contrast to humans, aromatase expression in lower mammals (rodents), birds, and fish is expressed in the brain and gonad by highly conserved promoters I.f and II, respectively (Simpson et al., 1994). In higher mammals, aromatase is expressed in placenta, skin, adipose tissue, and bone.

Regarding the human brain aromatase enzymatic activity, immunoreactivity and gene expression (mRNA) were reported in studies of specific brain regions, including the temporal cortex (Steckelbroeck et al., 1999), hypothalamic and ventral forebrain nuclei (Ishunina et al., 2005), hippocampus (Stoffel-Wagner et al., 1999), and thalamus (Sasano et al., 1998). The rat and monkey brain has low aromatase expression, with high expression present only in the preoptic area, ventromedial nucleus of the hypothalamus, medial amygdala, and the bed nucleus of the stria
While the aromatase expression in various tissues is regulated by the use of tissue-specific promoters throughout the action of different transcription factors and signaling pathways, the aromatase brain expression regulation is unclear and complex due to its abundance in many brain regions. Thus, it is suggested that the aromatase expression regulation is region-specific (Roselli et al., 1985, Zhao et al., 2007). A study of rat brain aromatase expression has shown that the aromatase expression in most brain areas, including the amygdala and hippocampus, is not regulated by gonadal steroids as the aromatase expression in preoptic area and hypothalamus (Abdelgadir et al., 1994). Thus, it was suggested that there may be both steroid-dependent and/or steroid-independent processes regulating aromatase expression in the different brain regions. Generally speaking, aromatase expression and specific estrogen receptors are crucial for many physiological processes, including cellular proliferation, reproduction, sexual behavior, aggression, cognition, memory and neuroprotection in various animal species (Garcia-Segura, 2008, Saldanha et al., 2009).

### 1.2.2 Aromatase deficiency

Aromatase deficiency is a condition characterized by high concentrations of circulating testosterone and low concentrations of estrogen. Models of aromatase deficiency, from reported human cases or the aromatase knockout mice model (ArKO mice), have highlighted the importance of the aromatase enzyme and estrogen in both sexes (Simpson, 2000, Grumbach and Auchus, 1999). The mutation in most individuals with aromatase deficiency is characterized by a single base pair change that leads to either a single amino acid substitutions or a premature stop codon (Carani et al., 1997, Morishima et al., 1995, Conte et al., 1994, Ito et al., 1993, Mullis et al., 1997). In another example, an exon-intron splice junction was disrupted (Shozu et al., 1991).
Although most reported cases have homozygous mutations, some are heterozygous mutations (Mullis et al., 1997, Conte et al., 1994). Recently, an aromatase deficiency case documented a patient that had a heterozygous aromatase mutation that disrupted the aromatase protein structure (Chen et al., 2015). In most reported cases, females have virilization during the pregnancy period that subsides postpartum. Therefore, manifestations of aromatase deficiency in females actually depend on development stage. During prenatal development, aromatase deficiency causes obvious masculinization of urogenital sinus and external genitalia and pseudohermaphroditism in the fetus. Whereas during puberty, defects in aromatase affects development of secondary sex characteristics and causes delayed skeletal maturation, virilization, polycystic ovaries and hypergonadotropin hypergonadism (Grumbach and Auchus 1999, Zirilli et al., 2008). The common phenotypes in men with aromatase deficiency are hypergonadotropism, macroorchidism, tall stature owing to failure of epiphyseal fusion, delayed bone maturation that resulted in osteopenia, elevation of low density lipoprotein (LDL) cholesterol, hyperinsulinemia, increased triglyceride levels, elevation of testosterone, FSH, LH, and reduced circulating estrogen (Sudeep et al., 2013, Chen et al., 2015, Baykan et al., 2013).

ArKO mice have allowed for further insight into the sex-, tissue- and developmental stage-dependence of estrogen homeostasis as mediated by aromatase (Hill and Boon, 2009, Britt et al., 2001). Male ArKO mice have altered spermatogenesis and impaired sexual behavior that leads to compromised fertility (Robertson et al., 2001, Conte et al., 1994). Also, they have displayed prostate enlargement, and elevation of circulating testosterone and 5α-dihydrotestosterone (DHT) levels (McPherson et al., 2001). Infertility of female ArKO mice is due to ovarian dysmorphosis and degeneration (Britt et al., 2000). Moreover, they have low estradiol, and high testosterone concentrations compared to the wild type females (Fisher et al., 1998). Both ArKO males and
females have obese phenotype with increased adipocyte volume and number in gonadal and infrarenal fat depots (Jones et al., 2000). Also their serum cholesterol, high density lipoprotein (HDL), leptin, and insulin concentrations are high. Despite both sexes of ArKO mice showing these metabolic syndromes, only male ArKO mice develop hepatic stenosis (Van Sinderen et al., 2014). Additionally, decreased trabecular bone volume and thickness in both ArKO males and females results in skeletal abnormalities mediated by estrogen deficiency due to the aromatase deficiency (Öz et al., 2000).

1.2.3 Estrogen homeostasis

It is well known that estrogen homeostasis is very important to many mammalian physiological processes including: reproduction (Gibson and Saunders, 2012, Brock et al., 2011, Akingbemi, 2005, Rochira et al., 2001), development (Fernández-Pérez et al., 2013, Baker, 2013), behavior (Balzer et al., 2015, McCall and Singer, 2012), and carcinogenesis (Hu et al., 2012, Suba, 2012). Estrogen is not only responsible for functional regulation of the uterus, ovary, and breast, but is also critical in normal metabolism of bone (Klein-Nulend et al., 2014, Suba, 2012, Khosla et al., 2012) and lipids (Mauvais-Jarvis et al., 2013, Kim et al., 2014), in vascular function (Tiyerili et al., 2012, Chen et al., 2015, Kim et al., 2014) and arteriosclerosis (Nofer, 2012, Barton, 2013).

Aromatase deficiency and postmenopausal conditions, which are both characterized by estrogen insufficiency, highlight the importance of estrogen. Most phenotypes, including osteoporosis, delayed bone maturation, hypergonadotropism, hyperinsulinemia, dyslipidemia, ovarian cyst in females, macroorchidism in males, and non-alcoholic fatty liver, resulting from estrogen deficiency in patients with aromatase deficiency can be restored by estrogen replacement
therapy (Bilezikian et al., 1998, Burckhardt et al., 2015, Baykan et al., 2013, Sudeep et al., 2013, Bulun, 1999). It is well known that postmenopausal women are at high risk to develop osteopenia, osteoporosis, and cardiovascular diseases. Whereas estrogen replacement therapy during the early postmenopausal period prevents reductions in bone density, osteoporosis (Lindsay et al., 2005, Bagger et al., 2004), and decreases risk of mortality from heart failure or myocardial infarction (Schierbeck et al., 2012).

1.2.4 Aromatase as a potential target for endocrine disrupting chemicals

Some environmental contaminants alter aromatase expression by either inhibition or induction. For example, organotin compounds like tributyltin (TBT) and dibutyltin, inhibit human placenta aromatase activity in vitro (Cooke, 2002). Also, TBT in combination with bisphenol-A, or nonylphenol, have synergistic inhibitory effects on aromatase activity (Benachour et al., 2007). In Denmark and Finland, there has been a reported association between newborn boys with congenital cryptorchidism and high concentrations of TBT in their mother’s placenta (Rantakokko et al., 2013). In a teleost fish, guppy (Poecilia reticulata), TBT inhibited male brain aromatase expression which was associated with elevated testosterone concentrations and a disturbance of reproductive behavior (Tian et al., 2015). Other environmental chemicals including methylmercury (Hinfray et al., 2006), triazole and imidazole fungicides (Tröskén et al., 2004), perfluorinated chemicals (PFCs) such as perfluorooctanesulfonate (PFOS), perfluorobutanesulfonate (PFBS), and perfluorooctanoic acid (PFOA) (Gorrochategui et al., 2014) have been suggested as aromatase inhibitors; whereas estrogenic compounds such as nonylphenol (Bonefeld-Jorgensen et al., 2007), bisphenol-A (Nativelle-Serpentini et al., 2003, Chung et al., 2011a) and ethynylestradiol (Roggio et al., 2014) induce aromatase expression. Therefore, the
possibility that aromatase expression and its downstream physiological events are affected by environmental contaminants has been a target of intense recent research (Sanderson, 2006, Cheshenko et al., 2008, Mills et al., 2014). Our previous work on Fundulus heteroclitus found that BaP-waterborne exposure inhibited both adult and embryo brain aromatase expression (Dong et al., 2008).

1.2.5 Therapeutic aromatase inhibitors

Aromatase inhibitors (AI) are generally classified based on their chemical structures as steroidal and non-steroidal AIs. Among reported AIs, 80% belong to the steroidal class because their chemical structures are related to the natural aromatase substrate. They include: formestane, exemestane, atamestane and 10-propargylandrostenedione. The steroidal class are able to inactivate aromatase enzyme by binding tightly or irreversible to the active site of the enzyme and preventing the endogenous aromatase substrates from the binding and converting to estrogen. Due to their strong binding, they are considered as selective AIs (Njar and Brodie, 1999). Unlike steroidal AIs, non-steroidal AIs including aminogluthethimide, fadrozole, anastrozole, and letrozole, have a heteroatom that interferes with steroid hydroxylations by binding to the heme iron of the cytochrome P450s (Brueggemeier et al., 2013). Also, AIs are classified as first, second, and third generation due to their priority in clinical use.

Briefly, AIs are clinically used for estrogen sensitive breast cancer in postmenopausal women (Brueggemeier et al., 2013, Dowsett et al., 2010) and gynecomastia in children and adolescents (Shulman et al., 2008). Other off-label uses of AIs have been reported such as treatment of impaired spermatogenesis in men with excess aromatase activity (Schlegel, 2012),
ovulation induction in infertile women (Palomba, 2015, Casper and Mitwally, 2012), delayed epiphysial maturation and to increase predicted adult height in boys with idiopathic short stature and constitutional delay of puberty (Palmert, 2015, Shams et al., 2014).

1.2.6 Developmental defects associated with aromatase disruption

The ability of endocrine-disrupting chemicals to interfere with the steroid hormone biosynthesis pathway, including aromatase, has been previously elucidated (Sanderson, 2006, Zoeller et al., 2012). Over the past two decades, several studies have reported human developmental defects in offspring of pesticides applicators living in rural Minnesota (Garry et al., 1996). Some of the pesticides applied included endocrine disrupting chemicals like triphenyl tin (TPT) (Garry et al., 2002), which inhibits aromatase (Saitoh et al., 2001). Central nervous, cardiovascular, gastrointestinal, urogenital, and musculoskeletal systems were major organ systems that were reported among birth defects in the Minnesota cohort (Garry et al., 2002). In rural areas in Argentina, glyphosate (a herbicide which also inhibits aromatase (Gasnier et al., 2009)) was reported to induce birth defects include neural defects and craniofacial malformations (Ho, 2010). These observed malformations were similar to developmental defects in frog embryos exposed to glyphosate in the laboratory (Ho, 2010). Developmental defects were also associated with prenatal exposure to other aromatase disrupting chemicals such as phthalates and bisphenol-A (Escamilla-Nunez et al., 2015, Philippat et al., 2012, Bustamante-Montes et al., 2013, Gascon et al., 2015, Axelsson et al., 2015). In animals, an aromatase modulator (letrozole) following gestational exposure showed toxic effects on prenatal development in rats like increased post-implantation loss and vertebral anomalies (Tiboni et al., 2008). Prenatal exposure of another aromatase modulator, diethylstilbestrol, caused malformation of the external genitalia of male and female mice (Mahawong et al., 2014).
1.3 Zebrafish as model organism

