Altered Nucleotide Excision Repair by Estrogens: Implications for Carcinogenesis

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ALTERATION OF NUCLEOTIDE EXCISION REPAIR BY ESTROGENS:

IMPLICATIONS FOR CARCINOGENESIS

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Estrogens and estrogen mimics represent a wide range of aquatic contaminants that elicit deleterious effects on exposed organisms. Despite well-characterized reproductive effects of environmental estrogens, less is known about non-reproductive impacts of exogenous estrogen exposure. Additionally, estrogens are known carcinogens, implicated in multiple human cancers. Little or no research has examined the effects of xenoestrogens on DNA repair despite being known carcinogens. The goal of this research was to test the hypothesis that aquatic estrogens enhance the effects of environmental mutagens by altering DNA repair. Of particular interest is nucleotide excision repair (NER), the only repair pathway to remove structurally diverse DNA lesions that cause helical distortion, such as DNA adducts caused by ubiquitous environmental carcinogens. Research presented here shows that 17α-ethinylestradiol (EE₂), a semi-synthetic hormone found in most oral contraceptives, alters hepatic mRNA levels of several key NER genes including XPC, XPA, XPD and XPF in adult male and female zebrafish exposed to environmentally relevant concentrations. Physiologically relevant concentrations of EE₂ suppressed rate and magnitude of bulky adduct repair in zebrafish liver (ZFL) cells. This
suppression of repair capacity in ZFL cells was not ameliorated in the presence of a complete estrogen receptor antagonist, which is known to antagonize the zebrafish estrogen receptor esr1. In adult zebrafish co-exposed to EE2 and benzo(a)pyrene (BaP), a prototypic polycyclic aromatic hydrocarbon and bulky adduct forming mutagen, hepatic mRNA levels of XPC and XPA were increased in comparison to fish exposed to EE2 alone. Regardless of hepatic NER transcript levels, fish co-exposed to EE2 and BaP exhibited increased BPDE-DNA adduct levels in comparison to both controls and fish exposed to BaP alone. In addition to single chemical exposures, adult zebrafish exposed to wastewater treatment effluent, a significant source of aquatic estrogens, exhibited altered NER transcript levels and decreased NER capacity. Collectively, this has significant implications for aquatic organisms living in contaminated environments, indicating the potential for higher mutation rates and increased neoplastic transformation with estrogen co-exposure than would be expected with mutagens alone. This research also presents an additional carcinogenic mode of action for estrogens, alteration of DNA repair.
Dissertation Acceptance Statement

On behalf of the Graduate Committee for Emily Notch, I affirm that this manuscript is the final and accepted dissertation. Signatures of all committee members are on file with the Graduate School at the University of Maine, 42 Stodder Hall, Orono, ME.

Dr. Greg Mayer, Assistant Professor

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“Energy and persistence conquer all things.” - Benjamin Franklin
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Chapter 1

INTRODUCTION

1.1 Estrogen

Steroid hormones are a broad class of small hydrophobic molecules that play an important role in vertebrate cell signaling. Three major classes of steroid hormones exist: adrenal steroids (including cortisol), sex steroids (including estrogen and testosterone), and Vitamin D$_3$. All steroid hormones are derived from cholesterol. Steroidogenesis starts with side chain cleavage of cholesterol to form pregnenolone, followed by metabolism of the side chain cleavage product into several intermediates including progesterone, androstenedione and testosterone [1]. The rate limiting step in steroid biosynthesis is release of cholesterol from the outer to inner mitochondrial membrane, which is controlled by steroidogenic acute regulatory protein (STaR) [2, 3]. Several other enzymes are involved in steroid biosynthesis including but not limited to cholesterol side cleavage chain enzyme (CYP11A1), 17α-hydroxylase, 3β-hydroxysteroid dehydrogenase (HSD), 17β-HSD, and aromatase (CYP19) [1]. Most organs cannot make steroids directly from cholesterol, but many are able to convert intermediates into appropriate steroid hormones [1]. Estradiol, which is a recurrent theme throughout this dissertation, is primarily synthesized in gonads, particularly granulosa cells in the ovaries, fat cells, brain, arteries, liver and skin.

In mammals, estrogens regulate growth, differentiation, development, maintenance and a wide range of other functions in various cell types [4, 5]. Estrogens are responsible for development of female secondary sex characteristics and function as reproductive hormones in both males and females. However, these are not the only roles
of estrogens. Pleiotropic, tissue specific responses to estrogen are seen in a wide range of cell types. Estrogen is also implicated in many human diseases including breast and other cancers, cardiovascular disease, osteoporosis and Alzheimer’s disease [6].

Estrogens play an important role in bone turnover in both males and females by inhibiting bone remodeling, suppressing bone resorption and stimulating bone formation [7]. Rapid increase in [Ca^{2+}] is seen after estrogen addition in endometrial cells, maturing oocytes and granulosa cells [8-10]. Estradiol also causes relaxation of coronary arteries and vessels in the aorta [11, 12]. Endothelial nitric oxide synthase is activated by estradiol in endothelial cells, resulting in vasodilation [13-15]. Estrogen also causes a variety of immune responses including induction of interleukin 1α (IL-1α), which in turn initiates a cascade of other cytokines, chemokines and growth factors [16-18]. In addition, estrogen is important for brain development, adult function, regulation and maintenance of several brain areas as well as aging processes including anatomy and connectivity of the hippocampus that may relate to Alzheimer’s disease [19].

Despite the liver not being commonly thought of as an estrogen responsive tissue, estrogens are metabolized in the liver to a variety of end products including estrone, catecholestrogens, quinones, estrone sulfate, estrone glucuronide and others [20, 21]. As such, higher concentrations of estrogen are necessary to elicit a response in the mammalian liver than other sex hormone responsive tissues including the breast, and uterus.

Steroidogenesis is well conserved between mammals and teleosts [22]. Estrogens control similar and additional functions in teleosts to those described in mammals. Similar to mammals, estrogens are primarily produced in the ovaries under the control of
pituitary hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and exert pleiotropic effects in many different tissue types [23]. However, neural tissue synthesis of estrogen is far higher in teleosts than in mammals [24, 25]. All three forms of zebrafish estrogen receptor (esr1, esr2a and esr2b) are found with differential distribution and partial overlap in the neuroendocrine regions of the brain including the preoptic region, mediobasal hypothalamus and posterior tuberculum [26]. As opposed to mammals, the zebrafish brain continues to grow in adulthood and increased aromatase expression in the zebrafish brain may indicate that estrogen plays an important role in this process [23].

Additionally, in teleosts the liver is a more important target organ for estrogen than in mammals [27, 28]. Production of vitellogenin (VTG), a phospholipoglycoprotein synthesized in all oviparous vertebrates, is regulated by estradiol in the liver of sexually mature female fish and this is a key step to egg production [29]. After production in the liver VTG is transported by the blood stream to the ovaries where it is cleaved into two daughter proteins, phosvitin and vitellin, which are the main components of the yolk [29].

In teleosts, estrogen also controls reproduction, sexual differentiation, gonadal morphology and calcium homeostasis [22, 30]. In zebrafish, as in many teleosts that are undifferentiated gonochorists, there is a “critical window” during development where the developing gonad resembles an immature ovary and signaling from either estrogens or androgens determine the appropriate gonad development [31, 32]. In zebrafish, complete sexual differentiation typically occurs by 30-40 days post hatch while sexual maturation does not occur until 120-160 days post hatch [32, 33]. Sex steroids regulate both of these processes.
1.2 Estrogen Receptors

The majority of estrogen’s effects are mediated through estrogen receptors (ER), which are members of the nuclear receptor super family of ligand activated transcription factors [34]. In humans there are two distinct nuclear estrogen receptor proteins, α and β. In contrast, in zebrafish and most other teleosts, there are three known functional nuclear ERs, esr1, esr2a and esr2b [35, 36]. Different estrogen receptors show differing tissue distribution and ligand affinities [37-40].

The general structure and functional domains of estrogen receptor are similar in both fish and humans [26]. At the N-terminus the A/B domain (AF1) regulates ligand independent gene transcription in a promoter specific fashion. The C domain contains 2 zinc fingers that allow for DNA binding at hormone response elements. The D domain is not well conserved, but helps to maintain tridimensional structure of the protein. The C terminal E/F domain contains the ligand binding domain as well as the ligand-dependent transactivation function (AF2). The most highly conserved regions of estrogen receptors are the DNA binding domain and the ligand-binding domain [5, 26]

Classical models of steroid action involve ligand entering the nucleus and binding to estrogen receptor causing dissociation of chaperone proteins and a conformational change in the receptor that reveals the DNA binding domain [34, 41]. The ligand bound receptors then homo- or hetero-dimerize and bind to cis-acting estrogen responsive elements (ERE) in the proximal promoter of estrogen regulated genes recruiting necessary co-factors and initiating transcription [34]. However, this is clearly not the only mechanism of action for estrogen as it has been shown that one-third of the genes that are regulated by estrogen in humans do not contain EREs in the proximal promoter [4, 42].
Ligand bound ERs can regulate genes with other *cis*-acting elements such as AP-1 or Sp1 through direct protein-protein interactions with other transcription factors [43-47].

In addition to nuclear estrogen receptors, recent research has revealed cytosolic, mitochondrial and membrane bound estrogen receptors [48, 49]. The membrane bound estrogen receptor, GPR30, is a seven transmembrane receptor hypothesized to account for the rapid response of cells after estrogen exposure via release of cAMP and other secondary signaling molecules [49-51]. In ligand unoccupied GPR30, a G-protein coupled receptor, the inactive conformation of the heterotrimeric G protein consists of GDP-bound Gα subunit associated with the βγ dimer. When ligand binds, a conformational change results in a GDP/GTP exchange with the release of GDP from the α subunit and subsequent binding of GTP resulting in dissociation from the βγ dimer promoting release of secondary signaling molecules [50]. GPR30 has been detected in multiple species of teleosts [51]. In particular, in both Atlantic croaker (*Micropogonias undulates*) and zebrafish, GPR30 plays an important role in regulation of oocyte maturation [52].

Due to the vital nature of estrogen in diverse physiological processes, regulation of the receptors that facilitate these pleiotropic responses is critical. Regulation of human estrogen receptor alpha is tightly controlled. Human ERα has 7 distinct transcriptional start sites that are utilized in different tissues for appropriate sex and tissue specific regulation [53]. Less is known about regulation of teleost estrogen receptors. Zebrafish esr1 contains several *cis*-acting promoter elements including multiple AP-1 sites, a non-canonical ERE and an additional half-ERE [27]. Research has shown that the half-ERE site in the promoter of zebrafish esr1 is integral for ligand bound esr1 to initiate
transcription [27]. Ligand bound zebrafish esr2a, but not esr2b, is also capable of binding to the promoter of esr1 and initiating transcription of the esr1 gene [27]. In rainbow trout multiple transcripts of esr1 are transcribed resulting in a long and short form that are expressed in different tissues [54, 55]. Similarly, multiple length transcripts of zebrafish esr1 are predicted, but have yet to be experimentally confirmed [26].

Specific pharmacological agonists and antagonists have been formulated for human estrogen receptors in order to better understand the role of specific receptors in various physiological processes. Type I antiestrogens such as ICI 182,780 are pure antagonists, functioning to inhibit all activity of estrogens in all tissues in mammals [56]. Type II and III antiestrogens, such as raloxifene and tamoxifen are selective estrogen receptor modulators (SERM) functioning differently depending on tissue type [57]. Not all of those agonists and antagonists exhibit the same function with teleost estrogen receptors (Table 1.1) [26, 36, 58] (Notch, unpublished data). Several of these compounds are also used in cancer treatments, particularly for estrogen receptor positive breast cancers [56].

<table>
<thead>
<tr>
<th>Compound</th>
<th>Agonist or Antagonist</th>
<th>Human Receptor</th>
<th>Functional in Zebrafish?</th>
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<td>ICI 182, 780</td>
<td>Antagonist</td>
<td>alpha &amp; beta</td>
<td>Yes – 1, 2a, and 2b</td>
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<tr>
<td>Tamoxifen</td>
<td>SERM</td>
<td>alpha &amp; beta</td>
<td>Yes – 1, 2a, and 2b</td>
</tr>
<tr>
<td>MPP dihydrochloride</td>
<td>Antagonist</td>
<td>alpha</td>
<td>No – 1, 2a, and 2b</td>
</tr>
<tr>
<td>PHTPP</td>
<td>Antagonist</td>
<td>beta</td>
<td>No – 1, 2a, and 2b</td>
</tr>
<tr>
<td>PPT</td>
<td>Agonist</td>
<td>alpha</td>
<td>No – 1 others TBD</td>
</tr>
<tr>
<td>DPN</td>
<td>Agonist</td>
<td>beta</td>
<td>No – 1 others TBD</td>
</tr>
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</table>

Table 1.1 Estrogen receptor agonists and antagonists.
1.3 Zebrafish as a Model Organism

The hypothalamic-hypophysis-gonad axis and endocrine systems are conserved among vertebrates [22, 23, 59, 60]. The molecular mechanisms of the zebrafish endocrine and hormonal signaling pathway are highly comparable to other vertebrates allowing for extrapolation of data from this model to other species [23, 61, 62]. This makes zebrafish an excellent model for research pertaining to estrogen and estrogen mimics [62].

Zebrafish also provide several technical advantages for research. They are easy and relatively inexpensive to maintain, optically clear during development, and knowledge of the genome allows for knock down of specific genes with morpholino or small RNA technologies. Zebrafish also have high fecundity and the length of time to sexual maturation is relatively short, allowing for multi-generational studies.

The zebrafish is a well-characterized model for toxicological studies [63]. Additionally, zebrafish are gaining acceptance in cancer research, particularly as a model for hepatocellular carcinoma [64]. Zebrafish hepatic neoplasias closely recapitulate human tumors and there is conservation of gene expression between humans and zebrafish during neoplastic transformation [65]. Zebrafish are also very susceptible to mutagen-induced tumors, but have a markedly low rate of spontaneous tumor initiation, making them an excellent model in which to examine tumor promotion [66]. In addition, the sequenced zebrafish genome makes them an ideal model for a variety of molecular applications. Combined, these characteristics make zebrafish an excellent model to elucidate impacts of estrogens in both environmental and human health.
1.4 Estrogens as Environmental Contaminants

Estrogens and estrogen mimics represent a wide range of contaminants in the aquatic environment and their presence in rivers and streams is well documented [67-73]. These include natural and synthetic hormones, industrial chemicals, pesticides, and naturally occurring contaminants such as arsenic [70, 72, 74-78]. Wastewater treatment plants constitute a significant entry source of anthropogenic pollutants to the aquatic environment including estrogens, particularly those of pharmaceutical origin [76, 79].

The most potent xenoestrogens in the aquatic environment are the synthetic and natural hormones, 17α - ethinylestradiol (EE2), 17β - estradiol (E2) and their metabolites estrone (E1) and estriol (E3) [38]. These estrogens can exert effects on aquatic organisms at concentrations in the low ng/L range [29]. A USGS survey of more than 100 U.S. streams revealed median concentrations of EE2 as 73 ng/L, and E2 as 30 ng/L, showing these compounds are present in the aquatic environment at sufficient concentration to exert biological effects [67]. Additionally, the semi-synthetic oral contraceptive EE2 is more resistant to degradation than naturally occurring estrogens such as 17-β estradiol [80]. Because of its greater stability and higher potency in vivo, EE2 may be of disproportional toxicological importance despite being found at much lower environmental concentrations than natural steroids and estrogen mimics [80].

Xenoestrogen exposure has been linked to a variety of deleterious reproductive endpoints in pertinent literature. These include increased plasma vitellogenin in male and female fish, increased intersex fish, decreased egg and sperm production, decreased egg and sperm quality, feminization of male fish and complete collapse of a fish population [81-89]. A wide range of xenoestrogens increase plasma vitellogenin in male fish [29, 84,
85, 90-95]. Additionally, increased bile estrogen levels and intersex fish have also been observed in rivers downstream from sewage treatment plants with known estrogenic discharge, indicating that estrogens can exert their effects at environmental concentrations and not just under laboratory conditions [84, 88, 96].

Despite the well-characterized reproductive effects of environmental estrogens, less is known about non-reproductive impacts of exogenous estrogen exposure. Fish are rarely exposed to just one hormone, but rather a chemical cocktail of hormones and other toxicants, including environmental carcinogens [67]. While the effects of estrogenic compounds are known to be additive, whether or not xenoestrogens potentiate the actions of other compounds is not clear [97]. Potential interactions of these complex mixtures and their role in carcinogenesis needs to be examined. Little is known about the effects of xenoestrogens on DNA repair despite estrogen being listed as a known carcinogen [98]. While 17-ß estradiol has been shown to promote mutagen induced hepatic tumors in medaka (Oryzias latipes) and rainbow trout (Oncorhynchus mykiss), the underlying mechanism has yet to be elucidated [99, 100].

1.5 Estrogens as Carcinogens

In addition to being aquatic toxicants of concern, estrogens are known carcinogens implicated in many different human cancers including: breast, prostate, testicular, ovarian, uterine, liver, colon and others [21, 101]. Estrogens are classified as non-mutagenic or epigenetic carcinogens and typically thought to be tumor promoters [21, 98]. The standard paradigm for estrogen’s role in carcinogenesis is based on increased cell proliferation caused via estrogen receptor resulting in higher probability of mutations occurring during DNA synthesis leading to a malignant phenotype [21, 101].
Despite a great deal of research in this area, the bulk of the carcinogenic effects of estrogen are still attributed to potent mitogenic effects resulting in increased cell cycle and the opportunity for random genetic errors, as well as production of reactive oxygen species, quinone metabolites that covalently bind to DNA, and chromosomal aberrations such as aneuploidy [21, 101-106]. However, collectively these effects don’t fully address the cancer risk posed by estrogens. While estrogen induced cell proliferation, generation of reactive oxygen species and production of reactive quinone metabolites undoubtedly represent an important role in estrogen induced carcinogenesis, other mechanisms likely contribute, especially in a tissue specific manner.

One additional mechanism for non-mutagenic carcinogens to increase cancer risk is by functioning as co-carcinogens, potentiating the effect of other mutagenic compounds. There is some previous research to suggest that estrogens may function as co-carcinogens by altering DNA repair. Estrogen inhibits ataxia telangiectasia-mutated and Rad3 related (ATR) kinase signal cascades, which are important cell signaling checkpoints for initiation of DNA repair [107]. 17β-estradiol has been shown to attenuate nucleotide excision repair in human keratinocytes [108]. Kang et. al. showed that human breast cancer cells co-exposed to E_2 and benzo(a)pyrene (BaP) exhibited increased BaP DNA adducts than cells exposed to BaP alone [109]. In vivo studies in mice showed that 100% of animals exposed to a DNA damaging agent, α-napthaflavone, in conjunction with estrogen developed hepatocellular carcinoma in comparison to only 30% in the group exposed to α-napthaflavone alone [110, 111]. Collectively these studies indicate an additional mode of action for estrogens in carcinogenesis may be alteration of DNA repair processes.
1.6 Nucleotide Excision Repair

Nucleotide excision repair is the key DNA repair pathway that removes a variety of environmentally triggered lesions, particularly bulky DNA adducts. NER recognizes and removes structurally diverse DNA lesions, particularly those that distort the DNA double helix [112]. Small sections of damaged DNA are removed in NER via a multi-step process involving the assembly of numerous proteins at the site of DNA damage [113-115]. This heterologous assembly of repair factors consists of initial damage recognition, damage verification and open complex formation, incision on either side of the lesion, DNA synthesis and ligation. Two sub-pathways exist in NER, global genome repair (GGR) and transcription coupled repair (TCR) (Figure 1.1 and 1.2) [114]. Many of the core proteins function in both pathways with major differences occurring in initial damage recognition. The rate limiting step in either pathway is detection of DNA damage [116].
Figure 1.1 Global genome repair sub-pathway of NER. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (Friedberg, E.C. How nucleotide excision repair protects against cancer. 1:22-33), copyright (2001)
Figure 1.2 Transcription coupled repair sub-pathway of NER. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (Friedberg, E.C. How nucleotide excision repair protects against cancer. 1:22-33), copyright (2001) a.) stalled RNA polymerase b.) recruitment of initial damage recognition TCR factors c.) removal of RNA template d.) recruitment of other NER or BER factors e.) repair of bulky adduct

In GGR, helical distortion attracts XPC-HR23B to damaged DNA, however excision does not occur unless a lesion is present, indicating a multi-step process
requiring damage verification [113, 115, 117, 118]. Once the XPC-HR23B complex has recognized damaged DNA, it recruits sequential repair factors to form an open complex, including XPA and the transcription factor IIH (TFIIH) complex containing XPB and XPD, 3’ and 5’ helicases, respectively [113, 118, 119]. XPA complexed with RPA, serves to verify damage prior to incision around the damaged site [120]. XPB and XPD unwind DNA around the lesion site and two nuclease s, XPG and XPF, cleave the 3’ and 5’ ends of the open complex [113, 115]. After the adducted segment of DNA is excised, DNA synthesis and ligation complete the process to replace the removed section of DNA.

In TCR, initial damage recognition occurs because helical distortion of the DNA results in inability of RNA polymerase II to progress past the lesion, resulting in recruitment of the heterodimer of CSB and CSA proteins that recognize the stalled polymerase [121]. From this point forward, TCR closely resembles GGR with recruitment of XPA-RPA, TFIIH, XPG and ERCC1-XPF to unwind the helix, and excise damaged DNA [122]. In comparison to GGR, TCR is able to more rapidly repair UV induced cyclobutane pyrimidine dimers (CPD), but is less proficient at repair of the other major UV adduct, 6-4 photoproducts (6-4PP) [121].

The ability to recognize damaged DNA is the most critical step for any repair process. With certain types of lesions, predominantly CPDs, the helical distortion is not significant enough to bind XPC-HR23b [121, 123]. An additional protein is involved in DNA repair in this situation, DDB2, which binds to DNA lesions to further distort the helix and allow for recognition by XPC-HR23b [123]. DDB2 is a heterodimer comprised of p127 and p48 sub-units [123]. Mutations in the p48 subunit result in loss of function
associate with the complementation group E form of the disease Xeroderma pigmentosum [124].

Loss or functional decrease of any NER associated protein is associated with increased cancer risk, as exemplified by the disease Xeroderma pigmentosum that confers significantly higher risk of skin cancer in afflicted humans [118]. This is likely more significant for the initial damage recognition proteins, XPC and XPA, since they are required for recruitment and assembly of the repair complex. In humans a greater than ten-fold decrease in XPA is necessary for XPA to be a limiting step in bulky adduct repair [125]. However, XPC is found at substantially lower concentrations in the cell and thus significant decreases in XPC may have more impact on overall repair capacity [125].