Zebrafish, *Danio rerio*, are tropical fresh water fish that are native to inland streams and rivers of India, and also distributed in North America, Africa, and Europe. They are a highly appreciated model in developmental biology (Grunwald and Eisen, 2002). More recently they have been successfully used in toxicology testing (Parng, 2005), biomonitoring (Liao et al., 2012), biomedical research (Brittijn et al., 2009), and drug development (Chakraborty et al., 2009). Additionally, they have become an attractive model for environmental risk assessments owing to their ability to provide small-scale and high-throughput analyses (Scholz et al., 2008, Bugel et al., 2014, Mandrell et al., 2012). A crucial advantage of fish is that they are particularly well suited for reproductive and developmental studies because of their transparent chorions, high fecundity and rapid development. Relevant to risk assessment and chemical screening, the developmental landmarks in zebrafish as they relate to adverse outcome pathway (AOP) development have also been recently established (Villeneuve et al., 2014). They develop similar organ systems and share common biochemical and molecular pathways with mammals (Patton and Zon, 2001, Bondesson et al., 2015, Kettleborough et al., 2013, Howe et al., 2013). Compared to other animal models, they are easy to maintain in the laboratory owing to their small size, hardiness, short generation time and low cost. Importantly, embryos are able to absorb compounds in water owing to the tiny pores of their chorion (Goldsmith, 2004). Many advantages make zebrafish the favorable model compared to other fish models, like *Fundulus*, including their well annotated genome (Woods et al., 2000, Kelkar et al., 2014), the precise description of their developmental stages and the seven periods of embryogenesis (Kimmel et al., 1995), and the availability of knockdown/out technologies (Kelly and Hurlstone, 2011, Timme-Laragy et al., 2012) and transgenic strains that allow for pinpointing critical molecular targets associated with toxicity phenotypes (Weinstein, 2002).
1.3.1 Aromatase in fish

Unlike most mammals, aromatase in fish is encoded by two distinct CYP19 genes (Sebastian and Bulun, 2001, Meinhardt and Mullis, 2002, Britt et al., 2000, Conley and Hinshelwood, 2001). These are CYP19A1 (cyp19a1a), which is mainly expressed in the ovary and CYP19A2 (cyp19a1b), which is expressed in brain. CYP19 isozymes and promoter regions have been cloned in many different teleosts such as Fundulus (Dong et al., 2008, Patel et al., 2006) and zebrafish (Kishida and Callard, 2001, Kazeto et al., 2001). Furthermore, consistent with its significant biological function, CYP19 is relatively highly conserved. There is about 50-90% peptide sequence identity between fish and mammalian forms with higher conservation in the heme binding site and the steroid pocket (Conley and Hinshelwood, 2001). CYP19 regulation of steroidal estrogens is very important for both sex determination and reproduction in fish (Meyer, 1999). Estrogen promotes hepatic vitellogenesis during ovarian follicular development while synthesis of estrogen in the brain is very important for developmental sex determination, sex-specific reproductive behaviors, neurogenesis, and brain repair (Melo and Ramsdell, 2001, Diotel et al., 2013). The significance of higher aromatase activities in teleost brain compared to gonad is not fully understood (Forlano et al., 2001, Coumailleau et al., 2015). These two genes are also differentially regulated during development.

Adult brain aromatase (cyp19a1b) was found exclusively expressed in radial glial cells (RGCs) of teleost fishes including toadfish (Forlano et al., 2001), rainbow trout, and zebrafish (Menuet et al., 2005, Menuet et al., 2003). These cells exist in the embryonic brain of all vertebrate species and remain abundant in the fish adult brain (Kriegstein and Alvarez-Buylla, 2009), whereas; they disappear in mammals at birth to become astrocytes (Malatesta and Götz, 2013, Pinto and Götz, 2007). In zebrafish, mRNA in situ hybridization, immunohistochemistry, and GFP
expression driven by \textit{cyp19a1b} promoter techniques indicated brain aromatase highest expression in the GRCs in the olfactory bulbs, telencephalon, preoptic area, and hypothalamus (Diotel et al., 2010, Pellegrini et al., 2007, Pellegrini et al., 2005, Menuet et al., 2005). Unlike medaka, sexual dimorphism in brain aromatase expression of zebrafish and European sea bass was not detected (González and Piferrer, 2003, Okubo et al., 2011). However, the \textit{cyp19a1b} expression was detected as early as 48 hpf in zebrafish embryos. Interestingly, increased brain aromatase (\textit{cyp19a1b}) expression was parallel with increased estrogen receptors expression (\textit{esr1}, \textit{esr2b} and \textit{esr2a}) (Mouriec et al., 2009b). Also, E2 induces brain aromatase (\textit{cyp19a1b}) expression in radial glial cells mainly in the preoptic area and mediobasal hypothalamus of 48 hpf and 108 hpf larvae. On other hand, blocking estrogen receptor action reduced \textit{cyp19a1b} expression, suggesting that estrogen regulates \textit{cyp19a1b} expression in the brain (Mouriec et al., 2009b, Pellegrini et al., 2005). Therefore, \textit{cyp19a1b} expression has been used as a biomarker for estrogenic endocrine disrupting chemicals (Brion et al., 2012). Different studies have shown that an alteration of \textit{cyp19a1b} expression, by pollutant or aromatase modulators, leads to negative impacts on early zebrafish development (Shi et al., 2008, Sreedevi et al., 2014, Cohen et al., 2014). Recent work in our laboratory found that BaP significantly decreased adult and embryonic brain aromatase mRNA expression, and ovarian aromatase activity (Patel et al., 2006, Dong et al., 2008).

\subsection*{1.3.2 Sex determination and gonad maturation}

Despite many studies into the mechanisms of sex determination, scientists still do not fully understand how sex is determined. The genes responsible for sex determination in some invertebrates, such as \textit{D. melanogaster} and \textit{C. elegans}, are well characterized (Cline and and Meyer, 1996, Goodwin and Ellis, 2002). While in vertebrates, sex determination is not well
characterized, and many mechanisms are involved. In mammals, females have two X chromosomes, whereas males have XY chromosomes. Male sex is determined by a dominant male determining gene on the Y chromosome called the sex-determining region Y gene (*sry*) that initiates an up-regulation of the sry-related HMG box gene 9 (*sox9*) expression. *sox9* expression ultimately suppresses *wnt4*, and leads to the establishment of testis-specific pathway (Eggers and Sinclair, 2012). Due to the absence of *sry* in XX individuals, transcription factors *wnt4* and *rspo1* are expressed leading to further downstream events that eventually suppress *sox9* expression and allow ovary-specific pathway to progress (Eggers and Sinclair, 2012, Polanco and Koopman, 2007). Differentiation of gonads mediates testicular and ovarian hormone production that induces anatomical and physiological differences of either fate, and also determines the sexual fate of other organs (Brennan and Capel, 2004). In non-mammalian vertebrates, *sry* is not conserved, but the genes functioning downstream of *sry*, like *sox9*, are conserved (Rodríguez-Mari et al., 2005). Polygenic, environmental factors such as temperature, and social architecture also can be involved in sex determination of many vertebrates including fish (Devlin and Nagahama, 2002, Godwin et al., 2003).

Sex determination in zebrafish is polygenetic and not well understood (Traut and Winking, 2001, Liew et al., 2012). Like other vertebrates, zebrafish sex determination is impacted by different environmental factors such as hypoxia, temperature, food availability, and population density (Shang et al., 2006, Spence et al., 2008, Tong et al., 2010, Uchida et al., 2004). However, histological differences in developing gonads are the first signs of zebrafish sex determination (Yamamoto, 1969). During early zebrafish development, all individuals show oogenesis and form an immature non-functional ovary that further differentiates to either mature ovary or testis (Wang et al., 2007, Maack and Segner, 2003). Germ line cells are very important for female sex
determination because absence of the germ line leads to suppressed expression of \textit{cyp19a1a} and male sex fate (Siegfried and Nüsslein-Volhard, 2008).

The impact of endocrine disrupting chemicals, especially aromatase modulators, on zebrafish sex determination has been partially elucidated (Andersen et al., 2003, Baumann et al., 2014, Caspillo et al., 2014, Segner, 2009, Örn et al., 2003). For example, exposing zebrafish during gonadal differentiation to an aromatase inhibitor, fadrozole, lead to masculinization with testicular morphology (Fenske and Segner, 2004, McAllister and Kime, 2003). However, zebrafish can exhibit sexual plasticity. After exposing adult females to aromatase inhibitors, retraction of the ovaries and testis-like organs formed (Takatsu et al., 2013). Together these studies highlight the importance of aromatase in sex determination and gonad maturation.

1.4 Morpholinos as a gene knockdown tool

Morpholino oligonucleotides (MOs) are the most broadly used anti-sense knockdown tool in different vertebrates models including zebrafish. They were first discovered by Dr. James Summerton (Summerton and Weller, 1997). MOs are typically short chains that are composed of about 25 morpholino subunits. Each subunit contains a nucleic acid base that is attached to the morpholine ring. These subunits are linked to each other by non-ionic phosphorodiamidate inter-subunit linkage (Fig 3) to form a complementary backbone to pair with its corresponding RNA. This uncharged linkage makes MOs very stable against intracellular nuclease activity. MOs do not exert their hindrance effect by degrading their RNA targets, but instead act via a RNAse H-independent steric blocking mechanism (Summerton 1999).
Figure 3. Morpholino oligonucleotide unit.

MOs have been used to: hasten gene discovery through large-scale screening (Yamada, Shoguchi et al. 2003), explore candidate gene function (Corey and Abrams 2001, Lan, Bayliss et al., 2007), verify mutant phenotypes (Dutton et al., 2001, Pickart et al., 2004, Sun et al., 2004), and reduce maternal and zygotic gene function (Ciruna et al., 2002, Lee et al., 2013). Because a morpholino oligo is able to specifically bind to its target site to block access of cell components to that target site, they can be used to block translation, splicing, miRNAs or their targets, and ribozyme activity. In splice blocking, MOs bind and inhibit pre-mRNA processing by inhibition of the splicesome components. RT-PCR is used to assess the effectiveness of MO to block or modify the splicing. Successful splice-modification would appear on an electrophoretic gel as changes in the RT-PCR product band size, intensity or disappearance (Draper et al., 2001, Wu et al. 2008). For translation blocking, MOs bind complementary mRNA sequences within the 5’
untranslated region (UTR) near the translational start site hindering ribosome assembly (Fig 4). Western blot can be used to assess the effectiveness of MOs to knockdown the target protein (Nasevicius and Ekker 2000. Also, the *in vitro* protein synthesis, TNT T7 Quick Coupled Reticulocyte Lysate System has been used to assess the effectiveness of MOs to exert its protein knockdown effect (Jenny et al., 2009).

![Diagram of translation process](image)

**Figure 4.** Mechanism by which morpholinos can block translation.

Although MOs are widely used as anti-sense knockdown tool, recent studies have highlighted some disadvantages of MOs such as mediating off-target effects like induction of p53. Furthermore there are concerns about the reliability of using MOs to assess genes function (Kok et al., 2015, Stainier et al., 2015) because of the absence of the same observed morphant phenotypes in genetic mutant embryos. However, about 300 observed morphant phenotypes in Zebrafish Information Network (ZFIN) were consistent with stated phenotypes in genetic mutant
embryos. Also, the mechanism of how MOs induce, for example, p53 as off-target effects is not known, but might be related to variability of MO preparations. Comparing our cyp19a1b-MO morphants with those treated with a known aromatase inhibitor (fadrozole) and rescuing morphant effects by exposures E2 supplementation helped establish the reliability of the phenotypes noted in the cyp19a1b-MO embryos.
1.5 Study specific aims and hypotheses

1.5.1 Central Hypothesis:

BaP deregulates the steroid hormone hypothalamus-pituitary-gonad feedback loop, alters estrogen homeostasis and adversely impacts developmental and reproductive physiology.

Figure 5. Hypotheses associated with potential adverse outcomes of BaP exposure as a result of disruption of the hypothalamus-pituitary-gonad axis.
1.5.2 Specific Aims

Aim 1. Identify waterborne BaP exposure effects on zebrafish embryonic brain aromatase (cyp19a1b mRNA expression). RT-qPCR will be used to assess brain aromatase (cyp19a1b) mRNA expression in 96 hpf zebrafish homogenates after BaP-waterborne exposure.

Hypothesis: BaP will decrease brain aromatase (cyp19a1b) mRNA expression.

Aim 2. Identify developmental phenotypes mediated by waterborne BaP exposure during early zebrafish development. Expose zebrafish embryos to low and high BaP concentrations during their early development and assess mortality, hatching efficiency, and morphological abnormalities up to 96 hpf.