1.7 Hepatocellular Carcinoma

Nucleotide excision repair plays a key role in human hepatocellular carcinoma (HCC) as one of the most causative agents involved in HCC worldwide is aflatoxin which creates bulky DNA adducts that are preferentially repaired by the NER pathway [126]. Several other factors are commonly associated with human HCC including alcohol-induced cirrhosis, Hepatitis B and C infection and other chronic inflammatory conditions [127]. Further highlighting the importance of NER in HCC is the fact that core proteins from both hepatitis B virus and hepatitis C virus impair DNA repair, in particular NER [128, 129]. HCC is also marked by a very long preneoplastic period, where accumulation of DNA damage and mutations may represent key events for initiation of neoplastic transformation [130].

Estrogen and estrogen receptors also play a key role in human HCC despite being a male dominated disease. Prolonged used of oral contraceptives results in increased risk
of HCC [131]. Altered expression and splice variants of ERα are common in human HCC, even in early stages of the disease [132]. Patients with alcohol induced cirrhosis and alcoholic hepatitis have increased hepatic ERα levels and show patterns of liver feminization [133, 134]. Splice variants of ERα are noteworthy in the prognosis of hepatocellular carcinoma patients [135]. Patients with variant ERα missing exon 5 have extremely poor prognosis and do not respond to anti-estrogen treatment [135, 136]. Exon 5 encodes the ligand-binding domain, rendering this variant form constitutively active which leads to altered estrogen signaling [132, 134, 137]. The most highly malignant and invasive tumors often express only the exon-5 variant form of ERα [132, 135]. While the liver is not classically defined as an estrogen responsive tissue, there is little doubt that estrogen commonly plays a role in liver cancer.

1.8 This Research

These diverse concepts presented thus far are brought together in this dissertation on the study of the effects of estrogens on hepatic nucleotide excision repair. The goal of this research was to test the hypothesis that aquatic estrogens enhance the effects of environmental mutagens by altering DNA repair. This was accomplished by 1.) examining gene expression of NER genes in adult zebrafish after estrogen exposure, 2.) determining the ability of zebrafish liver cells to repair DNA damage after estrogen exposure and 3.) determining if co-exposure of estrogen and mutagenic compounds results in increased levels of DNA damage than those exposed to mutagenic compounds alone. These studies were done using zebrafish as an excellent model to elucidate impacts of estrogens in both environmental and human health.
Chapter 2

17α-ETHINYLESTRADIOL DECREASES EXPRESSION OF MULTIPLE HEPATIC NUCLEOTIDE EXCISION REPAIR GENES IN ZEBRAFISH

(DANIO RERIO)

2.1 Abstract

Waterborne 17α-ethinylestradiol (EE₂) alters hormone-mediated biological indicators in fish. These alterations include increased plasma vitellogenin, increased intersex individuals, decreased egg and sperm production, reduced gamete quality, and complete feminization of male fish. Together, these observations implicate aquatic estrogens in a broad range of detrimental effects on fish reproduction and fitness. In addition to impairing reproductive processes, EE₂ is also a strong promoter of hepatic tumor formation. Since many ubiquitous, aquatic hepatocarcinogens form DNA adducts that are preferentially repaired by nucleotide excision repair (NER) processes, we hypothesized that EE₂ may exert co-carcinogenic effects by reducing an organism’s ability to repair DNA adducts via this mechanism. The present study used fluorescence-based quantitative RT-PCR to examine effects of environmentally relevant concentrations of the semisynthetic estrogen, EE₂, on hepatic NER gene expression. Adult male and female zebrafish (Danio rerio) were exposed to 1 ng/L, 10 ng/L or 100 ng/L concentrations of EE₂, or to a solvent control (0.05%, v/v ethanol), for 7 days with static water renewal every 24 h. Effectiveness of EE₂ exposure in the liver was confirmed by examining hepatic expression of two estrogen-responsive biomarkers, vitellogenin-1 and cytochrome P450-1A1 (CYP1A1). Quantitative analysis confirmed that exposure to 100 ng/L EE₂ caused significant decreases in transcript abundance of several hepatic
NER genes in male zebrafish, including XPC (>17-fold), XPA (>7-fold), XPD (>8-fold), and XPF (>8-fold). Adult female zebrafish exhibited a four-fold decrease in XPC mRNA abundance at all exposure concentrations. Decreased mRNA abundance of NER genes was also seen to a lesser degree at lower concentrations of EE₂. Adult male zebrafish showed greater reduction of hepatic NER transcript levels than their female counterparts, which is consistent with the sexually dimorphic incidence of hepatocellular carcinoma in many species. Decreased transcript levels of NER genes have been shown to be an important epidemiological marker for increased cancer risk and decreased repair capacity in humans.

2.2 Introduction

Documentation of pharmaceuticals and personal care products in surface waters has stimulated diverse research examining their environmental impacts [138]. As a result of numerous studies on a wide variety of pharmaceuticals, organizations nationwide are beginning to implement drug recovery and disposal plans to help alleviate impacts of improperly disposed prescription drugs. Such proactive efforts are a reasonable step toward reduction of environmental inputs of pharmaceuticals and personal care products via public sewers and landfills. However, pharmaceuticals and their metabolic products will remain a constant source of wastewater contamination as long as pharmaceuticals are consumed and excreted [79, 139, 140].

One group of environmentally relevant pharmaceuticals found in wastewater is comprised of endocrine active compounds such as sex steroids and chemical hormone mimics. The most potent of these xenoestrogens in the aquatic environment is 17α-ethinylestradiol (EE₂), the semisynthetic hormone found in numerous oral contraceptives
and hormone replacement therapies [38]. A recent survey of more than 100 streams in the U.S. revealed a median EE\textsubscript{2} concentration of 73 ng/L [67]. Given that estrogens elicit responses in aquatic organisms at concentrations in the low ng/L range, the survey suggests that EE\textsubscript{2} is present in sufficient amounts in the aquatic environment to induce biological effects [67]. Additionally, EE\textsubscript{2} is more resistant to degradation than natural steroids such as 17\beta-estradiol (E\textsubscript{2}) [80]. Due to its greater stability and higher potency \textit{in vivo}, EE\textsubscript{2} may be of disproportional toxicological importance despite being found at much lower concentrations in surface waters than natural steroids such as estrone (E\textsubscript{1}) [80].

The effects of estrogens on a variety of reproductive processes in teleosts have been well delineated. These include increased plasma vitellogenin in male and female fish, increased proportions of intersex fish, decreased egg and sperm production, reduced gamete quality, and complete feminization of male fish [141-146]. A wide range of estrogens and estrogen mimics are known to increase plasma vitellogenin in male fish [29, 31, 93, 146, 147]. Increased plasma vitellogenin, altered estrogen levels in bile, and increased intersex proportions have been found in fish downstream of sewage treatment plant outflows with known estrogenicity [84, 88, 96]. This suggests that estrogens affect reproductive processes in the environment in addition to laboratory investigations.

In contrast to the wealth of information about effects of exogenous estrogen exposure on fish reproduction, very little is known about effects on non-reproductive processes. Estrogens, including EE\textsubscript{2}, are known to promote mutagen-induced hepatic neoplasia in medaka (\textit{Oryzias latipes}) and rainbow trout (\textit{Oncorhynchus mykiss}) [99, 100]. However, the mechanism of action of estrogens in increased rates of mutagen-
induced neoplastic transformation is not known [99, 100]. Increased somatic mutations, regardless of resultant neoplastic transformation, can lead to genomic instability of individuals and decreased fitness of populations [148]. One biological process that circumvents mutations caused by DNA lesions is DNA repair. Previous research has not examined the effects of xenoestrogens on DNA repair processes in fish, despite known carcinogenic effects of estrogen including attenuated nucleotide excision repair in human cells [98, 108].

Nucleotide excision repair (NER) is the primary DNA repair pathway responsible for removing a variety of lesions caused by bulky adduct forming mutagens [113, 115]. Bulky adduct forming mutagens, such as benzo(a)pyrene, are ubiquitous in the environment and are concentrated in areas impacted by anthropogenic pollution [67, 148, 149]. NER removes small sections of adducted DNA via a multiple step process involving the assembly of numerous proteins at the site of DNA damage [113, 115, 117]. This heterologous assembly of repair factors consists of proteins that carry out initial damage recognition, damage verification and open complex formation, incision on either side of the lesion, DNA synthesis, and DNA ligation.

Two sub-pathways exist in NER: global genome repair and transcription coupled repair. Many of the core NER proteins are functional in both sub-pathways with the primary difference occurring in initial damage recognition. The rate-limiting step in either pathway is the initial detection of DNA damage [116]. In global genome repair, XPC and XPA work in conjunction to recognize and verify DNA damage and initiate open complex formation prior to excision of damaged DNA [113]. Helical distortion attracts one XPC-HR23B heterodimer to the site of DNA damage but dual incision of the
adducted oligomer only occurs if a lesion is present [150]. Thus, a multi-step process involving damage verification is necessary before damage excision. Once the XPC-HR23B complex has recognized damaged DNA, sequential repair factors are recruited to form an open complex around the damaged site [113, 115, 121]. Formation of the open complex begins with association of the damage verification heterodimer XPA-RPA, and recruitment of the TFIIH complex which contains helicases XPB and XPD that unwind DNA in 3′–5′ and 5′–3′ directions, respectively [113, 119]. Two nucleases, XPG and XPF, then cleave 3′ and 5′ ends of the open complex [115]. After the adducted segment of DNA is removed, DNA synthesis and ligation complete the process to replace the excised DNA oligomer.

The present study examined effects of EE2 exposure on hepatic gene expression of NER damage recognition, damage verification, helicase, and endonuclease proteins in sexually mature zebrafish (*Danio rerio*). Concentrations of nucleotide excision repair mRNAs that code for proteins involved in progressive steps of the NER pathway were quantified in zebrafish livers after 7-day exposure to environmentally relevant concentrations of EE2. Results from this investigation showed a significant, sexually dimorphic alteration of NER gene expression after EE2 exposure and indicate a novel synergistic mechanism for estrogens in environmental carcinogenesis.

2.3 Materials and Methods

2.3.1 Adult Zebrafish Exposures

One-year-old zebrafish were maintained at the University of Maine zebrafish facility with a light:dark cycle of 14:10 h. Prior to EE2 exposure, 20 male and 20 female fish were placed in separate 3.5 L tanks for each exposure regime with water from the
University of Maine zebrafish facility (carbon filtered and UV-treated Orono, ME town water, with 7.5 mg/L dissolved oxygen and 42 mg/L hardness) and maintained at 27.6°C. Aqueous 17α-ethinylestradiol (CAS 57-63-6, Sigma E4876) was diluted in ethanol to produce a stock concentration of 2 mg/L and added to tanks to yield final EE₂ concentrations of 1 ng/L, 10 ng/L or 100 ng/L. Maximum ethanol levels were 0.05%, two orders of magnitude below the lowest observed effect concentration of ethanol for zebrafish [151]. Although no discernable difference in transcript abundance of NER genes could be detected between 0.05% ethanol exposed and unexposed zebrafish (data not shown), control fish were exposed to 0.05% ethanol under the same conditions as 17α-ethinylestradiol exposed fish for proper vehicle control. Experimental and control fish were exposed for 7 days in static water with complete renewal once per day. During daily water renewal, fish were visually inspected for overall health. Fish were fed commercially available fish food daily, 2 h prior to water renewal to minimize any adherent interactions between food and 17α-ethinylestradiol.

2.3.2 RNA Isolation

Total RNA was isolated from pooled samples of five livers from the same sex adult fish using phenol-free total RNA isolation procedures (RNAqueous, Ambion). Fish were anesthetized by a brief immersion in ice water and immediately euthanized by a sharp blow to the head [152]. Liver and intestinal tissues were surgically removed, after which the liver was separated from intestinal tissue. Liver samples were lysed with 500 µL cold guanidinium thiocyanate lysis/binding solution, manually homogenized and diluted with an equal volume of ethanol. Samples were then bound to a glass fiber filter and washed three times with ethanol. Total RNA was eluted with 50–80 µL of 75°C
DNAse/RNase free water (Invitrogen) and stored at −80°C. Three to five distinct RNA samples were collected for each experimental and control exposure. RNA integrity and concentration was assessed utilizing microcapillary electrophoresis with an Agilent 2100 bioanalyzer (Agilent). One microliter of total RNA from each sample was compared to 1µL of RNA ladder (RNA 6000 ladder, Ambion) with a known concentration of 150 ng/µL and six RNA transcripts of various sizes. RNA quality was verified by comparing corresponding 18S and 28S peaks on electropherograms for each sample tested. Only intact RNA was used for further analysis.

2.3.3 Primer Design

Sequences for zebrafish NER genes were obtained from GenBank and Ensembl whole genome databases. cDNA sequences from multiple organisms were aligned and used to validate NER sequences in the Ensembl D. rerio genomic database. Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3www.cgi) was used to design primers with appropriate quantitative RT-PCR specifications: 18–25 nucleotide length and GC content of 40–65%. NCBI’s basic local alignment search tool (BLAST) was used to verify primer specificity. Amplicons from RT-PCR reactions were sequenced to ensure correct gene products. Primers used for amplification of their corresponding gene products are listed in Table 1.
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<thead>
<tr>
<th>Gene product</th>
<th>Accession number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Size (bp)</th>
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<td>AF210727</td>
<td>CCTGGGCGGTTGTCATCT</td>
<td>TGAGGAATGGTGAAGGAAG</td>
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<tr>
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<td>TTTGAACGAGCAACGAC</td>
<td>AGTCCGCTGGATTTGATG</td>
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<td>XPC</td>
<td>XM694603</td>
<td>GCCAACATCCGTTCAGAAT</td>
<td>GAACGGTGGGAAAACAAAG</td>
<td>239</td>
</tr>
<tr>
<td>HR23B</td>
<td>BC056578</td>
<td>GAGGAGAACACCACGCTGA</td>
<td>GGGATACCCGTGACGAGATA</td>
<td>235</td>
</tr>
<tr>
<td>XPA</td>
<td>BC055179</td>
<td>GCTGGGGAGACATGAAAATC</td>
<td>TGCTGATGAAATGCTGGTGC</td>
<td>192</td>
</tr>
<tr>
<td>XPD</td>
<td>BC049410</td>
<td>AACCGGACAATAGCACAATC</td>
<td>ATCATTTCGTGAACCAACAA</td>
<td>228</td>
</tr>
<tr>
<td>XPF</td>
<td>BC054895</td>
<td>AACTCAAGAAACCGGCAAA</td>
<td>GGGTCAGACTGTAGGGGTC</td>
<td>229</td>
</tr>
<tr>
<td>18s</td>
<td>AC139725</td>
<td>CATGGGCGTTCTAGTTGTT</td>
<td>CGGACATCTAAGGGCATCAC</td>
<td>180</td>
</tr>
</tbody>
</table>

Table 2.1 Primer sets used for quantitative RT-PCR. Primers used for specific zebrafish gene amplification, NCBI accession numbers for sequences used in primer design and amplicon size of PCR product.

2.3.4 Quantitative RT-PCR

Fluorescence-based quantitative RT-PCR was performed using the MX4000 Multiplex Quantitative PCR system (Stratagene). Each reaction contained SYBR green RT-PCR master mix (0.2mM each dNTP, MgCl₂, Taq polymerase, 10nM fluorescein, SYBR green dye and stabilizers; BioRad), forward and reverse primers (30–150nM final concentration), ROX reference dye (Invitrogen), 25 ng total RNA, iScript reverse transcriptase (BioRad) and nuclease free water. cDNA synthesis was carried out at 50°C for 10 min, followed by 5 min at 95°C for reverse transcriptase inactivation. Forty cycles of amplification and fluorescence data collection were carried out with a two-step PCR of 10 s at 95°C and 30 s at 55°C. Dissociation curves were created with a 1 min denaturation step at 95°C, followed by a ramp of 41 cycles starting at 55°C for 30 s and increasing 1°C every cycle. Relative change in transcript abundance was normalized to 18S rRNA and calculated utilizing the $2^{-\Delta \Delta CT}$ analysis method [153]. Prior to analysis, amplification efficiency was examined using LinRegPCR software, which calculates
efficiency based on raw real-time PCR data [154]. Efficiencies for normalizing gene (18S) and all other transcripts were the same (1.8±0.1). Control expression levels were normalized to a value of 1. Each RNA sample was run in triplicate with three to five samples per exposure regime. A single peak in all dissociation curves verified production of a single amplicon per primer pair.

2.3.5 Statistics

Quantitative RT-PCR data were analyzed using one-way analysis of variance (ANOVA). Equal variance and normality were validated on raw Ct values prior to ANOVA. Normality of error was assessed with Lillifors test. Equal variance of samples was assessed with plots of estimates versus studentized residuals and modified Levene’s test. One-way ANOVA was performed on ΔΔCt values for a given gene for all treatments. When statistically significant differences were found between treatment groups (p < 0.05), Dunnett’s test was used to determine which experimental treatments were significantly different from controls. To validate that EE2 exposure did not alter 18S rRNA abundance, Ct values were analyzed by one-way nested ANOVA and p > 0.8 was used to determine no significant difference between treatments. All statistical analyses were done using SigmaStat 3.0 (SYSTAT Inc.) or SYSAT 11 software (SYSTAT Inc.).

2.4 Results

2.4.1 Alterations in vitellogenin-1 and CYP1A1 mRNA levels verify hepatic response to EE2

Exposure to 17α-ethinylestradiol affected hepatic vitellogenin-1 expression in both male and female zebrafish. Seven days of exposure to 1 ng/L EE2 caused a wide variation in vitellogenin-1 transcript in male fish, ranging from levels similar to control
fish, to 1000-fold higher than controls. Adult male zebrafish exposed to 10 ng/L EE₂ and 100 ng/L EE₂ had 42,055- and 46,235-fold increases in vitellogenin-1 transcript levels, respectively (p < 0.05) (Figure 2.1a). The significant increase in male VTG expression after 10 ng/L and 100 ng/L EE₂ exposure represents similar transcript levels to those of female control fish. Adult female zebrafish exposed to 17α-ethinylestradiol showed a mild increase in vitellogenin-1 transcript. After 100 ng/L EE₂ exposure, females had a 2.8-fold increase in transcript, which was significantly different from controls (p < 0.05) (Figure 2.1b).
**Figure 2.1.** Hepatic VTG mRNA abundance. Relative levels of hepatic vitellogenin-1 transcript abundance ± standard error (S.E.M.) in (A) 1-year-old male and (B) 1-year-old female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE2 for 7 days ($n = 4$). *Statistically significant ($p < 0.05$).
Adult male zebrafish exposed to 1 ng/L or 10 ng/L of 17α-ethinylestradiol exhibited no significant change in hepatic CYP1A1 mRNA levels in comparison to controls. However, exposure to 100 ng/L EE2 caused a 14.7-fold decrease in CYP1A1 mRNA, which was significantly different from controls (p < 0.001) (Figure 2.2).

Exposure of females to 1 ng/L, 10 ng/L or 100 ng/L 17α-ethinylestradiol resulted in 3.5-, 4.5-, 17.3-fold decreases in CYP1A1 message, respectively. All decreases in hepatic CYP1A1 transcript in adult female zebrafish were significantly different from controls (1 ng/L and 10 ng/L p < 0.05 and 100 ng/L p < 0.001).

**Figure 2.2.** Hepatic CYP1A1 mRNA abundance with EE2. Relative levels of hepatic CYP1A1 transcript abundance ± S.E.M. in 1-year-old male and female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE2 for 7 days (n = 4). *Statistically significant (p < 0.05), ***Statistically significant (p < 0.001).
2.4.2 EE₂ decreases gene expression of the DNA damage recognition protein, XPC

Male fish exposed to 1 ng/L EE₂ exhibited no change in XPC transcript levels. Adult male fish exposed to 10 ng/L and 100 ng/L EE₂ had 2.8- and 17.3-fold decreases in XPC expression, respectively (Figure 2.3). The decreases observed at 10 ng/L and 100 ng/L EE₂ were significantly different from controls (p < 0.05). While the male fish showed greater reductions in XPC expression with increasing EE₂ concentration, female fish showed a remarkably consistent four-fold decrease in XPC transcript levels across all concentrations. Adult female zebrafish exposed to 1 ng/L, 10 ng/L or 100 ng/L EE₂ showed 3.6-, 4.4- and 4.0-fold decreases in hepatic XPC mRNA, respectively. All of these decreases in XPC transcript after exposure to EE₂ were significantly different from controls (p < 0.05).
Figure 2.3. Hepatic XPC mRNA abundance after EE$_2$ exposure. Relative levels of hepatic XPC transcript abundance ± S.E.M. in 1-year old male and female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE$_2$ for 7 days (n = 4). *Statistically significant (p < 0.05).

2.4.3 No change was observed in HR23B mRNA levels

HR23B is a heterodimeric partner of the initial damage recognition protein, XPC. Both male and female zebrafish exposed to EE$_2$ exhibited no change in hepatic HR23B expression at any of the exposure concentrations (Figure 2.4).
Figure 2.4. Hepatic HR23B mRNA abundance after EE₂ exposure. Relative levels of hepatic HR23B transcript abundance ± S.E.M. in 1-year-old male and female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE₂ for 7 days (n = 4). *Statistically significant (p < 0.05).

2.4.4 Expression of the damage recognition protein, XPA, is reduced after EE₂ exposure

Similar to XPC, exposure of male zebrafish to 1 ng/L EE₂ caused no change in hepatic XPA transcript. A 5.4- and 7.7-fold decrease in hepatic XPA message levels were observed in adult males exposed to 10 ng/L or 100 ng/L EE₂, respectively (Figure 2.5). XPA transcript levels in the 10 ng/L and 100 ng/L exposure were significantly different from control unexposed males (p < 0.05). In adult females lower concentrations of EE₂ caused slight decreases in hepatic XPA message. Exposure to 1 ng/L, or 10 ng/L EE₂ caused 2.9- and 2.8-fold decreases in XPA transcript, respectively, which were
significantly different from controls (p < 0.05). In contrast, adult females exposed to 100 ng/L EE₂ showed no change in XPA expression from unexposed control fish.

![Figure 2.5](image.png)

**Figure 2.5.** Hepatic XPA mRNA abundance after EE₂ exposure. Relative levels of hepatic XPA transcript abundance ± S.E.M. in 1-year old male and female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE₂ for 7 days (n = 4). *Statistically significant (p < 0.05).

### 2.4.5 EE₂ exposure reduces expression of the 5’ helicase, XPD

Adult male zebrafish exposed to 1 ng/L EE₂ exhibited a 4.1-fold increase in hepatic XPD transcript, which was significantly different from control expression levels (p < 0.05). In adult male zebrafish, exposure to 10 ng/L or 100 ng/L EE₂ resulted in a 6.2- and 8.3-fold decrease in hepatic XPD transcript, respectively (Figure 2.6). Both decreases were significantly different from unexposed control expression levels (p < 0.05). In adult females 1 ng/L EE₂ caused no change in hepatic XPD transcript in comparison to controls. Exposure to 10 ng/L EE₂ resulted in a 3.4-fold decrease in hepatic XPD
expression in adult females, which was significantly different from controls (p < 0.05). Higher concentrations of EE₂ resulted in no alteration of XPD gene expression in adult females.