Hypothesis: Waterborne BaP exposure will dose-dependently cause developmental abnormalities and adverse impacts on the reproductive system.

Aim 3. Transiently knockdown zebrafish brain aromatase (cyp19a1b) during early development and compare resulting phenotypes and molecular consequences with the phenotypes and molecular consequences caused by BaP-waterborne exposure. Design a morpholino oligonucleotide sequence that blocks translation of cyp19a1b protein and assess developmental defects at 96 hpf.

Hypothesis: Zebrafish brain aromatase (cyp19a1b) knockdown will cause phenotypes and molecular consequences in zebrafish larvae similar to BaP-mediated effects.

Aim 4. Identify developmental phenotypes mediated by waterborne fadrozole exposure during early zebrafish development and compare resulting phenotypes and molecular consequences with morphant phenotypes and molecular consequences. Expose zebrafish embryos to low and high fadrozole concentrations during their early development.
Hypothesis: Waterborne fadrozole exposure will cause developmental abnormalities similar to cyp19a1b morphant mediated effects.

Aim 5. Generate cyp19a1b antibody to assess cyp19a1b-MO effectiveness by western blot. Hypothesis: cyp19a1b-MO would knockdown cyp19a1b protein expression.

Aim 6. Further validate the morpholino effectiveness with the in vitro protein synthesis, TNT Quick Coupled Reticulocyte Lysate System expressing cyp19a1b. Hypothesis: The cyp19a1b-MO will knockdown zebrafish brain aromatase activity in vitro.

Aim 7. Evaluate the ability of estradiol to rescue zebrafish larval toxicity caused by BaP-waterborne exposure, cyp19a1b knockdown, fadrozole exposure. In BaP+E2, cyp19a1b-MO +E2, and fadrozole+E2 co-exposed zebrafish larvae, mortality, hatching efficiency, and morphological abnormalities will be measured. Hypothesis: E2 co-treatment with BaP-waterborne, cyp19a1b-MO, and fadrozole will alleviate BaP, cyp19a1b knockdown, fadrozole-mediated morphological deformities in 96 hpf zebrafish larvae.

Aim 8. Evaluate the effect of BaP, cyp19a1b knockdown, and fadrozole on steroid hormone (estrogen) concentrations. Use reverse phase-HPLC to measure zebrafish embryo estrogen concentrations after BaP-waterborne exposure and cyp19a1b-MO injection. Hypothesis: BaP, CYP19a1b-MO, and fadrozole will decrease larval estrogen hormone concentrations.
Aim 9. Evaluate BaP and *cyp19a1b* knockdown effects on zebrafish gonad maturation. Histological assessment will be used to evaluate the gonad maturation of 52 days post-fertilization zebrafish exposed to BaP or injected to *cyp19a1b*-MO during their early development.

Hypothesis: BaP-waterborne exposure and *cyp19a1b* knockdown during early development will interfere with gonad maturation.
CHAPTER 2: METHODS and MATERIALS

2.1 Zebrafish culture

Both the AB wild-type zebrafish that were purchased from Zebrafish International Resource Center (ZFIN, Eugene, OR) and the *Fli-EGFP* transgenic zebrafish that were gifted by Dr. Tanguay (OSU) were raised under IACUC-approved conditions. Fish were kept in Aquatic Habitats ZF0601 Zebrafish Stand-Alone Systems (Aquatic Habitats, Apopka, FL) with zebrafish water (pH 7.0-7.5, 60 parts per million (ppm) Instant Ocean, Cincinnati, OH) at 24-30°C, 14:10 light-dark cycle. Adult fish were fed twice daily with tropical flake fish food and live brine shrimp. Larvae were fed with ArteMac-0 powered food (20-80 micron size, Bio-Marine, Hawthorne, CA) and/or live brine shrimp depending on their age. Sexually mature fish were selected as breeders and their eggs were collected for the studies.

2.2 Zebrafish embryos BaP and BaP+E2 exposure

Fertilized eggs were cleaned and disinfected with 0.4 ppm methylene blue for 1-2 min and then randomly sorted into six treatment groups (4-8 replicates per group), namely control dimethylsulfoxide (DMSO, 0.01% v/v), control 17β-estradiol (E2 10 nM, 2.72 μg/L), 10 μg/L (40 nM) or 50 μg/L (200 nM) BaP (stock solution 0.0025 g/5mL in DMSO; final DMSO concentration was 0.01% in all treatment groups), 10 BaP+E2, and 50 BaP+E2. Fifty fertilized eggs were pooled randomly and raised in 50 mL of zebrafish water (60 ppm, pH 7.0-7.5) in glass petri dishes. During
the exposure period (2.5-96 hpf), 0.4 ppm methylene blue was added to zebrafish water to inhibit fungus growth. Exposures for each experimental treatment began at approximately 2.5 hpf. Water was changed and embryos were re-dosed every day. Embryos were pooled (15 larvae/pool, 3 replicates/treatment) at 96 hpf (embryos would typically hatch at 48-72 hpf) for RNA extraction and larvae were stored in 0.5 ml RNAlater at -80°C immediately.

2.3 Measurement of BaP concentration in water samples by GC-MS

Both control and BaP water were sampled once from each solution preparation (for a total of 4 replicates/treatment). Water samples (25-200 mL) were collected after dosing and analyzed to confirm control and BaP concentrations from each preparation of embryo zebrafish exposure. Water samples were passed slowly through Sep-Pak C18 3 cc Vac RC Cartridge (500 mg) (Waters Corp., Milford, MA) that had been pre-washed with 50 mL of 75% methanol. Methylene chloride (7.5 mL, 2X) was added to the columns to elute BaP. Solvents were evaporated under a gentle flow of nitrogen gas. Samples were re-constituted in iso-octane. BaP concentrations in the water extracts were measured by gas chromatography (Agilent 6890) coupled with mass spectrometry (Agilent 5973N) in selected ion monitoring mode for ions 252 and 253. BaP standards (0.1, 0.2, 0.5, 1, and 2 ppm) were prepared in iso-octane to build a standard curve.

2.4 RNA extraction, purification and reverse transcription

Pooled 96 hpf larvae (15 larvae/pool) were homogenized with a pellet pestle cordless motor (Sigma-Aldrich) in QIAzol Lysis Reagent (Qiagen, Valencia, CA). RNA was isolated and purified with RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s
protocols. Total RNA (250 ng) was reverse transcribed to double stranded cDNA libraries by using TaqMan® Reverse Transcription Reagents (Applied Biosystems). Each reaction contained random hexamers, multiscribe RT, RNase inhibitor, deoxynucleotide triphosphate mix, 25 mM MgCl₂, and 10X RT buffer.

2.5 Quantitative reverse transcription real time (RT-qPCR)

RT-qPCR primers were designed with Primer Express® Software v2.0 (Applied Biosystems) and selected based on their specificity (checked with NCBI Primer-Blast, http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). Relative abundance of target genes to 18S rRNA transcripts in the cDNA libraries was determined by qPCR with SYBR®Green in a GeneAmp 7500 Sequence Detection System (Applied Biosystems) and calculated with the 2⁻ΔΔCt method. Statistical differences between treatments or was determined on the linearized 2⁻ΔCt values. Each sample was measured in two separate reactions on the same plate. Amplification efficiencies of the cyp19a1b and 18S rRNA primer pairs were tested to ensure that they were not statistically different.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Slope</th>
<th>Efficiency</th>
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<td>-4.0345</td>
<td>76.95</td>
<td></td>
<td>Brain</td>
</tr>
<tr>
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<td>F: 5'-ATA CCA CCT GGC AGC AAA AGA GC-3' R: 5'-CCA CAA GCT TTC CCA TTT C-3'</td>
<td>-3.601</td>
<td>89.54</td>
<td>0.2568</td>
<td>Brain</td>
</tr>
<tr>
<td>18S</td>
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<td>-3.963</td>
<td>78.79</td>
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<td>96 hpf</td>
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<tr>
<td>cyp19a1b 807/838</td>
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<td>-4.2805</td>
<td>71.24</td>
<td>0.1603</td>
<td>96 hpf</td>
</tr>
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</table>
2.6 Zebrafish embryo fadrozole and fadrozole+E2 exposure

Fertilized eggs were cleaned and disinfected with 0.4 ppm methylene blue for 1-2 min and then randomly sorted into six treatment groups (4-8 replicates per group), namely control, dimethylsulfoxide (DMSO, 0.01% v/v), control 17β-estradiol (E2 10 nM), 10 (38.5 nM) or 50 μg/L (193 nM) fadrozole (Sigma-Aldrich, stock solution 0.0025 g/5mL in DMSO; final DMSO concentration was 0.01% in all treatment groups), 10 F+E2, and 50 F+ E2. Embryos/larvae were subsequently raised as described above in Section 2.3.

2.7 Morpholino knockdown

Gene-Tools (Philomath, OR) designed the 25 base morpholino sequence to block initiation of translation of zebrafish cyp19a1b by overlapping the translational start codon. The cyp19a1b-MO sequence was 5’-TTACCACATGCTCCATCATCACCTC-3’ and was fluorescein-labeled. The designed sequence was aligned (Fig. 6) with the cyp19a1a (gonad form) sequence to confirm minimal similarity in the region by the start codon. A standard control morpholino provided from Gene-Tools (control MO, 5’-CCTCTTACCTCAGTTACAATTTATA-3’) was used as injection control. All MOs were diluted to 250 μM stock (stored at -20 °C) with RNase-free water for injection, vortexed well and briefly centrifuged before using. A standard microinjection set up was used in our study. Aluminosilicate capillary needles were pulled on a model P-97 needle puller with the following program: Heat 550, Pull 190, Velocity 170, Time 170, and Pressure 500. The needle was loaded with MOs using a microloader tip (Eppendorf, Hamburg, Germany) and inserted into a 3-axis micromanipulator (Narshige, Greenvale, NY). A MDI PM 1000 Cell Microinjector (MicroData Instrument Inc. S. Plainfield, NJ) was used to control the injection time.
and pressure. Incoming pressure varied between 19 to 24 psi depending on the needle opening size. Embryos were lined up on the edge of a microscope slide placed in petri-dish and embryo injection was conducted at the one to two cell-stage with MO volume around 3-5 nL. Incorporation of injection was confirmed under a fluorescence microscope (Nikon 90i Eclipse) (Fig. 7).

```
MO         ctccactactacctcgtacaccatt
 cyp19alb  gaggtgatgatggagcatgtgtaaaggatg
 cyp19ala  gtctttatggcaggtgatctgctccagccct
```

**Figure 6.** Alignment MO, *cyp19alb*, and *cyp1ala* sequences in the region by the translational start codon.

**Figure 7. Morpholino incorporation.** Non-injected embryo at 5 hpf (A). ZF embryo with morpholino at 5 hpf (B) and 4 dpf (C). These pictures confirm effective injection and incorporation of morpholino.
2.8 Developmental deformities

At 96 hpf, photos were captured with a MicroFire® camera (Optronics, Goleta, CA) attached to a Zeiss Stemi 2000-C Stereo Microscope (Jena, Germany) using Picture FrameTM Application 2.3 software (Optronics). Five larvae per replicate at a time (ultimately 20 per group) were anesthetized in 300 mg/L tricaine methanesulfonate (MS-222) and 600 mg/L sodium bicarbonate. Larvae were immediately placed on a microscope slide with a chamber containing 5% methyl cellulose and two photos were taken per fish: dorsal view and lateral view. Anatomical structures to determine morphological development were recorded as previously described (Brannen et al., 2010) with modifications (Corrales et al., 2014b). Feature analysis included body length, tail shape, optic vesicle, pectoral fins, heart, swim bladder, abdomen, and craniofacial morphology (Fig. 8).
Figure 8. Representative measured morphological endpoints from supplemental materials of (Corrales et al., 2014b).

Blind to treatment measurements and scoring of the anatomical structures were recorded using ImageJ software (Schneider et al., 2012). The scale was set to the number of pixels per millimeter using a 1-mm micrometer scale. The total body length along the spine was measured followed by the area of the swim bladder, area of the pericardial and yolk sac edema when present,
area the optic vesicle (eye). Scores for tail and pectoral fin shapes were assigned following specific criteria in Table 2.

<table>
<thead>
<tr>
<th>Table 2. Larval tail and pectoral fin shape scoring criteria</th>
</tr>
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<tbody>
<tr>
<td>Score</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>1</td>
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</tbody>
</table>

Scoring criteria: 4 = normal, 3 = mild abnormality, 2 = moderate abnormality, and 1 = severe abnormality.

2.9 Histology and gonad maturation

BaP-exposed and cyp19a1b-MO injected zebrafish were euthanized at 52 dpf and fixed in Dietrich’s fixative (30% ethanol, 10% formalin, and 2% glacial acetic acid v/v) for two weeks at room temperature, followed by dehydration in increasing gradients of ethanol (70-100%). After cleared in Clearify™ (American Master Tech Scientific, Inc., Lodi, CA), fish were embedded in melted paraffin (Paraplast X-tra, Sigma-Aldrich) and sectioned (5 μM thickness) with a microtome (Olympus American Inc., San Jose, CA). For histopathological examination, sections were stained with hematoxylin and eosin with the following procedure: Sections were deparaffinized in Clearify™ and hydrated with a gradient of ethanol series (100% twice, 95%, 90%, 80%, 70%) each for 2 min and placed in flowing tap water for 5 min. After staining in Harris’s hematoxylin for 1 min and washing in tap water for 3 min, slides were incubated in 1% acid alcohol solution (2 mL HCl + 200 mL 100% ethanol) for 21 seconds. Sections were rinsed in water and placed in 0.125% ammonium hydroxide solution (200 mL deionized water + 0.25 mL ammonium hydroxide) for 1 min until tissues turned blue. After rinsing 2 min in water and 6 min in 95% ethanol, sections were stained in eosin for 30 sec and rinsed in 100% ethanol at least twice until
color differentiation was correct. Cover slipped slides were assessed under the microscope, and gonad maturation were scored to either immature ovary, transition male, mature testis, or mature ovary (Fig. 26) based on (Kallivretaki et al., 2007).

2.10 Sample preparations and HPLC quantitation of estrogen concentrations

Non-exposed zebrafish embryos were collected at 4, 48, and 72 hpf. Also, exposed embryos, either to BaP (10 and 50 µg/L), fadrozole (50 µg/L), or injected with cont-MO or cyp19a1b-MO were collected at 48 hpf (n=3, 25 embryos/pool). Embryos/larvae were washed with deionized water three times then sorted in 1.5 mL ultra-centrifuge epitubes. Water was completely removed, and 0.5 mL of 0.2 N perchloric acid (Sigma-Aldrich) was added. Then embryos/larvae were homogenized on ice for three cycles (each cycle consisted of 10 sec homogenization followed by 10 sec rest), and sonicated for 20 min (Branson 3510). After centrifugation at 40,000 rpm (98,400 x g) for 30 min at 23°C using an Optima Max Ultracentrifuge, the supernatant was transferred to a test tube and an additional 0.5 mL of 0.2 N perchloric acid was added. Two additional extractions were performed as described. All combined supernatants were evaporated to dryness using a stream of nitrogen gas at 45°C in a water bath. After evaporation, the dried residue was reconstituted in 100 µL of the mobile phase consisting of HPLC grade acetonitrile:water (40:60% v/v). For sample cleanup, a Waters HPLC system consisting of 717 plus autosampler, 600 pump, and 2489 UV detector, and Empower 3 software was used. A volume of 96 µL of each sample was injected onto a C18 column (100 mm length, 4.6 mm diameter, and 3 µm particle size, Phenomenex # 00D-0075-E0) using a 0.6 mL/min flow rate (pressure was 1560±30 psi) and a 15 min run time. The wavelength of UV detector was 280 nm. The eluent was collected manually 1 min before the retention time of the E2 standard peak and 2
min after for each sample. This eluent was dried and then reconstituted with 60 µL of mobile phase (HPLC grade acetonitrile:water (50:50% v/v)). Because the limit of detection of E2 using UV detection was only 500 nM, a different system with more sensitive fluorescence detection was used for ultimate E2 quantitation. The second system was a Waters HPLC system with 717 plus autosampler, 515 isocratic pump, and 2475 fluorescent detector and a HP 3395 integrator system. Detection of E2 concentrations was done by injecting 50 µL of cleaned-up sample, onto a C18 column (150 mm length, 4.6 mm diameter, and 3 µm particle size, Phenomenex # 00F-4311-E0) at 0.6 mL/min flow rate (Pressure was 1340±150 psi) and a 14 min run time. The fluorescent detector was used at 280 nm for excitation wavelength, 310 nm for emission wavelength, 30 nm for gain, and 600 nm for EUFS. The retention time of E2 was 8.479±0.130 min. The external standard curve of known E2 concentrations ranged from 2.344 to 300 nM and was analyzed as described above with the fluorescence system (Table 3, Fig. 9). To verify the recovery of E2 during the clean-up method, a 225 nM stock of E2 was processed as described above. The E2 recoveries (n = 3) for E2 during sample clean-up were 95.8 ± 1.23 %.
Table 3. Estradiol standard curve concentrations.

<table>
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<th>Area under the curve</th>
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<td>135639</td>
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</table>

The correlation coefficient was 0.999895.

Figure 9. Estradiol standard curve graph.
2.11 Statistics

Results were analyzed using GraphPad Prism 5.0 (La Jolla, CA) and presented as mean ± S.E. Mortality, hatching, qRT-PCR, and data of developmental deformities were analyzed using the 1-way ANOVA followed by Neuman–Keulls post hoc test. Deformity incidence by treatment across score classifications was analyzed by 2-way ANOVA. Statistical significance was accepted at $p \leq 0.05$. 
CHAPTER 3: RESULTS

3.1 BaP Waterborne Embryo-Larval Results

3.1.1 GC/MS BaP water concentration confirmation.

Actual BaP concentrations of BaP waterborne exposures were 7.6 ± 1.19 µg/L (for 10 µg/L), and 37.5 ± 2.15 µg/L (for 50 µg/L). Collected at t = 0 for n=4 independent exposures.

3.1.2 Effect of BaP on cyp19a1b mRNA expression in 96 hpf zebrafish larvae homogenates.

The RT-qPCR results showed that 10 and 50 µg/L BaP significantly decreased cyp19a1b mRNA expression of whole larvae extracts compared to controls at 96 hpf (Fig. 10).

![Bar chart showing mRNA fold change compared to control for different BaP concentrations](image)

Figure 10. cyp19a1b mRNA expression in 96 hpf zebrafish larvae homogenates. Both BaP concentrations significantly reduced cyp19a1b mRNA-expression compared to control. Bars with
different letters are statistically significant (ANOVA, n = 3 replicates/treatment, 15 larvae/pool, p<0.05).

3.1.3 Impacts of BaP exposure and BaP+E2 on mortality of zebrafish embryos/larvae

Zebrafish embryos/larvae exposed to waterborne concentrations of DMSO, BaP (10 and 50 μg/L) alone or BaP + E2 (10 nM, 2.72 μg/L) for 96 hpf showed that 10 and 50 μg/L BaP significantly increased the mortality compared to control at 24 and 96 hpf. Co-treatment of 10 nM E2 significantly decreased the mortality caused by 10 BaP. The highest mortality was in the 50 BaP + E2 treatment group (Fig. 11).

![Cumulative mortality of zebrafish larvae.](image)

**Figure 11. Cumulative mortality of zebrafish larvae.** BaP (10 and 50 μg/L) significantly increased the mortality compared to control at 24 and 96 hpf. At 24 hpf, 10 BaP + E2 (10 nM) significantly decreased the mortality that was caused by 10 BaP alone, but 50 BaP + E2 significantly increased the mortality at 96 hpf compared to all treatment groups (ANOVA; n = 4 replicates/treatment, 50 larvae/pool, p<0.05).
3.1.4 Impacts of BaP exposure and BaP+E2 on hatching efficiency of zebrafish embryos

Hatching efficiency was significantly decreased by 10 and 50 μg/L BaP compared to control group at 48 hpf (Fig. 12). This effect was also observed in the BaP+E2 co-exposure, but no significant change in hatching efficiency was observed by any treatment at 72 hpf.

Figure 12. Hatching efficiency of zebrafish larvae. BaP (50 and 10 μg/L) significantly decreased the hatching efficiency compared to control at 48 hpf. Also, 10 and 50 BaP + E2 (10 nM) significantly decreased the hatching efficiency at 48 hpf (ANOVA; n = 4 replicates/treatment, 50 larvae/pool, p<0.05).
3.1.5 Impact of BaP exposure and estradiol co-exposure on larvae body length, optic vesicle, and swim bladder

Body length of 96 hpf larvae was significantly decreased from 3.68 ± 0.043 mm in control group to 3.26 ± 0.089 mm and 2.79 ± 0.09 mm by 10 and 50 µg/L BaP, respectively (Fig. 13A). While decreased body length by 10 BaP was restored by estradiol co-exposure, the 50 BaP + E2 did not rescue the decreased body length mediated by 50 BaP alone.

Optic vesicle (eye) area of 96 hpf larvae was significantly reduced from 0.07 ± 0.0009 mm² in control group to 0.052 ± 0.004 and 0.041 ± 0.001 mm² by 10 and 50 µg/L BaP, respectively (Fig. 13B). Following 10 BaP + E2 co-exposure, E2 significantly restored the decrease in optic vesicle area mediated by 10 BaP exposure. However, the 50 BaP + E2 co-exposure did not change the optic vesicle area reduction mediated by 50 BaP alone.

Control larvae had swim bladders with average areas of 0.024 ± 0.01 mm² but in larvae from the 10 and 50 µg/L BaP groups, 100% of the larvae had uninflated swim bladders (Fig. 13C). E2 treatment alone also caused a decreased swim bladder area, with 15 of the 20 larvae having uninflated swim bladders.
Figure 13. Morphological changes in larvae caused by BaP waterborne exposure at 96 hpf. Both BaP 10 and 50 μg/L concentrations significantly reduced body length (A), optic vesicle area (B), and swim bladder (C). Co-treatment with 10 nM E2 significantly counter-acted larval body length and optic vesicle size caused by 10 BaP (ANOVA, n = 4 replicates/treatment, 50 larvae/pool, p<0.05).
3.1.6 Impact of BaP exposure and estradiol co-exposure on larvae heart and yolk sac

Both BaP concentrations, 10 and 50 μg/L, caused pericardial edema (Fig. 14). Yolk sac edema area was significantly increased by BaP in a dose-dependent manner. Both pericardial and yolk sac edema caused by 10 μg/L BaP was significantly alleviated by estradiol co-exposure.

Figure 14. Morphological changes in larval heart and yolk sac caused by BaP waterborne exposure at 96 hpf. Both BaP concentrations significantly increased pericardial and yolk sac edema. E2 co-exposure significantly counter-acted the increased pericardial and abdominal edema caused by low but not the high BaP concentrations (ANOVA, n = 4 replicates/treatment, 5 larvae/pool, p<0.05).
3.1.7 Impacts of BaP and estradiol co-exposure on larvae tail and pectoral fin shapes

Incidence of normal tail shape significantly decreased from 95% in the control group to 20% in the 50 BaP and 50 BaP + E2 (Fig. 15A). Mild tail deformity incidence was significantly increased by 50 BaP and 50 BaP + E2, while the moderate tail deformity incidence was significantly increased by only 50 BaP + E2. Both BaP concentrations and BaP + E2 co-exposure significantly decreased incidence of normal pectoral fin from 90% in control group to 50% and 0%, respectively (Fig. 15B), moderate pectoral fin deformity incidence was significantly increased by 50 μg/L BaP. Only 50 μg/L BaP significantly increased the incidence of severe pectoral fin deformity.