![Figure 2.6. Hepatic XPD mRNA abundance after EE₂ exposure. Relative levels of hepatic XPD transcript abundance ± S.E.M. in 1-year old male and female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE₂ for 7 days (n = 4). *Statistically significant (p < 0.05).](image)

**2.4.6 Expression of the 5’ nuclease, XPF, was diminished after EE₂ exposure**

Similar to XPD expression, male fish exposed to 1 ng/L EE₂ showed a 4.1-fold increase in hepatic XPF expression, which was significantly different from controls (p < 0.05). Adult males exposed to 10 ng/L or 100 ng/L EE₂ exhibited 4.3- and 8.1-fold decreases in hepatic XPF expression, respectively (Figure 2.7). Both decreases were significantly different from unexposed male control expression levels (p < 0.05). Adult
female zebrafish exposed to EE₂ exhibited similar patterns of hepatic XPF expression as XPD expression. Exposure to 1 ng/L or 100 ng/L EE₂ caused no change in hepatic XPF message levels in comparison to unexposed female controls. Exposure to 10 ng/L EE₂ resulted in a 2.7-fold decrease in XPF transcript, which was significantly different from control transcript levels (p < 0.05).

**Figure 2.7.** Hepatic XPF mRNA abundance after EE₂ exposure. Relative levels of hepatic XPF transcript abundance ± S.E.M. in 1-year old male and female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE₂ for 7 days (n = 4). *Statistically significant (p < 0.05).

### 2.5 Discussion

In this study we used two known biomarkers of estrogen exposure, vitellogenin and CYP1A1, to validate our experiments examining the effects of EE₂ on DNA repair. Vitellogenin is an egg yolk precursor protein made in the liver of teleosts before transport to the oocytes. Transcription of this protein is prototypically upregulated via estrogen-
responsive elements in the proximal promoter region of the gene [28]. We found that vitellogenin mRNA levels were greatly increased after estrogen exposures, similar to previous studies [28, 93, 155]. CYP1A1 is known to be repressed after estrogen exposure as evidenced by reduced EROD activity [156, 157]. In addition, CYP1A1 mRNA abundance and activity have been shown to decrease after estrogen exposure and our results were consistent with this trend [158]. We feel the response of these two genes in our studies validates normal estrogen response for zebrafish exposed to EE_2. In our estrogen responsive model we tested the hypothesis that estrogen alters regulation of DNA repair. We observed significant alteration in hepatic transcript abundance of multiple nucleotide excision repair (NER) genes after differing EE_2 exposures. This gives rise to many questions regarding estrogen exposure in environmental carcinogenesis.

Induction of hepatic vitellogenin-1 mRNA validated our 17\(\alpha\)-ethinylestradiol exposures in adult zebrafish [29, 31, 93, 95, 141]. As in previously published studies, exposure to a range of EE_2 concentrations caused significant increases in vitellogenin-1 transcript levels in adult male fish [28, 31, 93, 95]. At 10 ng/L and 100 ng/L EE_2 male fish exhibited vitellogenin-1 expression levels similar to those seen in female control fish. In contrast, female fish exposed to EE_2 exhibited a mild vitellogenin induction, which is also consistent with other data [28, 95]. Mild responses in vitellogenin-1 induction in female fish after EE_2 exposure has been attributed to higher levels of endogenous estrogens, and normally fluctuating vitellogenin during the reproductive cycle [29].

Previous studies have shown decreased CYP1A1 activity after estrogen exposure in rainbow trout hepatocytes, carp (Cyprinus carpio) and seabream (Sparus aurata) [156-158]. While decreased activity of CYP1A1 has been examined in other aquatic organisms
after estrogen exposure, it has not yet been established in zebrafish. We show that CYP1A1 mRNA abundance is significantly decreased in both male and female fish after EE₂ exposure. Decreased CYP1A1 mRNA abundance in rainbow trout hepatocytes corresponds with decreased CYP1A1 activity [158]. Our results indicate that zebrafish would likely have decreased CYP1A1 activity after estrogen exposure due to decreased CYP1A1 mRNA abundance, as previously described in rainbow trout hepatocytes.

Bulky DNA adducts caused by PAH metabolites are repaired via the NER pathway. Oxidation of benzo(a)pyrene and other ubiquitous PAHs by cytochrome p450 enzymes, such as CYP1A1, is necessary for these compounds to form bulky DNA adducts [148, 149, 159]. Decreased transcript levels of CYP1A1 seen in this study after EE₂ exposure suggest that carcinogenic PAH metabolites may be formed at a much lower rate, making impaired NER function less of a concern. However, Navas and Segner showed estrogen only impacted basal levels of CYP1A1, and that CYP1A1 could still be induced in estrogen co-exposure with the AhR agonist β-napthaflavone [158]. This provides evidence that estrogen would not likely interfere with the metabolism of PAHs to carcinogenic metabolites.

In addition to causing deleterious reproductive effects, estrogen promotes hepatic tumors in a variety of aquatic organisms [99, 100]. To date there is not a clear understanding of the mechanisms involved in estrogen-induced tumorigenesis. Our novel finding that EE₂ decreases abundance of multiple hepatic NER transcripts highlights a non-reproductive response to estrogens in aquatic organisms. Decreased NER gene expression may, in part, explain increased rates of neoplastic transformation that have been documented in cases of estrogen co-exposure [99, 100]. Alteration of DNA repair
processes by estrogen could present an increased risk of fixed mutations and genomic instability in aquatic organisms.

The most consistent alteration of NER gene expression in both male and female zebrafish after EE2 exposure was in XPC, part of the heterodimer responsible for initial damage recognition. Male zebrafish exhibited a dose-dependent response to EE2 in XPC expression with the most significant decrease at 100 ng/L. Females did not show as great of a response in XPC expression as their male counterparts after EE2 exposure. However, females showed a remarkably consistent four-fold decrease in XPC transcript at all EE2 concentrations tested. Loss or decrease of any NER associated protein may lead to an increased incidence of uncorrected DNA lesions. This is likely more significant for the initial damage recognition proteins, XPC and XPA, since they are required for recruitment and assembly of the repair complex [113]. Normal intracellular XPC protein levels are significantly lower than those of XPA, making decreases in XPC more likely to impact repair capacity [125]. Decreases in XPC mRNA abundance seen after 10 ng/L and 100 ng/L EE2 exposure in both male and female zebrafish are far greater than those suggested to have implications for impaired NER function [125]. HR23B, the heterodimer partner to XPC, showed no change in expression levels in either sex fish after EE2 exposure. HR23Bs role in NER is stabilization of XPC [113, 115]. So it is likely that regardless of HR23B expression remaining unchanged, initial damage recognition would be altered after EE2 exposure due to decreased XPC mRNA abundance. In addition, the lack of alteration in HR23B indicates that this phenomenon is not global repression by EE2, but rather a gene specific response.

In addition to decreased XPC transcript, adult male zebrafish had significantly
decreased hepatic XPA expression after exposure to 10 ng/L and 100 ng/L EE2. Adult female zebrafish also had decreased XPA mRNA abundance after exposure to 1 ng/L and 10 ng/L EE2. XPA is responsible for damage verification and open complex assembly in NER [113, 115]. Studies have shown that XPA protein levels must be decreased to <10% of normal concentrations in order to impact NER function [125]. While it is not known whether transcript levels of NER genes are directly proportional to protein levels, we observed a reduction in XPA transcript similar to those noted as having deleterious effects on repair capacity in human cells [125].

In contrast to other NER genes where little change was seen in male zebrafish at 1 ng/L EE2, XPD and XPF mRNA abundance was increased. XPD, a 5’ helicase, is part of the TFIIH complex that also plays a role in normal transcription and cell cycle regulation. It is probable that at low concentrations of EE2 increased metabolic rates in these fish results in increased transcription leading to the higher levels of XPD observed [113]. XPF is a 5’ nuclease, which also plays a role in cross-link repair and homologous recombination. The mechanisms governing upregulation of XPF transcript abundance by EE2 are not presently known.

Although it is not yet understood whether transcriptional decreases in NER genes are sufficient to alter repair capacity, expression levels of NER genes are currently used as important epidemiological markers for increased cancer risk in humans [160, 161]. Additionally, Kang et al. showed increased benzo(a)pyrene induced DNA adduct formation with co-exposure of BAP and 17β-estradiol in human breast cancer cells [109]. They hypothesized that the increased adduct formation was due to increased free radical generation with estradiol metabolism [109]. Our research presents an alternate hypothesis
for increased adduct formation, which is decrease in damage recognition and repair through decreased expression of key NER genes. Preliminary data from our laboratory indicate that EE2 exposure does in fact delay bulky adduct repair rates in human and zebrafish liver cell lines (data not shown); however, further investigation is necessary to ascertain the degree of significance.

Another interesting aspect of this study is the dichotomy of response between male and female fish. In contrast to male fish, female zebrafish exposed to EE2 showed less alteration of NER gene expression similar to the milder response in vitellogenin induction. The lesser response by females to EE2 may be due to increased levels of endogenous estrogen in female fish. One additional explanation is female fish possess greater metabolic capacity for exogenous estrogens than male fish, allowing estrogens to be more rapidly converted into biologically inactive metabolites.

In addition to implications for aquatic organisms, data acquired from this research model have potential human health implications. Zebrafish are rapidly gaining acceptance as a cancer model, in particular for hepatocellular carcinoma (HCC) [63, 64, 162]. Zebrafish hepatic neoplasias are similar to humans [63, 64, 162]. Further studies have shown zebrafish gene expression to be very similar to humans in liver tumors and tumor progression [65]. As estrogen has also been implicated in development of HCC, this research has significant ramifications for the role of estrogen in increased cancer risk. The more pronounced effect of decreased NER transcripts in male fish exposed to estrogen is of great interest given the sexually dimorphic incidence of human HCC [127].

The greatest cause for concern, if estrogen indeed potentiates the effects of carcinogenic PAHs, is failure to remove adducts allowing for increased mutation rates or
cell mortality. Studies have shown increased hepatic neoplasias in freshwater and estuarine fish living in contaminated environments [148, 163-165]. While estrogen contamination was not examined in these studies, estrogenic compounds are present in a majority of rivers and streams due to effluent from sewage treatment plants and other industrial sources [67, 69, 79]. This study gives rise to the question of whether or not estrogenic compounds in polluted environments potentiate the effects of carcinogenic PAHs, ultimately leading to higher incidence of hepatic or other neoplasias than would normally be expected from PAH contamination alone.
Chapter 3

17α-ETHINYLESTRADIOL HINDERS NUCLEOTIDE EXCISION REPAIR IN ZEBRAFISH LIVER CELLS

3.1 Abstract

Nucleotide excision repair (NER) is the primary mechanism that removes bulky DNA adducts such as those caused by ubiquitous environmental mutagens including benzo(a)pyrene and other polycyclic aromatic hydrocarbons. Recent data suggest that exposure to environmentally relevant concentrations of estrogen decreases hepatic mRNA abundance of several key NER genes in adult zebrafish (*Danio rerio*). However, the impact of decreased hepatic NER expression on NER function was not investigated in the previous study. The goal of this study was to examine the effect of the potent estrogen receptor agonist 17α-ethinylestradiol (EE₂) on rate and magnitude of bulky DNA adduct repair. Here we show that exposure of zebrafish liver (ZFL) cells to physiologically relevant concentrations of EE₂ resulted in reduced ability of ZFL cells to repair damaged DNA in comparison to control cells. Co-exposure to EE₂ and a complete estrogen receptor antagonist (ICI 182,780) also resulted in reduced NER capacity, whereas ICI 182,780 alone did not affect the ability of ZFL cells to repair UV damage. These results indicate that estrogen exposure can decrease cellular NER capacity and that this effect can occur in the presence of an estrogen receptor antagonist, suggesting that EE₂ can affect NER processes through mechanisms other than nuclear estrogen receptor activation.
3.2 Introduction

Nucleotide excision repair (NER) is the primary DNA repair pathway responsible for removing a variety of DNA lesions caused by bulky adduct forming mutagens [113, 115]. Bulky adduct forming mutagens, including polyaromatic hydrocarbons (PAH) such as benzo(a)pyrene, are ubiquitous in the environment and concentrated in areas heavily impacted by anthropogenic pollution [148, 149, 166]. Aquatic organisms living in contaminated freshwater and estuarine sites have high incidence of hepatic neoplasia, indicating the importance of NER [148, 149, 165].