Figure 15. Degree of larval morphological changes caused by waterborne BaP exposure at 96 hpf. Incidence of normal tail shape (A) was significantly decreased by 50 μg/L BaP. Both the 10 and 50 μg/L BaP concentrations significantly decreased the incidence of normal pectoral fins (B). Treatment bars with different letters are statistically significant within a category (ANOVA, n = 4 replicates/treatment, 5 larvae/pool, p<0.05). Treatment groups and bar colors as in figure 11.
Figure 16. Representative morphological anomalies mediated by BaP exposure in larvae at 96 hpf. Red arrows demonstrate changes in swim bladder. Yellow arrows show changes in larval tail. Brown arrows represent the abnormal pectoral fins. Purple and blue arrows indicate pericardial and yolk sac edema, respectively.
3.2 *cyp19a1b*-MO Knockdown Results

3.2.1 Impacts of *cyp19a1b*-MO knockdown and *cyp19a1b*-MO+E2 on zebrafish embryos/larvae mortality

Embryos/larvae mortality significantly increased from about 20% in control-MO group to about 40% by *cyp19a1b*-MO at all-time points (24, 48, 72, and 96 hpf) (Fig. 17). E2 (10 nM) significantly prevented the increased mortality mediated by *cyp19a1b* knockdown.

![Graph](image)

**Figure 17. Cumulative mortality of zebrafish larvae.** Injection of *cyp19a1b*-MO significantly increased the mortality compared to injection of control MO at 24, 48, 72, and 96 hpf. E2 (10 nM) significantly reduced the mortality caused by *cyp19a1b* MO at all-time points (ANOVA; n = 8 replicates/treatment, 30 larvae/ pool, p<0.05).
3.2.2 Impacts of *cyp19a1b*-MO knockdown and *CYP19a1b-MO+E2* on hatching efficiency of zebrafish embryos

At 48 hpf, *cyp19a1b*-MO significantly decreased the percent of hatched larvae from 84% in control-MO group to 35% (Fig. 18). *cyp19a1b* Morphants that were treated with E2 hatched similarly to controls. At 72 hpf, there was no significant change in hatching percentage by any of the treatments.

![Figure 18. Hatching efficiency of zebrafish larvae.](image) Injection of *cyp19a1b*-MO significantly decreased the hatching efficiency compared to injection of control-MO at 48 hpf. Decreased hatching efficiency mediated by *cyp19a1b*-MO was rescued by E2 treatment (ANOVA; n = 4 replicates/treatment, 30 larvae/pool, p<0.05). Treatment groups and bar colors as in Figure 17.
3.2.3 Impacts of \textit{cyp19a1b}-MO knockdown and \textit{cyp19a1b}-MO+E2 on larval body length, optic vesicle, and swim bladder

\textit{cyp19a1b}-MO significantly decreased larval body length from 3.93 ± 0.07 mm in the control-MO group to 3.31 ± 0.06 mm (Fig. 19A). Estradiol treatment significantly restored the decreased larval body length in \textit{cyp19a1b} morphants.

Larval optic vesicle area significantly decreased from 0.079 ± 0.001 mm$^2$ in control-MO group to 0.063 ± 0.001 mm$^2$ in \textit{cyp19a1b} morphants (Fig. 19B). The \textit{cyp19a1b}-MO + E2 treatment significantly counteracted the reduction in larval optic vesicle area.

In all treatment groups (10 nM E2, \textit{cyp19a1b}-MO and \textit{cyp19a1b}-MO +E2) deflated larval swim bladders were observed (Fig. 19C).
Figure 19. Larval morphological changes at 96 hpf caused by injection of cyp19a1b-MO. cyp19a1b knockdown significantly reduced body length (A), optic vesicle size (B), and swim bladder inflation (C), while 10 nM E2 treatment significantly restored both body length and optic vesicle area (ANOVA, n = 4 replicates/treatment, 50 larvae/pool, p<0.05).
3.2.4 Effects of *cyp19a1b*-MO and *cyp19a1b*-MO+E2 on zebrafish larval heart and yolk sac.

Significantly increased pericardial and yolk sac edema was measured in *cyp19a1b* morphants with respective average areas of 0.062 ± 0.018 and 0.084 ± 0.0264 mm² (Fig. 20). Both types of edemas were significantly alleviated by E2 treatment.

**Figure 20.** Larval morphological changes at 96 hpf caused by injection of *cyp19a1b*-MO. *cyp19a1b*-MO knockdown significantly increased pericardial edema and yolk sac edema. In *cyp19a1b*-MO + 10 nM E2 larvae, pericardial and yolk sac areas were not different than in controls (ANOVA, n = 4 replicates/treatment, 50 larvae/pool, p<0.05).
3.2.5 Impacts of cyp19a1b-MO and cyp19a1b-MO+E2 on larvae tail and pectoral fin shapes

Incidence of normal tail shape was significantly decreased from 80% in the control-MO group to 25% by cyp19a1b-MO (Fig. 21A). Also, cyp19a1b morphants had significantly increased incidence of severe tail shape deformities compared to the control-MO group. In morphants co-treated with E2, no significant reduction in normal tail shape was noted. There were no significant changes in pectoral fin shape observed in cyp19a1b morphants (Fig. 21B).

**Figure 21. Degree of larval morphological changes at 96 hpf caused by injection of cyp19a1b-MO.** Incidence of normal tail was significantly decreased by injection of cyp19a1b-MO. Also, injection of cyp19a1b-MO significantly increased the incidence of severe tail deformity (A). Treatment of cyp19a1b morphants with E2 restored incidence of normal tail and decreased the incidence of severe tail to control levels (A). Treatment bars with different letters are statistically significant within a category (ANOVA, n = 4 replicates/treatment, 5 larvae/pool, p<0.05). Treatment groups and bar colors as in Figure 17.
Figure 22. Representative morphological anomalies mediated by *cyp19a1b*-MO observed in larvae at 96 hpf. Red arrows demonstrate changes in swim bladder. Yellow arrows show changes in larval tail. Purple and blue arrows display pericardial and abdominal edema, respectively.
3.3 Fadrozole waterborne embryo-larval results

3.3.1 Effects of fadrozole and fadrozole+E2 on zebrafish embryos/larvae mortality

At all-time points (24, 48, 72, and 96 hpf), mortality of embryos/larvae was significantly increased by 10 and 50 μg/L (38.5 and 193 nM) fadrozole compared to control (Fig. 23). Mortality mediated by 10 μg/L fadrozole was significantly rescued by 10 fadrozole+E2 co-treatment. In contrast, 50 fadrozole+E2 significantly enhanced the mortality from 5% in 50 fadrozole alone to 11%.

![Cumulative mortality graph](image)

Figure 23. Cumulative mortality of zebrafish larvae exposed to fadrozole and fadrozole+E2.

Both 10 and 50 μg/L fadrozole significantly increased mortality compared to control at 24, 48, 72, and 96 hpf. While 10 nM E2 significantly rescued the mortality caused by 10 μg/L fadrozole, 50 μg/L fadrozole + E2 co-treatment significantly increased the mortality compared to all treatment groups (ANOVA; n = 4 replicates/treatment, 50 larvae/pool, p<0.05).
3.3.2 Effects of fadrozole and fadrozole+E2 on zebrafish hatching efficiency

At 48 hpf, the percentage of hatched larvae significantly decreased from 69% in the control group to 2%, 4%, 11%, and 2% by 10, 50 μg/L fadrozole and 10 fadrozole+E2, 50 fadrozole+E2, respectively (Fig. 24). Only the 10 μg/L fadrozole treatment group had a significantly decreased percent hatched (97%) at 72 hpf.

Figure 24. Hatching efficiency of zebrafish larvae. Both the 10 and 50 μg/L ± E2 fadrozole groups had significantly decreased hatching efficiency compared to control at 48 hpf. Only 10 μg/L fadrozole significantly decreased the hatching efficiency at 72 hpf (ANOVA; n = 4 replicates/treatment, 50 larvae/pool, p<0.05). Treatment groups and bar colors as in Figure 23.
3.3.3 Effects of fadrozole and fadrozole+E2 on larval body length, optic vesicle, and swim bladder

Both 10 and 50 μg/L fadrozole significantly decreased larval body length from 3.76 ± 0.0204 mm in the control group to 3.34 ± 0.04 and 3.20 ± 0.12 mm, respectively (Fig. 25A). E2 co-treatment significantly restored the decrease in larval body length mediated by 10 and 50 μg/L fadrozole.

Larval optic vesicle area was significantly decreased from 0.07 ± 0.002 mm² in controls to 0.056 ± 0.003 and 0.053 ± 0.003 mm² by 10 and 50 μg/L fadrozole, respectively (Fig. 25B). These decreases in larval optic vesicle were significantly counteracted by E2 co-treatment.

Deflated swim bladder was observed in all treatment groups (Fig. 25C). Larval swim bladder area was 0.0114 ± 0.001 mm² in controls. One hundred percent of the 10 and 50 μg/L fadrozole-treated larvae had uninflated swim bladders. The swim bladder area was significantly increased by fadrozole + E2 co-treatments but not to the area of controls.
Figure 25. Morphological changes in larvae caused by fadozole waterborne exposure at 96 hpf. Both 10 and 50 μg/L fadozole concentrations significantly reduced body length (A), optic vesicle area (B), and swim bladder area (C). The 10 nM E2 co-treatment significantly counteracted the decreases in larval body length, optic vesicle, and swim bladder area caused by both fadozole concentrations (ANOVA, n = 4 replicates/treatment, 50 larvae/pool, p<0.05).
3.3.4 Effects of fadrozole and fadrozole+E2 on larval heart and yolk sac

Pericardial and yolk sac edema were detected in zebrafish larvae following 10 and 50 μg/L fadrozole exposure. Average pericardial edema area was 0.0199 ± 0.008 and 0.025 ± 0.005 mm² in the 10 and 50 μg/L treatment groups, respectively (Fig. 26). E2 co-treatment significantly decreased pericardial edema area. The average yolk sac edema area in 10 μg/L fadrozole group was 0.035 ± 0.012 and 0.043 ± 0.005 mm² in the 50 μg/L fadrozole group. Yolk sac edema mediated by both fadrozole treatments was significantly alleviated by E2 co-treatment.

![Figure 26. Morphological changes in heart and yolk sac edema caused by fadrozole waterborne exposure at 96 hpf.](image)

Both fadrozole concentrations significantly increased pericardial and yolk sac edema. E2 co-exposure significantly counteracted both types of edema caused by fadrozole alone (ANOVA, n = 4 replicates/treatment, 5 larvae/pool, p<0.05).
3.3.5 Effects of fadrozole and fadrozole+E2 on larval tail and pectoral fin shapes

Incidence of normal tail shape was decreased from 90% in the controls to 45% and 50% by 10 and 50 μg/L fadrozole, respectively (Fig. 27A). Estradiol co-treatment significantly counteracted the reduction of normal tail shape mediated by fadrozole treatments, but the incidence of pectoral fin shape was not changed by any fadrozole treatment (Fig. 27B).

Figure 27. Degree of larval morphological changes caused by waterborne fadrozole exposure at 96 hpf. Incidence of normal tail shape (A) was significantly decreased by 10 and 50 μg/L fadrozole. E2 co-treatment significantly restored the decreased incidence of normal tail shape in larvae from both fadrozole concentrations (A). Treatment bars with different letters are statistically significant within a category (ANOVA, n = 4 replicates/treatment, 5 larvae/pool, p<0.05). Treatment groups and bar colors as in Figure 23.
3.4 Effects of BaP, fadrozole, and cyp19a1b knockdown on estrogen concentrations in zebrafish embryos.

Clean-up chromatography followed by reverse phase-HPLC with fluorescent detection was used to quantitate the concentration of E2 in control zebrafish embryos/larvae and treated-embryos with either BaP (10 and 50 µg/L), fadrozole (50 µg/L), or injected with cyp19a1b-MO. Estradiol significantly increased during development in a time-dependent manner from 78.57 ± 4.08 pg/embryo in 4 hpf to 137 ± 6.55, and 170 ± 9.31 pg/larvae at 48 and 72 hpf, respectively (Fig. 28A).

Estradiol concentrations (137 ± 6.55 and 131 ± 4.23 pg/embryo) of 48 hpf in control groups (control-DMSO and cont-MO) significantly decreased to 66.7 ± 10.8, 86.0 ± 19.0, 77.4 ± 14.5, and 57.2 ± 0.51 pg/embryo by 10 BaP, 50 BaP, 50 fadrozole, and cyp19a1b-MO, respectively (Fig. 28B)
Figure 28. E2 concentrations in normal and treated zebrafish embryos/larvae. Estradiol concentrations in non-exposed zebrafish embryos significantly increased in a time-dependent manner from 4 hpf to 72 hpf (A). Both BaP concentrations, 50 fadrozole, and cyp19a1b-MO significantly decreased E2 in 48 hpf larvae compared to control groups (B) (ANOVA, n = 3 replicates/treatment, 25 embryos or larvae/pool, p<0.05).
3.6 Preliminary data of effects of BaP and cyp19a1b knockdown on zebrafish gonad maturation

Histological assessments of gonad maturation of 52 dpf zebrafish that were exposed to 10 and 50 μg/L BaP from 2 – 96 hpf and matured in clean water or had transient cyp19a1b knockdown during early life are shown in Table 4 and Fig. 29. The number of biological replicates was not enough to evaluate the gonad maturation end point due to increased mortality observed during maturation mediated by either BaP exposure or cyp19a1b knockdown. In future studies designed to assess the effects of BaP exposure and cyp19a1b inhibition on gonad maturation, lower concentrations of BaP and aromatase inhibitor are suggested.

<table>
<thead>
<tr>
<th>Gonad Maturation</th>
<th>Cont. DMSO (n=15)</th>
<th>10 BaP (n=13)</th>
<th>50 BaP (n=13)</th>
<th>cyp19a1b-MO (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature ovary</td>
<td>86%</td>
<td>23%</td>
<td>69%</td>
<td>55%</td>
</tr>
<tr>
<td>Transition male</td>
<td>0%</td>
<td>23%</td>
<td>0%</td>
<td>11%</td>
</tr>
<tr>
<td>Mature ovary</td>
<td>0%</td>
<td>7%</td>
<td>7%</td>
<td>0%</td>
</tr>
<tr>
<td>Mature testis</td>
<td>13%</td>
<td>46%</td>
<td>30%</td>
<td>33%</td>
</tr>
</tbody>
</table>
Figure 29. Representative zebrafish gonad maturation at 52 dpf. Immature ovary containing perinucleolar oocytes (arrows) (A), ovary transition to testicular tissue distinguished by deteriorating oocytes (arrow) and spermatogonial cysts (double arrows) (B), mature testis (C), and mature ovary with perinucleolar (single arrow) and cortical alveolar (double arrows) oocytes (D).
CHAPTER 5. DISCUSSION

The focus of this study was on brain aromatase *cyp19a1b*. As discussed in the introduction, teleosts are unique from most mammals because they express two distinct aromatase genes, one that is primarily expressed in the brain and one that is primarily expressed in the gonad. Yet when nucleotide sequences are compared, little functional divergence among vertebrate aromatases suggests similar functionality, though transcription of these genes can be highly variable (Wilson et al., 2005). In fish, brain aromatase activity is higher up to one thousand times compared to brain aromatase activity in mammals (Callard et al., 1981, Pasmanik and Callard, 1985). The ability to synthesize estrogen in the fish brain has been suggested to be associated with continuous neurogenesis (Pellegrini et al., 2007). Other proposed roles of neuronal aromatase in various fish species include reproduction-related vocalizations (midshipman, (Forlano et al., 2001, Dong et al., 2008) and sex determination (sea bass, medaka, pejerrey and wrasse, Blazquez and Pieferre 2004; Marsh et al., 2006; Melo and Ramsdell 2001; Strobl-Mazzulla et al., 2005). Early developmental expression of *cyp19a1b* has been reported in zebrafish (Brion et al., 2012, Trant et al., 2001, Sawyer et al., 2006, Menuet et al., 2005) and in *Fundulus* by our lab (Dong et al., 2008).

Soon after the recognition of the unique brain form of aromatase, it was discovered that its expression was inducible by estrogenic compounds (Kuhl et al., 2005; Tchoudakova et al., 2001). By now, the effects of many environmental contaminants on brain aromatase have been elucidated. For example, 10 nM E2 induced *cyp19a1b* mRNA expression and enzyme activity in zebrafish embryos/larvae (Menuet et al., 2005). Also ethynylestradiol (EE2), a potent estrogenic
contaminant in the aquatic environment, induced cyp19a1b protein in zebrafish larvae in both dose- and time-dependent manners (Vosges et al., 2010). cyp19a1b mRNA was also induced in male goldfish exposed to EE2 (10 nM) (Martyniuk et al., 2006). Male-to-female sex reversal in medaka was associated with induction of cyp19a1b expression and activity following dichlorodiphenyltrichloroethane (DDT) exposure (Kuhl et al., 2005). With respect to BaP’s ability to disrupt aromatase gene expression and activity, our previous studies have used the environmentally relevant estuarine fish Fundulus heteroclitus. Initial studies with RT-qPCR showed no significant effect on cyp19a1b expression in either BaP–exposed adult or embryo Fundulus which was attributed to high inter-fish variability (Patel et al., 2006). In contrast, in situ hybridization showed that BaP significantly decreased cyp19a1b expression in both Fundulus adults and embryos (Dong et al., 2008).

While Fundulus is a useful model for studying potential consequences of direct environmental contamination and ecosystem effects (e.g. Superfund sites like the Elizabeth River, Virginia (Wills et al., 2010)), they prove harder to use for mechanistic analyses for several reasons. Their genome is not fully annotated and available. Also, Fundulus develop more slowly, and MO studies are harder to perform in this model. For these reasons, in this study zebrafish were used to further investigate the effects of cyp19a1b inhibition by BaP on the early development. Both species show similar developmental deformities (e.g. cardiac and body shape defects) upon exposure to BaP (Wassenberg and Di Giulio, 2004, Corrales et al., 2014b, Fang et al., 2013, Fang et al., 2015, Goodale et al., 2013, Andreasen et al., 2002, Knecht et al., 2013, Clark et al., 2010, Wills et al., 2009, Wassenberg et al., 2002). Furthermore, in both species cyp19a1b gene expression was decreased following BaP exposure (Fig. 10 and Dong et al., 2008). RT-qPCR showed significantly decreased cyp19a1b mRNA expression in 96 hpf zebrafish larvae exposed to
nominal BaP-waterborne concentrations of 10 and 50 µg/L. Upregulation of brain aromatase 
\textit{cyp19a1b} by E2 in \textit{Fundulus heteroclitus} (Greytak et al., 2005) has been suggested to be mediated by estrogen response element (ERE) and estrogen receptor (ER) binding sites in the \textit{cyp19a1b} promotor (Dong et al., 2008, Dong and Willett, 2008). Likewise, the brain aromatase \textit{cyp19a1b} promotor in zebrafish contains ERE and ER binding sites (Kazeto et al., 2003, Mouriec et al., 2009b, Le Page et al., 2008, Le Page et al., 2006, Menuet et al., 2005). Together ERE and ER are responsible for the neuronal upregulation of \textit{cyp19a1b} by estrogen and androgen hormones (Mouriec et al., 2009a, Menuet et al., 2005, Handa et al., 2008). Expression of \textit{esr1} and \textit{esr2b} were temporally and locationally parallel with early expression of \textit{cyp19a1b} in zebrafish embryos, and blocking these receptors by ICI182,780 (ER antagonist) decreased the expression of \textit{cyp19a1b} suggesting that \textit{esr1} and \textit{esr2b} are important in the regulation of the basal expression of \textit{cyp19a1b} (Mouriec et al., 2009b). Furthermore, knockdown of zebrafish \textit{esr1} and \textit{esr2b} has shown that E2-inducible \textit{cyp19a1b} expression was specifically mediated by \textit{esr2b} (Griffin et al., 2013).

BaP is lethal and teratogenic to zebrafish embryos with LC$_{50}$ of 5.1 µM (1285 µg/L) and an EC$_{50}$ of 0.52 µM (131 µg/L), respectively (Weigt et al., 2011). Our previous work found that environmentally relevant concentrations of BaP (2.4 and 24 µg/L) increased the larval mortality to 38.5% and 25%, respectively compared to control (Fang et al., 2013). Comparatively, in this study both BaP concentrations 10 and 50 µg/L increased the mortality by 7.5% and 21%, respectively. The mortality typically increased within the first 24 hpf, and then remained stable until 72 hpf. In another study, zebrafish parents and their embryos were exposed continuously to 42 µg/L BaP, and the mortality of zebrafish embryos significantly increased to 55.2% within 24 hpf, and to 68.5% at 96 hpf (Corrales et al., 2014a). The higher mortality of zebrafish embryos in Corrales study was likely due the multigenerational aspects of the exposure. Increased
embryo/larval mortality mediated by BaP in our study is a well-established impact of PAHs waterborne exposure (Carls and Thedinga, 2010, Barron et al., 2004, Hawkins et al., 2002, Carls et al., 1999, Fang et al., 2013, Bugiak and Weber, 2010). Because mortality is a relatively blunt measure of developmental toxicity, our study was designed to measure more subtle developmental deformities that might in turn contribute to either reduced overall fitness and/or subsequent mortality.

That said, the potential role for cyp19a1b to be mediating a role in survival was shown by the fact that cyp19a1b knockdown and both aromatase inhibitor doses (10 and 50 µg/L fadrozole) significantly increased mortality of zebrafish embryos and larvae at all time points compared to control groups. This finding was consistent with the increased mortality of zebrafish larvae that were exposed to aromatase inhibitors including fadrozole (Santos et al., 2014, Allgood et al., 2013).

Because of aromatase’s role in estrogen synthesis, embryos/larvae were provided supplemental E2 to mechanistically validate and rescue the aromatase inhibition caused by either BaP, cyp19a1bMO or fadrozole. With HPLC analyses, it was confirmed that all three of these aromatase inhibitory mechanisms decreased E2 concentrations in 48 hpf larvae by 43 – 63 % (Fig. 28b). Similarly, co-treating embryos/larvae with E2 (10 nM) was able to decrease mortality mediated by 10 BaP, 10 fadrozole and cyp19a1b-MO knockdown.

It is known that estrogen is important in many physiological processes. In humans, E2 concentrations vary based on gender and developmental stage. In adult men, the normal E2 concentrations range from 21-30 pg/mL (parts per trillion) (Baykan et al., 2013). Men with aromatase deficiency had significantly lower concentrations ranging from undetectable to 7 pg/mL (Morishima et al., 1995, Chen et al., 2015). In adult women, E2 concentrations vary. Typical
premenopausal women have a baseline of 55 pg/mL of E2 that dramatically increases during the menstrual cycle phases to reach up to 106 pg/mL at the mid luteal phase (Rothman et al., 2011). Also, postmenopausal women or those with aromatase deficiency have low E2 concentrations in the range of 5 - 10 pg/mL (Conte et al., 1994, Rothman et al., 2011). Similarly, aromatase knockout mice (ArKO) showed a significant reduction in E2 concentrations down to 6-8 pg/mL (Ling et al., 2004). Adult male and female zebrafish have reported serum E2 concentrations of ~5 and 11 ng/mL, respectively (ppb) (Deng et al., 2010). For comparison, we found that during early zebrafish development E2 concentrations increased in time-dependent manner from 78.57 ± 4.08 pg/embryo in 4 hpf to 137 ± 6.55 and 170 ± 9.31 pg/larvae at 48 and 72 hpf, respectively. When expressed on a per weight basis, whole embryo E2 was ~650 ppb in controls. Like in the ArKO mice, the E2 concentration of cyp19a1b-morphant 48 hpf embryos was significantly reduced to 57.2 ± 0.51 pg/embryo (a ~60% reduction). Also, 10 μg/L BaP, 50 μg/L BaP, and 50 μg/L fadrozole significantly decreased embryo/larval E2 concentrations. We believe that the HPLC method used herein was more reliable and specific than some of our previous unpublished work that quantitated E2 in 7 dpf larvae and 21 dpf zebrafish by ELISA methods. In those studies we found E2 concentrations in control fish to be 250-300 pg/larvae and 500 pg/fish respectively (data not published). Compared to another study that quantified E2 concentrations in 48 hpf embryos by HPLC- PDA detection, our control fish E2 concentrations were slightly lower (137 vs. 500 pg/embryo) (Trickler et al., 2014) potentially because FLU detection provided more specificity.