In addition to bulky adduct forming mutagens estrogenic contaminants are prevalent in many aquatic environments [67, 73, 74]. Surveys have consistently shown concentrations of xenoestrogens in the ng/L range in rivers and streams [67]. Since estrogens exert biological effects in the low ng/L range, these findings indicate xenoestrogens are present at sufficient concentrations to elicit biological effects [67]. The reproductive effects of aquatic estrogens on teleosts have been well delineated in the literature in terms of biomarker analysis, population dynamics and gonadal morphology [31, 84, 85, 88, 89]. More recent studies have indicated that synthetic estrogens may alter NER process in zebrafish, implicating estrogens in deleterious non-reproductive effects [167].

Estrogens have been implicated in the etiology of a variety of human cancers [21, 130]. While the majority of research on the role of estrogen in carcinogenesis focuses on increased cell proliferation, generation of reactive oxygen species and oxidative DNA damage by quinone metabolites, there is evidence to suggest that an additional mode of action for estrogen may be alteration of DNA repair processes [21, 168-170]. 17β-
estradiol (E₂) has been shown to attenuate nucleotide excision repair in human keratinocytes and co-exposure of E₂ and benzo(a)pyrene in human breast cancer cells results in increased DNA adducts as compared with benzo(a)pyrene exposure alone [108, 109]. Both of these studies specifically looked at adducts that are preferentially repaired by NER. Additionally, recent research has shown decreased hepatic mRNA abundance of several key NER genes including XPC, XPA, XPD and XPF, after 17α-ethinylestradiol (EE₂) exposure in adult zebrafish [167]. Taken together, these studies indicate that estrogens have the potential to alter NER, and this may be an important carcinogenic mode of action in certain tissues.

Loss of NER function is associated with increased cancer risk, as exemplified by the disease Xeroderma pigmentosum that confers significantly higher risk of skin cancer in afflicted humans [118]. Two critical steps in the NER process are initial damage recognition and damage verification. XPC is the protein responsible for initial damage recognition in the global genome repair sub-pathway of NER [171]. XPA is responsible for damage verification and initiation of repair complex assembly in both sub-pathways of NER; global genome and transcription coupled repair [113, 115]. Both XPC and XPA are necessary for subsequent recruitment of repair factors for removal of damaged DNA [115]. Alteration of DNA repair can lead to accumulation of DNA damage resulting in higher mutation rates. If aquatic estrogens do in fact alter NER processes, this may potentiate effects of environmental mutagens leading to higher rates of neoplasia and influence genetic variation between populations living in contaminated versus pristine environments [149, 165].
Since previous research has shown that EE$_2$ decreases hepatic mRNA abundance of key NER genes in adult zebrafish, we hypothesized that EE$_2$ may also alter repair of DNA lesions in zebrafish liver (ZFL) cells [167]. The goal of this study was to examine the effect of EE$_2$ pre-exposure on overall nucleotide excision repair capacity in zebrafish liver cells. This is the first study to examine NER capacity in liver cells after estrogen exposure. Here we show that EE$_2$ exposure does alter NER mRNA abundance and overall bulky DNA adduct repair in zebrafish liver cells. Furthermore, we show that decreased NER after EE$_2$ exposure can occur in the presence of the complete nuclear estrogen receptor antagonist, ICI 182,780.

3.3 Materials and Methods

3.3.1 Cell Culture

Zebrafish liver cells were maintained at 28°C with ambient CO$_2$. Normal culture media (LDF media) was comprised of 50% L-15 (Invitrogen), 35% DMEM (Invitrogen), 15% Ham’s F-12 Nutrient Mix (Invitrogen) with 50 ng/mL murine Epidermal Growth Factor (Invitrogen), Insulin-Transferrin Selenium Supplement (Invitrogen), 0.5% Trout sera (East Coast Biologics) and 5% FBS (Invitrogen). ZFL cells were passed when they reached 90–95% confluence at a ratio of 1:5 into T25 tissue culture flasks (Corning 430168) approximately every 5 days. Cells were subcultured by first washing the flask with sterile phosphate buffered saline (Invitrogen) two times and then adding 1mL of 0.05% trypsin (Invitrogen). Once cells had detached, 4mL of serum containing media was added and each of five flasks were seeded with 1mL of cell suspension and an appropriate amount of fresh media.
3.3.2 RNA Isolations

Cells were plated in 6-well plates (CoStar 3516) at 5×10^5 cells/well in 2mL of LDF media. After plating, cells were allowed to adhere for 24 h and then exposed to 1, 10 or 100nM EE_2 (CAS 57-63-6, Sigma E4876), 100 nM ICI 182,780 (Fulvestrant, CAS 129453-61-8 Tocris International) or combination of 100 nM EE_2 and 100 nM ICI 182,780 for 24 h. EE_2 and ICI 182,780 stocks were made in 95% ethanol and diluted in cell culture media to the appropriate concentration, making final ethanol concentration in each well to 0.1%. Control wells were exposed to solvent control (1:1000 dilution of 95% ethanol in media). No difference in mRNA abundance was found between solvent exposed cells and unexposed controls, so for all studies control wells were exposed to 0.1% ethanol for proper vehicle control. After 24 h exposure total RNA was isolated using phenol free RNA isolation procedures (RNAqueous kit, Ambion/ABI). Briefly, media was aspirated and each well rinsed with 500µl of sterile PBS to remove any residual media. Cells were lysed with 500µl of cold guanidinium thiocyanate lysis/binding solution, and diluted with equal volume ethanol. Samples were then bound to a glass fiber filter and washed three times with ethanol. RNA was eluted with 60µl of 75°C DNAse/RNAse free water (Invitrogen) and stored at −80°C. Three distinct samples were collected per exposure concentration. RNA integrity and concentration was assessed utilizing micro-capillary electrophoresis with the Agilent 2100 bioanalyzer (Agilent). One microliter total RNA from each sample was compared to 1µl RNA ladder (RNA 6000 ladder Ambion) with 6 RNA transcripts of varying sizes and known concentration of 150 ng/µl. RNA quality was verified by observation of corresponding 18S and 28S
peaks on electropherograms for each sample tested. Only intact RNA was used for further analysis.

3.3.3 Quantitative RT-PCR

Fluorescence based quantitative RT-PCR was performed using primer sets and methodology previously reported [167]. Relative change in transcript abundance was normalized to 18S rRNA and calculated utilizing the $2^{-\Delta\Delta Ct}$ analysis method [153]. Prior to analysis, amplification efficiency was examined using LinRegPCR software, which calculates efficiency based on raw real-time PCR data [154]. Efficiencies for normalizing gene (18S) and all other transcripts were the same (1.8±0.1). Control expression levels were normalized to a value of 1. Each RNA sample was run in triplicate with three samples per exposure regime. A single peak in all dissociation curves verified production of a single amplicon per primer pair. Data shown are representative of 2 separate experiments with 3 wells per treatment type per experiment.

3.3.4 UV Damage

pHRL-CMV (Promega) was UV damaged with a Stratalinker UV Crosslinker (Stratagene). Undamaged pHRL plasmid was diluted to a final concentration of 50 ng/μl in DNAse/RNAse free water. An aliquot of undamaged plasmid was collected and stored at −80°C. The remainder of plasmid was placed in a 6 well cell culture plate without the lid at a volume of 1500μl per well. Plates containing pHRL-CMV were subjected to 500, 1000, or 2000 J/m² UV light. Plasmid was then aliquoted and stored at −80°C until use.

3.3.5 Transient Transfections

Cells were plated in 6-well plates at 5×10⁵ cells/well in 2mL of LDF media to yield 40% confluence at time of transfection. After plating, cells were allowed to adhere
for 24 h and were then exposed to 1, 10 or 100 nM EE$_2$, 100nM ICI 182,780 or combination of 100 nM EE$_2$ and 100 nM ICI 182,780 for 24 h as described above. After 24 h, cells were transfected with damaged and undamaged plasmid. Briefly, transfection mixtures with 500 ng UV damaged pHRL-CMV, 2µg of pGL3-Control (Promega) as a transfection control and 6µl of Lipofectin (Invitrogen) were combined in serum free media. After washing each well with 2mL of serum free media (50% L-15, 35% DMEM, 15% Ham’s F-12 Nutrient Mix with Insulin-Transferrin Selenium Supplement), the transfection mixture was added to each well. Cells were allowed to incubate with the transfection mixture for 5 h and then the transfection mixture was replaced with clean media containing serum (50% L-15, 35% DMEM, 15% Ham’s F-12 Nutrient Mix with 50 ng/mL Epidermal Growth Factor, Insulin-Transferrin Selenium Supplement, 0.5% Trout sera and 5% FBS). Wells were assayed for luciferase after 12, 24 and 36 h of incubation.

3.3.6 Luciferase Assay

To measure both firefly and renilla luciferase activity the Dual-Glo Luciferase Assay system (Promega) was utilized. Briefly, cell culture media was aspirated from each well. Cells were rinsed with 500µl of PBS to remove any residual media containing phenol red. PBS was aspirated from each well. 100µl of clean PBS was added to each well, followed by 100µl of Dual-Glo Luciferase Reagent. Cells were allowed to lyse for 10 min, and then contents were transferred to a 96-well plate prior to assessing luminescence. Plates were read with a GLOMAX 96-well plate luminometer (Promega). Each well was read for 10 s and each plate was read five times per luciferase assay type. After measuring firefly luciferase (pGL3), 100µl of Stop & Glo Luciferase Reagent was
added to each well. Renilla luciferase (pHRL) signal was allowed to develop for 10min prior to measuring luminescence. pGL3 measurements were used to normalize for transfection efficiency. The ratio of pHRL to pGL3 was first normalized to protein. Percent repair was calculated as normalized damaged luciferase expression divided by normalized undamaged luciferase expression. Data shown are representative of 3 separate experiments with 3 wells per treatment type per experiment.