While E2 was able to rescue mortalities associated with the low BaP and fadrozole concentrations, the 50 BaP+E2 and 50 fadrozole+E2 significantly increased the mortality of zebrafish embryos and larvae compared to the mortality caused by either 50 BaP and 50 fadrozole alone. BaP is a ligand of aryl hydrocarbon receptor (AhR), which is a member of the basic-helix-loop-helix Per (Period)–ARNT (aryl hydrocarbon nuclear translocator)–SIM (single minded) (bHLH-PAS) family (Gu et al., 2000). After activation by ligands like BaP or TCDD, AhR binds
ARNT and associates with AhR response elements (XRE) on the target genes, such as cyp1a1, and cyp1b1 (Hankinson, 1995). These genes were induced due to the induction of AhR pathway in 96 hpf zebrafish exposed prenatally and developmentally to waterborne BaP (Fang et al., 2015). Induction of these genes is mechanistically involved in BaP’s carcinogenicity and teratogenicity (Mandal, 2005). However, it is also established that CYP1A1 and CYP1B1 enzymes can metabolize E2 (Lee et al., 2003), and this is one of the proposed mechanisms in the cross talk between AhR and ER pathways (Matthews and Gustafsson, 2006). In fact, the larval E2 concentrations were decreased in BaP compared to control 48 hpf larvae in this study.

Cross-talk has also been found because some E2-induced genes are inhibited by activation of AhR (reviewed in: Safe and Wormke, 2003). As mentioned previously, the promoter of zebrafish cyp19a1b has an ERE which is a mediator of cyp19a1b upregulation by E2, but the cyp19a1b promotor also has AhR recognition site (Tong and Chung, 2003). A recent study specifically evaluated the cross talk between the AhR and ER pathways in goldfish by measuring the effects of E2, BaP, and the combination of E2+BaP on the expression of ahr2, ERα, and cyp1a as well as circulating vitellogenin concentrations and CYP1-enzyme activity. BaP induced ahr2 and cyp1a in a dose-dependent manner but antiestrogenic activity was noted in E2 + lower concentrations of BaP (20 and 50 µg/L) reflected by inhibited ahr2 and cyp1a expression and a decrease in vitellogenin concentrations and, thus, a “reciprocal inhibiting mode of ER-AhR interaction” was suggested (Yan et al., 2012).

Similar to the Yan study, the effects caused by the lower concentrations of BaP were rescued by E2 treatment, whereas the higher doses in co-treatment often showed enhanced toxicity. Our co-treatment studies further support the potential for AhR and ER cross-talk (Fig. 30). Yet, the reason for enhanced incidence of mortality by co-exposure of E2 and high doses of BaP is not
understood. One possibility is E2-mediated inhibition of \textit{cyp1a}. It is well known from knockdown and CYP1A-inhibitor studies that CYP1A is protective against PAH-mediated developmental toxicity (Billiard et al., 2006). While it is speculation, and \textit{cyp1a}, \textit{er\alpha}, and \textit{AhR2} expression were not measured in this study, perhaps the higher dose BaP toxicity was enhanced by ER\alpha-mediated inhibition of AhR2 which in turn CYP1 expression (Fig. 30).

\textbf{Figure 30.} A scheme of the potential AhR2 and ER cross talk that could explain enhanced larval toxicity seen following E2 + 50 \mu g/L BaP co-treatments.

In a transcriptomic analysis of differential gene expression in 96 hpf zebrafish exposed parentally and developmentally to waterborne BaP, organismal death was the most highly significant disease pathway impacted and included 212 differentially regulated molecules (Fang 2015). Based on BaP-mediated differential expression, inhibition of key mediators related to activation of organismal death included: transforming growth factor (\textit{tgf}) beta, bone morphogenetic protein 2 (\textit{bmp2}), and growth differentiation factor 2 (\textit{gdf2}) (Fang et al., 2015).
Further studies are needed to investigate these candidate genes/pathways to determine the impact of aromatase modulation on gene expression. Importantly, estrogen is important in both the activation of tgf pathway by liberating it from its latent complex during implantation period in mouse (Ma et al., 2013) and in upregulation of bmp2 protein (Kousteni et al., 2007).

Normally, zebrafish embryos start to hatch out of the chorion between 48 – 72 hpf (Kimmel et al., 1995). The potential of environmental contaminants, such as PAHs including BaP, to impact zebrafish embryos hatching efficiency has previously been observed. Hatching can occur earlier (Carls and Thedinga, 2010, Colavecchia et al., 2004, Corrales et al., 2014b, Carls et al., 1999) or longer compared to respective controls (Colavecchia et al., 2004, Carls and Thedinga 2010). In this study, waterborne exposure to both low and high BaP doses significantly increased hatching time compared to control (at 48 hpf) that was consistent with a previous study that has shown same effect (Fang et al., 2013). Also, fadrozole exposure and knockdown of cyp19a1b showed significantly increased time to hatch. In zebrafish, there are conserved molecular mechanisms for hatching including: the hatching gland cells secrete hatching enzyme 1 (ZHE1) that in turn cleave the chorion glycoproteins, zona pellucida glycoproteins 2 and 3, that soften the chorion (Sano et al., 2008), so that the embryo’s contractile movements burst the chorion and the embryos hatch (Okada et al., 2010). Although this study did not assess the effects of BaP, fadrozole, and cyp19a1b on specific molecular or physical hatching-associated mechanisms, it is hypothesized that because of general reduced fitness (reflected in decreased length, edemas, body and fin axis defects) that the delayed hatching could be due to decreased contractile movements. Delayed development and fitness of zebrafish embryos has been previously suggested to account for alterations in hatching period (Danzmann et al., 1989, Pakkasmaa and Jones, 2002). Importantly, by 72 hpf all treatment
groups completely hatched so the overall impact of delayed hatch may not be physiologically significant.

In addition to mortality, six developmental phenotypes were negatively impacted by BaP and fadrozole waterborne exposure and cyp19a1b knockdown in 96 hpf zebrafish larvae including: body length; optic vesicle; swim bladder inflation; pericardial and abdominal edema; and incidence of normal larval tail. Many of these phenotypes were consistent with those reported in other mammalian models following exposure to PAHs and/or BaP.

For example, human epidemiological studies suggest that when comparing offspring of smoking and non-smoking mothers, babies of smoking moms have significantly lower birth lengths (Wang et al., 1997, Prabhu et al., 2010, Vardavas et al., 2010). Furthermore, studies of fetal exposure to PAH via ambient pollution has found that newborns had a significantly decreased birth-length (Perera et al., 1998, Perera et al., 2003), and this birth-length deficient persisted into their childhood (Jedrychowski et al., 2015). Likewise, mallard duck (Anas platyrhynchos) embryos exposed to crude oil had reduced growth, body weight, crown-rump length, and bill length (Hoffman and Gay, 1981). In fish, BaP significantly decreased length of seabass (Dicentrarchus labrax) juveniles (Gravato and Guilhermino, 2009). Also, decreased in body length was one of phenotypes that resulted of zebrafish parental dietary BaP exposure in F1 generation (Corrales et al., 2014b). Here, we found that both BaP and fadrazole waterborne exposure and cyp19a1b knockdown significantly decreased zebrafish larvae body length. This finding is consistent with a study that reported aromatase inhibitors (aminoglutethimide and 4-hydroxyandrostenedione) and selective estrogen receptor modulators (tamoxifen and clomiphene) treatments decreased zebrafish larvae body length (Hamad et al., 2007). Many studies have shown the importance of steroid hormones, especially estrogen, in bone formation and growth. Also, the pubertal growth spurt of
both sexes is driven primarily by estrogen (Cutler, 1997, Kini and Nandeesh, 2012, Singh et al., 2011). Therefore, we found that estradiol rescued zebrafish larvae body length reduction mediated by 10 BaP, 10 and 50 fadrozole, and cyp19a1b knockdown in co-treatment. Likewise, decreased body length caused by tamoxifen treatment alone was rescued by E2 co-treatment (Hamad et al., 2007). In the larval zebrafish transcriptomic study mentioned above, deactivation of apolipoprotein E (ApoE) pathway by BaP is suggested as a possible mechanism mediating decreased body size (Fang et al., 2015). Accordingly, estradiol is a key element in activation of ApoE pathway (Srivastava et al., 1997). Future work could study the effect of aromatase inhibition on the ApoE activity and receptor expression and the role of this pathway as a possible explanation of ability of estradiol to rescue decreased body-length mediated by BaP, fadrozole, and cyp19a1b knockdown.

Deformity of tail shape, such as spinal curvature, is a common development alteration resulting from environmental contaminant exposure in, for example, medaka, zebrafish, and fathead minnow larvae (Nassef et al., 2010, Oliveira et al., 2009, Parrott and Bennie, 2009). In humans similar spinal deformities, like idiopathic scoliosis, have been reported (Wong and Tan, 2010). Environmental factors, estrogen hormone reduction, and estrogen receptor polymorphism are suggested as causes of idiopathic scoliosis (Wang et al., 2011, Esposito et al., 2009). Here, we found that 50 µg/L BaP, (10 and 50 µg/L) fadrozole, and cyp19a1b knockdown significantly decreased the incidence of normal tail shape. This is consistent with inducing spinal curvature of sea bass, Dicentrarchus labrax L, and zebrafish larvae by exposure to PAH or fadrozole, respectively (Santos et al., 2014, Danion et al., 2011). Although 10 µg/L BaP decreased the incidence of normal tail shape from 90% in control to 70%, this effect was not significant. Interestingly, E2 co-treatment significantly rescued the severe tail shape abnormality that was caused by 50 µg/L fadrozole and cyp19a1b knockdown, and also increased the incidence of normal
shape in zebrafish larvae that were exposed to 10 or 50 µg/L fadrozole, or had cyp19a1b knockdown. In addition, incidence of normal tail shape in the 10 µg/L BaP group increased from 70% to almost 90%. This suggests the importance of E2 in prevention of tail shape deformities.

Decreased optic vesicle (eye) area (microphthalmia) is one of the phenotypes that was observed in our studies. This negative effect could be due to dysfunctions in eye development or just be correlated to the overall decreased body size previously discussed. BaP-waterborne exposure caused microphthalmia in rainbow trout alevins (Hose et al., 1984). When considering eye development, it is possible to use morphological or cytochemical criteria to distinguish most retinal cell types and layers (retinal pigment epithelium RPE, outer nuclear layer ONL, inner nuclear layer INL, inner plexiform layer IPL, ganglion cell layer GCL) at 72-96 hpf (Malicki et al., 1996, Morris and Fadool, 2005). A recent study found that BaP (5, 50, and 504 µg/L) reduced the length of RPE, ONL, INL, IPL, GCL, diameter of the lens, and the cellular density in GCL. Furthermore, the above morphological changes were accompanied by differential expression of 15 genes involved in eye development and visual perception by either induction (cry5, per5, hspb6, chrna1, cyp1b1, cryba4, atoh8, and zgc:73142) or inhibition (arr3l, guk1, lin7a, gnat2, opn1sw1, opn1mw1, and LOC 100004285). Also, protein levels of arr3l, guk1, lin7a, and opn1mw1 were reduced by BaP exposure (Huang et al., 2014). Another study has shown that retinoic acid deficiency lead to microphthalmia in zebrafish due to inhibition of retinaldehyde dehydrogenase (Le et al., 2012). We reported that BaP exposure altered gene expression of aldehyde dehydrogenase (aldh1a1) in 96 hpf zebrafish larvae (Fang et al., 2015).

Aromatase inhibitors like aminoglutethimide also cause reductions in zebrafish larvae eye diameter that was accompanied with decreased thicknesses of the ONL, OPL, IPL, and GCL. Moreover, selective estrogen receptor modulators tamoxifen and clomiphene decreased the retina
thickness and IPL in larvae, respectively (Hamad et al., 2007), suggesting that estrogen hormone and its receptors are important in eye development. In our study, estradiol co-treatment with 10 µg/L BaP, both fadrozole doses and cyp19a1b knockdown countered the decreased zebrafish larvae eye area. Because different studies have found that estrogen up-regulated retinoid synthesis and retinoic acid receptors (Li et al., 2004, Prins et al., 2002), and increased aldehyde dehydrogenase ADH activity and mRNA expression (Simon et al., 2002), more studies are needed to clarify the interaction between all pathways mentioned above and their involvement in the eye development.