3.3.7 Statistics

Quantitative RT-PCR data were analyzed using one-way analysis of variance (ANOVA). Both equal variance and normality were validated on the raw Ct values prior to ANOVA. Normality of error was assessed with Lillifors test. Equal variance of the samples was assessed with plots of estimates versus studentized residuals and modified Levene’s test. One-way ANOVA was performed on raw Ct values and allowed for analysis between all treatments for a given gene. When statistically significant differences were found between treatment groups, Dunnett’s test was used to determine which treatments were significantly different from unexposed controls. To validate that EE2 exposure did not alter normalizing gene expression, Ct values were analyzed by one-way nested ANOVA and p > 0.8 was used as a threshold. Host cell reactivation data were analyzed using t-tests for individual time points and exposures. Normality of error was assessed with modified Levene’s test. All statistical analyses were done using SigmaStat 3.0 or SYSAT 11 software (SYSTAT Inc.).
3.4 Results

3.4.1 Exposure to EE₂ decreases NER mRNA abundance

Transcript levels of two NER genes responsible for initial damage recognition in global genome repair and damage verification in both global genome and transcription-coupled repair, XPC and XPA, were examined in ZFL cells after 24 h exposure to 1, 10 or 100nM EE₂ (Figure 3.1). XPC transcript levels were 0.5, 0.6 and 0.4 relative to control after 1, 10 and 100 nM EE₂ exposures, respectively. These transcript levels were significantly different from untreated control cells (p < 0.05). XPA transcript levels were 0.5, 0.5 and 0.4 relative to control after 1, 10 and 100 nM EE₂ exposures respectively, which were also significantly different from control (p < 0.05). This reduction in NER transcripts in ZFL cells corroborates previous in vivo results and validates this model for mechanistic investigations.
Figure 3.1. XPC and XPA mRNA in ZFL cells after EE\textsubscript{2} exposure. XPC and XPA mRNA abundance in ZFL cells after 24 h exposure to 17\textalpha-ethinylestradiol as measured with fluorescence based quantitative RT-PCR. Graphs represent average values from 2 separate experiments ± standard error of the mean (S.E.M.) with n = 3 for each experiment. *Significantly different from control p < 0.05.

3.4.2 Pre-exposure to EE\textsubscript{2} results in decreased NER over time

Zebrafish liver cells pre-exposed to 1, 10 or 100nM EE\textsubscript{2} for 24 h prior to transfection of UV damaged plasmid exhibited significantly decreased NER capacity after 24 h (p < 0.05) (Figure 3.2). Control ZFL cells were able to repair 39.6% of the UV damage while all EE\textsubscript{2} exposed cells were only able to repair 14.1–17.6% after 24 h. After 36 h, control cells repaired 53.9% of the UV damage. Cells exposed to 1nM EE\textsubscript{2} had repair capacity similar to that of control cells after 36 h. In contrast, cells exposed to 10
and 100 nM EE₂ persisted in significantly decreased NER capacity in comparison to controls after 36 h exhibiting 39.3% and 27.6% repair respectively (p < 0.05).

**Figure 3.2.** UV damage repair capacity in ZFL cells after EE₂ exposure. UV damage repair capacity in ZFL cells after pre-exposure to 17α-ethinylestradiol as measured with host cell reactivation. Plasmid damaged with 1000 J/m² UV light. Graphs represent average values ± S.E.M. from 3 independent experiments with n = 3 for each experiment. Filled circle, control; open triangle, 1 nM EE₂; filled square, 10nM EE₂; open diamond, 100 nM EE₂. *Significantly different from control at same time point p < 0.05.

**3.4.3 Pre-exposure to EE₂ decreases NER capacity regardless of UV damage amount**

The DNA plasmid vector, pHRL-CMV, was exposed to varying amounts of UV light to examine whether decreased NER capacity after EE₂ exposure was affected by the amount of DNA damage present. Control ZFL cells transfected with plasmid damaged with 500, 1000, or 2000 J/m² had 72.2%, 39.6% and 1.8% repair after 24 h (Figure 3.3).
Cells transfected with plasmid damaged with 2000 J/m² were unable to substantially repair UV damage with or without estrogen exposure. With the exception of ZFL cells transfected with plasmid damaged with this highest dose of UV, pre-exposure to EE₂ significantly reduced NER capacity. ZFL cells pre-exposed to 1nM EE₂ showed 44.7% and 17.2% repair for 500 and 1000 J/m² of UV damage after 24 h, which were significantly different from controls (p < 0.05). Cells pre-exposed to 10 nM EE₂ exhibited 36.8% and 16.5% repair for the same amount of UV damage after 24 h, which were significantly different from controls (p < 0.05). ZFL cells pre-exposed to 100 nM EE₂ showed 22.6% and 14.1% repair for 500 and 1000 J/m² of UV damage after 24 h, which were also significantly different from controls (p < 0.05).
Figure 3.3. Repair capacity with multiple UV damage amounts. Ability of ZFL cells with pre-exposure to 17α-ethinylestradiol to repair differing concentrations of UV damage after 24 h. Graphs represent average values ± S.E.M. from 3 independent experiments with n = 3 for each experiment. *Significantly different from control with same UV treatment p < 0.05, **p < 0.01, ***p < 0.001.

3.4.4 Decreased XPC mRNA abundance is not ameliorated by ICI 182,780

To examine the role of estrogen receptor in decreased NER mRNA abundance after EE₂ exposure, cells were exposed to the estrogen receptor antagonist ICI 182,780. ZFL cells exposed to 100nM EE₂ with or without 100 nM ICI 182,780 exhibited decreased XPC transcript levels (Figure 3.4). In contrast, cells exposed to 100nM ICI 182,780 alone had XPC transcript levels similar to that of control. Cells exposed to 100nM EE₂ alone had XPC transcript levels of 0.5 relative to control, which were significantly different from controls (p < 0.05). Co-exposure of EE₂ and ICI 182,780
resulted in transcript levels of 0.6 relative to control, while cells exposed to ICI 182,780 alone had XPC mRNA abundance levels of 1.0 relative to control. XPC mRNA abundance in ZFL cells after co-exposure of ICI 182,780 and EE2 was significantly lower than controls (p < 0.05) while cells exposed to ICI 182,780 alone were not significantly different than controls.

Figure 3.4. XPC mRNA in the presence of ICI 182,780. XPC mRNA abundance in ZFL cells after 24 h exposure to 17α-ethinylestradiol with or without the estrogen receptor antagonist ICI 182,780. Graphs represent average values ± S.E.M. from 2 separate experiments with n = 3 for each experiment. *Significantly different from control p < 0.05.
3.4.5 Decreased NER capacity after EE₂ exposure is not altered by ICI 182,780

ICI 182,780 was also used to investigate the role of estrogen receptor in decreased bulky adduct repair capacity of ZFL cells after EE₂ exposure. Cells exposed to 100nM ICI 182,780 alone showed 35.5% repair after 24h, which was similar to control cells at 39.6% repair (Figure 3.5). ZFL cells exposed to 100nM EE₂ or 100 nM EE₂ with 100 nM ICI 182,780 exhibited 12.7% and 12.8% repair after 24h respectively, which was significantly different from control cells (p < 0.001). Co-exposure of EE₂ and ICI 182,780 did not result in significantly different repair capacity from ZFL cells exposed to 100nM EE₂ alone.
**Figure 3.5.** UV damage repair capacity in the presence of ICI 182,780. The estrogen receptor antagonist ICI 182,780 does not alter the effect of 17α-ethinylestradiol on DNA repair capacity with 24 h pre-exposure. Plasmid damaged with 1000 J/m² UV light. Graphs represent average values ± S.E.M. from 3 independent experiments with n = 3 for each experiment. ***Significantly different from control p < 0.001.

3.5 Discussion

NER is a vital DNA repair pathway in the liver since many environmental mutagens are metabolized in this tissue to form products capable of causing bulky DNA adducts. While previous data indicate that EE₂ acts to reduce abundance of NER gene transcripts, it was not known how such a reduction in availability of template would affect repair of DNA lesions. The goal of this study was to examine effects of EE₂ on overall NER capacity in zebrafish liver cells. Since it is not currently possible to perform host cell reactivation assays in an *in vivo* model, this study made use of a suitable *in vitro* system. While this *in vitro* assay answers the mechanistic question of whether EE₂ affects
repair, caution must be used in extrapolation of this data to EE$_2$ exposures *in situ*. However, it is worth noting that during the validation of our *in vitro* model, mRNA levels of both XPC and XPA were more mildly suppressed than in previous *in vivo* studies. This suggests that the decreased removal of lesions may be more pronounced *in vivo* and that our model may in fact underestimate the effects of EE$_2$ on DNA repair seen in the environment.

Here we report that EE$_2$ exposure decreases repair of DNA lesions in ZFL cells in a dose responsive manner to differing estrogen concentrations after 24h. In the first 24 h post-transfection (29h post-exposure) there is a suppression of NER capacity at all estrogen concentrations tested. While there is a temporal recovery of repair levels similar to that of controls in the lowest dose of EE$_2$, the higher EE$_2$ doses show persistent suppression in the magnitude of NER capacity after 36h. Regardless of increased repair levels at later time points, initial hindrance of NER at 24h in all doses of EE$_2$ may be sufficient to increase mutation frequency.

In this study we also show that the effect of EE$_2$ on NER capacity in ZFL cells is not altered by co-exposure with the complete estrogen receptor antagonist ICI 182,780. Conflicting results with use of ICI 182,780 in aquatic organisms have been published and therefore results from this study must be interpreted cautiously. In rainbow trout opposing responses have been shown in regards to ICI 182,780 exposure. Latonnelle et al. showed ICI 182,780 did not inhibit estradiol (E$_2$) induced vitellogenin production in rainbow trout hepatocytes [172]. Other studies in the rainbow trout hepatoma cell line (RTH-149) indicated that exposure to high concentrations (1µM) of ICI 182,780 completely blocked E$_2$ induction of an ERE-luciferase vector, but at lower concentrations
(1 nM) of ICI 182,780 was unable to block effects of E₂ [173]. In other fish species ICI 182,780 was able to completely block the effects of E₂ [58, 172]. However in both these studies at least a 10-fold higher concentration of ICI 182,780 in comparison to estrogen was necessary to inhibit the response [58, 172]. This raises the question that the concentrations of ICI 182,780 used in this study were not sufficient to block the effect of EE₂ on NER. However, if the effect of EE₂ on NER is mediated by ERα we would expect to see some difference between the co-exposure of EE₂ and ICI 182,780 and EE₂ exposure alone. In this study, the NER capacity was almost identical in the EE₂ exposure with or without ICI 182,780 indicating ICI 182,780 had no impact on alteration of NER by EE₂.

Additionally, studies have shown in largemouth bass that ICI 182,780 is only able to completely inhibit ERα [58]. In the same study ICI 182,780 had no effect on the activity of ERβa and only inhibited ERβb by half [58]. This raises the question of whether or not the effect we see in ZFL cells is mediated by one of the other estrogen receptor isotypes. Further exploration of the role of ERα, ERβa, ERβb, and the membrane bound GPR30 in EE₂ induced alteration of NER capacity in zebrafish is required to fully understand the necessity of estrogen receptors in this process.

There are other potential mechanisms by which estrogens may have elicited decreased NER capacity that do not directly involve nuclear estrogen receptors. Estrogens have been shown to increase expression of high mobility group proteins (HMG), particularly HMG1 [174]. Overexpression of HMGA1 protein, either naturally or induced, in human breast cancer cells results in decreased TCR and GGR, suggesting that HMGA1 overexpression inhibits one or more steps common to both NER pathways
Another possible mechanism for estrogen induced decrease in rate and magnitude of NER capacity is alteration of the tumor suppressor/oncogene tp53. Tp53 is known to regulate several NER genes, including XPC, DDB2 and XPB [176, 177].