Epidemiological studies have linked maternal smoking and infant congenital heart defects (Alverson et al., 2011). Likewise, many studies have reported an increased risk of ischemic heart disease and cardiovascular mortality due to cardiovascular disease in employees who are occupationally exposed to high concentrations of PAHs (Burstyn et al., 2005, Tüchsen et al., 1996). Cardiac anomalies, such as pericardial edema, due to PAHs exposure is a well-recognized pathology in fish including zebrafish, Japanese medaka, and rainbow trout (Oncorhynchus mykiss) (Carls et al., 1999, Rhodes et al., 2005, Billiard et al., 1999, Incardona et al., 2004). Additionally, decreased ventricular length, increased ventricular wall thickness and increased blood vessel diameter were reported in zebrafish co-exposed to BaP and α-naphthoflavone (Bugiak and Weber, 2010). This is consistent with our finding both BaP concentrations, 10 and 50 µg/L, significantly caused pericardial edema. A potential mechanism of this cardiac defect mediated by BaP exposure has been previously clarified by Incardona and his colleagues when they found that cardiac toxicities were accompanied with induction of myocardial and endocardial CYP1A, and these toxicities were decreased following AhR2 knockdown suggesting that BaP exerts its cardiotoxicities through induction of CYP1A via AhR2 (Incardona et al., 2011). Additionally, via
pathway analysis, inhibition of ACTC4, KAT6A, NOTCH2, and PKD2 in 96 hpf zebrafish exposed parentally and developmentally to waterborne BaP were predicted to activate atrial septal defects (Fang et al., 2015). Further studies are needed for assessing the action of co-treatment of 10 BaP+E2 on the above candidate regulated molecules and the atrial septal pathway.

Likewise, fadrozole exposure, and cyp19a1b knockdown significantly caused pericardial edema. This negative impact might be related to the decreased body E2 concentrations mediated by both fadrazole and cyp19a1b knockdown (Fig. 27B). Reduction of E2 has been shown to have deleterious effects that promote cardiovascular diseases in, for example, postmenopausal women, while E2 replacement therapy decreases cardiovascular diseases risk in this population (Schierbeck et al., 2012). Correspondingly, an aromatase inhibitor (4-hydroxyandrostenedione) caused congestive heart failure-like symptoms (pericardial edema and decreased heart rate) in zebrafish larvae, and that was ameliorated by 10 nM E2 co-treatment (Allgood et al., 2013). This preventative effect mediated by E2 did not occur when embryos were treated with aromatase inhibitor+E2+nitric oxide inhibitor (NOI) suggesting that E2 prevents cardiotoxicities mediated by AIs through its action on the nitric oxide synthetase (NOS) pathway (Allgood et al., 2013). Furthermore, cardiotoxicity mediated by AI was alleviated when embryos were co-treated with AI + NO. Enhancement of NOS by E2 supported this finding (Weiner et al., 1994). In our fadrozole+E2, and cyp19a1b knockdown+E2 co-treatment experiments, we found that E2 significantly diminished cardiotoxicity mediated by fadrozole and cyp19a1b knockdown.

In many ways the fish swim bladder is believed to be an evolutionarily similar to the mammalian lung (Zheng et al., 2011). Development of the swim bladder in zebrafish starts as early as 48 hpf by formation of an epithelial bud that is followed by differentiation and growth that forms mesodermal layers (Winata et al., 2009). After 72 hpf, the swim bladder of a zebrafish larva
begins to inflate (Robertson et al., 2007). This inflation is critical for larvae to decrease their body density and maintain neutral buoyancy to be able to capture food and escape predators (Li et al., 2011a). Endothelial cells and blood circulation are important in the both the differentiation and inflation of the swim bladder (Winata et al., 2010). In our study, non-inflated swim bladders were observed among BaP, fadrozole, E2 only and cyp19a1b knockdown treated larvae. Non-inflated swim bladders were also reported in zebrafish offspring following a parental dietary exposure to the BaP (Corrales et al., 2014b) and in zebrafish embryos exposed to fadrozole (Santos et al., 2014). Furthermore, the non-inflated swim bladder phenotype following 3,3′,4,4′,5-pentachlorobiphenyl PCB126 exposure was AhR2 dependent, but was independent from cyp1 or cox signaling (Jönsson et al., 2012). However, in fish exposed to PAH- and oxy-PAH contaminated soil extracts incidence of non-inflated swim bladder was not definitively rescued by AhR2 knockdown (Wincent et al., 2015). In our study, exogenous E2 treatment did not rescue the non-inflated swim bladder phenotype mediated by BaP exposure and cyp19a1b knockdown. In larvae co-treated with either fadrozole concentration plus E2, there was an increased percentage of fish that had inflated swim bladders (~60%) compared to those exposed to fadrozole concentrations alone, but this percentage was still low compared to the control. Together these data suggest that E2 homeostasis is important in swim bladder formation and inflation. Some genes that have been reported to play role in development of swim bladder tissues include: nkxcl, prl, shha, ihaa, ptc1, ptc2, fgf10a, and acta2 (Winata et al., 2009, Abbas and Whitfield, 2009, Li et al., 2011a). Future work analyzing promoter regions of these target genes for AhR and/or ERE response elements, measurement of potential BaP or E2-mediated differential expression of target genes, and/or quantitation of embryo/larval E2 concentrations may further resolve the adverse outcome pathway associated with the non-inflated swim bladder phenotype.
In this study, pectoral fin deformities (e.g., short or missing fin) were observed only in BaP exposed larvae. This finding is parallel with the multigenerational impact of dietary BaP on F1 and F2 pectoral fin formation (Corrales et al., 2014b). Also, it is consistent with the perturbation of pectoral fin development due to oxygenated PAHs exposure (Knecht et al., 2013). Estradiol co-treatment did not rescue these fin morphological defects suggesting that BaP exposure disrupts larval pectoral fin development through pathways that are not associated with aromatase and E2-mediated pathways. The transcriptomic study of 96 hpf zebrafish larvae that were exposed prenatally to BaP showed downregulation of exon expression corresponding to genes involved in the fin development for example lama5 and skiv2l2 (Fang et al., 2015). Lama5 encodes laminin alpha 5 protein that is involved in establishing and elongation of the apical fold (Dane and Tucker, 1985, Webb et al., 2007) that emerges from the apical epidermal ridge and is critical for fin morphogenesis (Yano et al., 2012). Further studies are needed to investigate the specific effects of BaP on laminin alpha 5 protein expression and potential linkages with human developmental defects (Colognato and Yurchenco, 2000).
CHAPTER 5: CONCLUSIONS and FUTURE WORK

In conclusion, this study has further highlighted the importance of neuronal aromatase (cyp19a1b) in the normal early development of zebrafish. cyp19a1b-morphants and embryos/larvae exposed to the aromatase inhibitor fadrozole had similar developmental deformities including decreased body-length, optic vesicle area, deformities in tail shape, non-inflated swim bladders, pericardial and yolk sac edemas. In addition to these morphological defects, aromatase inhibition caused increased mortality and delayed hatch. These phenotypes were also associated with decreased embryonic E2 concentrations at 48 hpf. Furthermore, all these phenotypes, except non-inflated swim bladders, were alleviated by E2 co-treatment further supporting the role of aromatase in the mechanism of toxicity.

Furthermore, zebrafish embryos exposed to nominal BaP concentrations (10 and 50 µg/L) exhibited both decreased cyp19a1b mRNA expression and many of the same phenotypic defects manifested by aromatase inhibition including increased mortality, delayed hatch, decreased body-length, decreased optic vesicle area, lower incidence of normal tail shape, non-inflated swim bladders, pericardial and yolk sac edemas. In addition, BaP exposure decreased the concentration of E2 in 48 hpf embryos. BaP+E2 co-exposure effectively rescued all phenotypes mentioned above mediated by 10 µg/L BaP. However, in contrast, co-exposure of 50 µg/L BaP with E2 enhanced the mortality compared to that caused by 50 µg/L BaP alone suggesting potential estrogen receptor and aryl hydrocarbon receptor cross-talk at the higher concentration of the AhR agonist. Pectoral fin defects were uniquely caused by BaP exposure, and E2 co-exposure did not rescue this
phenotype suggesting that non-aromatase dependent molecular pathways are responsible for BaP-mediated pectoral fin deformities.

This research has centered on identifying the potential for PAHs, BaP specifically, and aromatase inhibitors to adversely affect development. There is a relatively new appreciation and associated area of scientific inquiry considering the developmental origins of health and disease (DOHaD). The underlying hypothesis is that environmental stress during key stages of development manifests in long term adverse outcomes and disease susceptibility. Future work should investigate the adult and potential multigenerational consequences of developmental aromatase inhibition. While our preliminary experiments were designed to assess impacts on sex determination, our sample numbers were limited because of mortalities as the developmentally exposed fish matured. Other predicted adverse outcomes that may occur in adults after aromatase inhibition include decreased reproductive fitness, cardiac defects and growth deficits. The zebrafish model is especially well suited to further investigate these longer term consequences of developmental exposures.

Our previous transcriptomic work (Fang et al., 2015) has identified a number of pathways that were differentially regulated by developmental BaP exposure and appear to be consistent with phenotypic deficits noted in the larvae (e.g. optic and cardiac defects, mortality). Future work can further probe the direct relationships between aromatase inhibition and the ApoE, AhR and Notch pathways. Relatively novel findings suggesting BaP-mediated eye and fin developmental toxicity need further mechanistic validation as well. Promoter analysis of candidate genes may indicate unexplored AhR and ER response elements. In turn, site directed mutagenesis of response elements and/or antisense knockdown of these candidate genes along with phenotypic anchoring will further
support the molecular mechanisms involved in PAH and estrogen dependent developmental toxicities.


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and Bone Densitometry in Childhood. *The Journal of Clinical Endocrinology & Metabolism*, 82, 1739-1745.


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RUN# 19908 JUN-20, 1985 17:24:39

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**TOTAL AREA = 1.3125E+08**

**MULT FACTOR = 1.0000E+00**

**BaP (10 µg/L)**
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<td>AREA FACTOR=1.8E+26</td>
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**TOTAL AREA=1.2999E+08**
**MUL FACTOR=1.0000E+08**

Fadrozole (50 µg/L)
Western blot using Spring Valley produced anti-CYP19b polyclonal antibody and commercially available b-actin (Anti-actin-beta (CT),Z-Fish™) (Anaspec) (for loading efficiency). In blot A, the protein concentration was 50 µg while in blot B, 35 µg was loaded. The incubation period for blocking solution, primary Ab and secondary Ab (Goat Anti-Rabbit IgG (H+L)-AP Conjugate, Bio-Rad) were each overnight. Primary Ab concentration was 1/100, secondary Ab was 1/2000 and β-actin was 1/100. MB = male brain from adult zebrafish. Larval samples represent 150 homogenized. The blue arrow indicated the hypothesized size of the CYP19a1b.
VITA KHALID ALHARTH Y

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Position
2009 until now, Teaching Assist in Department of Pharmacology and Toxicology, Prince Sattam bin Abdulaziz University at Alkharj, Saudi Arabia.

Education
2015, PhD Department of BioMolecular Sciences, Division of Pharmacology, University of Mississippi.
2009, B.S. in Pharmaceutical Sciences from Pharmacy School, King Saud University, Saudi Arabia.

Honors
2014, second place in platform competition of South Central Chapter of Society of Toxicology annual meeting.

Training
2008, medical representative in Abbott pharmaceutical company for two months.
2009, pharmacist trainee in King Fahad medical city hospital for four months in different sections such as drug and poison information center, iv-room, out-patient and in-patient pharmacies.
2014, completed online course titled “Epigenetic Control of Gene Expression”, provided by The University of Melbourne on coursera web site.
2013, attended “Gonadal Development, Function, and Toxicology” continuing education course provided by Society of Toxicology.
2013, attended 5th 1-day Zebrafish Behavioral Neuroscience and Neurophenotyping Workshop.

Memberships:
Since 2009, member of American Society for Pharmacology and Experimental Therapeutics.
Since 2009, member of Society of Toxicology.