These data show that EE2 decreases bulky adduct repair capacity and may represent one mechanism by which estrogens promote tumorigenesis. Estrogens have been previously shown to promote mutagen-induced tumorigenesis in rainbow trout and medaka without a complete understanding of the mechanisms involved [99, 100]. This finding also indicates that estrogen may potentiate the effects of environmental mutagens, resulting in higher mutation rates or greater incidence of hepatic neoplasia in organisms living in contaminated environments.
Chapter 4

17α-ETHINYLESTRADIOL ALTERS BENZO(A)PYRENE TOXICITY IN ZEBRAFISH

4.1 Abstract

Pharmaceutical hormones and other xenoestrogens are common aquatic contaminants that elicit a variety of deleterious effects on exposed aquatic fauna. In addition to a wide range of reproductive effects, estrogens alter nucleotide excision repair (NER) processes. One such pharmaceutical estrogen, 17α-ethinylestradiol (EE2), decreases hepatic mRNA abundance of several NER genes in adult zebrafish and alters rate and magnitude of bulky adduct repair in zebrafish liver cells. The question remains whether alteration of NER by estrogens results in increased DNA damage after co-exposure with environmental mutagens such as the prototypical polycyclic aromatic hydrocarbon, benzo(a)pyrene (BaP). Seven-day exposures of adult zebrafish to EE2, BaP or co-exposure of both toxicants were carried out to examine hepatic CYP1A and NER gene expression and BPDE DNA adduct formation. BaP alone induced CYP1A mRNA levels, while EE2 alone decreased CYP1A transcript. Co-exposure of these two compounds suppressed BaP induction of CYP1A in adult males. In the same co-exposures, mRNA abundance of two key NER genes, XPC and XPA, were increased in comparison to exposure to BaP alone, potentially indicating higher incidence of bulky DNA adducts. DNA adducts were very low in control male livers, and increased significantly with BaP exposure. In adult males co-exposed to EE2 and BaP, levels of hepatic DNA adducts were higher than those of fish exposed to BaP alone. This has significant implications for aquatic organisms living in contaminated environments and indicates the potential for
higher mutation rates and increased neoplastic transformation with estrogen co-exposure than would be expected with mutagens alone.

4.2 Introduction

Aquatic organisms are exposed to a variety of toxicants from both natural and anthropogenic sources. Pharmaceutical hormones and other xenoestrogens are common aquatic contaminants that elicit an array of deleterious effects, particularly reproductive impacts on exposed fauna. In addition, polycyclic aromatic hydrocarbons (PAH) and other environmental carcinogens are commonly found in the aquatic environment from incomplete combustion of organic matter and other anthropogenic sources [149]. Co-exposure to multiple types of toxicants is important for understanding potential interactions in the aquatic environment.

Xenoestrogens have been linked to a variety of deleterious endpoints in aquatic organisms. In particular, disruption of reproductive processes, including increased plasma vitellogenin, decreased fecundity, presence of ovo-testis and complete feminization of male fish have been linked to estrogen exposures [75, 83, 85, 178-180]. Less research has focused on non-reproductive impacts of aquatic estrogens. However, estrogenic contaminants have been shown to impact DNA repair, immune function, promote tumorigenesis and alter hepatic biotransformation enzyme function in aquatic organisms [99, 145, 167, 181-184].

Benzo(a)pyrene (BaP) is a common PAH found in many environments from incomplete combustion of organic matter [149]. BaP is also frequently used as a prototypical PAH in laboratory studies. BaP is not overtly toxic until it is metabolized by one or more Phase I enzymes, including cytochrome p4501a (CYP1A) and epoxide
hydrolase (EH). CYP1A seems to have a dual purpose related to BaP, the first is that of detoxication and the second metabolic activation [165, 185-187]. The amount of both BaP and CYP1A present in the cell will dictate which process is dominant [185]. The goal of metabolism of BaP is to increase water solubility to aid in excretion, however this process can also convert non-toxic BaP to its ultimate carcinogenic metabolite, benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), which is able to covalently bind DNA causing bulky DNA adducts that distort the double helix [188, 189]. These bulky DNA adducts are preferentially repaired by nucleotide excision repair (NER).

NER is the only repair pathway to remove structurally diverse DNA lesions that cause helical distortion, such as those caused by ubiquitous environmental carcinogens. The pathway is a stepwise progression including initial damage recognition, damage verification, DNA unwinding, excision, DNA synthesis and ligation [115]. Two important steps in this process are initial damage recognition and damage verification. In global genome repair, the heterodimer of XPC-HR23b is responsible for initial damage recognition [171]. In both global genome and transcription coupled repair, XPA is necessary for damage verification prior to recruitment of the rest of the repair complex [190].

Previous research has shown that environmentally and physiologically relevant concentrations of 17α-ethinylestradiol (EE₂) can impact hepatic NER at both the mRNA and functional levels in adult zebrafish (Danio rerio) [167, 191]. Additionally, estrogens, including EE₂, have been shown to decrease CYP1A at both the message and functional level in many fish species [167, 192-194]. This gives rise to the question of whether xenoestrogens, in particular EE₂, potentiate the effects of bulky adduct forming mutagens
such as BaP due to alteration of DNA repair. The goal of this research was to examine the impact of co-exposures of EE2 and BaP on nucleotide excision repair processes in adult zebrafish by measuring hepatic mRNA expression of two key NER genes and to determine how these relate to abundance of BPDE adducts in the genome.

4.3 Materials and Methods

4.3.1 Adult Zebrafish Exposures

One year old zebrafish (Danio rerio, AB strain) were maintained at the University of Maine zebrafish facility with a light:dark cycle of 14:10 hours. Prior to exposure, male and female fish were separated, placed in 3.5L tanks in groups of 8 according to sex and maintained in University of Maine zebrafish facility water at 27.6°C (carbon filtered and UV treated Orono, ME city water with 7.5mg/L dissolved oxygen and 42mg/L hardness). Eight-liter head tanks were set up above the exposure tanks, with each feeding two fish tanks. Head tanks were replenished twice daily, allowing for complete water renewal in the exposure tanks twice per day. Aqueous 17α-ethinylestradiol and benzo(a)pyrene were diluted in acetone to yield stock solutions of 100µg/L EE2, 1mg/L EE2, 10mg/L BaP and 500mg/L BaP and added to the head tanks to yield final concentrations of 10ng/L or 100ng/L EE2 and 1μg/L or 50μg/L BaP. Control fish were exposed to 0.01% acetone (v/v) under the same conditions as the chemical exposed fish for proper vehicle control. No difference between unexposed fish and 0.01% acetone fish was observed at any of the endpoints measured (data not shown). Experimental and control fish were exposed for 7 days. Fish were visually inspected daily for overall health and fed commercially available fish food once daily, 1h prior to head tank replenishment to minimize food – chemical interactions.
4.3.2 RNA Isolations

After 7 day exposure, fish were sacrificed and livers surgically removed. Total RNA was isolated using phenol free RNA isolation procedures (RNAqueous kit, Ambion/ABI) from single adult fish livers as previously described [195]. Briefly, liver tissue was manually homogenized in 300-500µL guanidinium thiocyanate lysis/binding solution and diluted with equal volume of ethanol. Samples were bound to a glass fiber filter and washed three times prior to elution with 80°C nuclease free water. Four samples of RNA were collected per sex per exposure regime. RNA integrity and concentration was assessed using micro-capillary electrophoresis with the Agilent 2100 bioanalyzer. One microliter total RNA from each sample was compared to 1µL RNA ladder with 6 RNA transcripts of varying sizes and known concentration of 150ng/µL. RNA quality was verified by observation of corresponding 18S and 28S peaks on the electropherograms for each sample tested. Only intact RNA was used for further analysis. Concentrations from the Agilent 2100 bioanalyzer were verified using a NanoDrop spectrophotometer.

4.3.3 Genomic DNA Isolations

After 7 day exposure, fish were sacrificed and livers were surgically removed. Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega). Briefly, livers were homogenized in 600µL cold nuclei lysis solution. After homogenization, 200µL of protein precipitation solution was added and samples were spun at 13000xg for 4 minutes in a room temperature centrifuge. The supernatant was kept and combined with 600µL of isopropanol to precipitate DNA. Samples were spun at 13000xg for 1 minute. Supernatant was discarded leaving pelleted DNA, which was
washed with 70% ethanol and spun down again at 13000xg for 1 minute. Genomic DNA was kept at 4°C under ethanol until time of use, at which point it was rehydrated in TE buffer (10mM Tris-HCL and 1mM EDTA, pH 8.0). Concentration and quality of the DNA was assessed with a NanoDrop spectrophotometer.

4.3.4 Quantitative RT-PCR

Fluorescence based quantitative RT-PCR was performed using primer sets and methodology previously reported [167]. Relative change in transcript abundance was normalized to 18S rRNA and calculated utilizing the $2^{-\Delta\Delta CT}$ analysis method [153]. Prior to analysis, amplification efficiency was examined using LinRegPCR software, which calculates efficiency based on raw real-time PCR data [154]. Efficiencies for normalizing gene (18S) and all other transcripts were the same (1.8 ± 0.1). Control expression levels were normalized to a value of 1. Each RNA sample was run in triplicate with four distinct RNA samples per exposure. A single peak in all dissociation curves verified production of a single amplicon per primer pair.

4.3.5 BPDE DNA Damage Standard Curve

Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) was purchased from the National Cancer Institute's Chemical Carcinogen Reference Standards Repository operated by Midwest Research Institute (Kansas City, MO). All reactions involving BPDE were performed under 620nM filtered light. BPDE was dissolved in tetrahydrofuran to a concentration of 2mg/mL and then diluted with 4mL of 100% ethanol to 0.4mg/mL. The diol epoxide solution was then mixed with 10mL of 1mg/mL calf thymus DNA solution and incubated overnight. Damaged DNA was exhaustively extracted with n-butanol and isoamyl alcohol until the BPDE peak at $A_{350}$ remained
constant. Concentration and purity of the DNA was assessed with a NanoDrop spectrophotometer.

Adducts were quantified with the following relationship that has been previously published [196, 197]:

\[
\% \text{ modification} = \frac{[\text{anti-BPDE}]}{[\text{DNA}]} \times 100
\]

where \([\text{anti-BPDE}] = A_{350}/2.9 \times 10^4\) and \([\text{DNA}] = (A_{260} - 0.18 \times A_{350})/6.65 \times 10^3\). Based on this formula, DNA damage in the highest standard was determined to be 2.55%. Dilutions of DNA standards were done with the addition of undamaged calf thymus DNA to maintain the same total DNA concentration per well.

**4.3.6 Immunoblotting**

Analysis of DNA adducts was done by immunoblotting with monoclonal antibodies targeting anti-BPDE DNA adducts using a BioRad Bio-Dot Microfiltration Apparatus. DNA was first heat denatured with 0.4M NaOH and 10mM EDTA and incubated at 100°C for 10 minutes. Reactions were neutralized by addition of equal volume of ice-cold 2M ammonium acetate. Samples were kept on ice during membrane preparation. Denatured DNA (200ng per well) was bound to a nitrocellulose membrane pre-soaked in 6x SSC buffer (900mM NaCl and 90mM sodium citrate) with vacuum filtration. Membranes were then washed with 500µL of 2x SSC buffer (300mM NaCl and 30mM sodium citrate) per well and baked for 10 minutes at 80°C prior to UV crosslinking with a Spectrolinker XC-1500 UV Crosslinker (Spectronics Corporation). Membranes were incubated in blocking solution of TBS-T (20mM Tris, 500mM NaCl, 0.1% Tween-20 pH 7.5) with 5% powdered milk (w/v) for 1h at room temperature. Membranes were washed three times in TBS-T and then incubated with primary antibody
(Mouse Anti-BPDE clone 8E11, Santa Cruz Biosciences) for 1h at room temperature with agitation. Membranes were again washed in TBS-T and incubated with secondary antibody (Rabbit anti-mouse horseradish peroxidase conjugate, AbCam) for 1h at room temperature with agitation. Membranes were washed again in TBS-T with a final rinse in TBS. Chemiluminescence was measured using ECL western blotting substrate kit (AbCam) and visualized on film. Film was scanned and densitometry analysis was performed with Image J.

4.3.7 Statistics

Quantitative RT-PCR data were analyzed using one-way analysis of variance (ANOVA). Both equal variance and normality were validated on the raw Ct values prior to ANOVA. Normality of error was assessed with Lillifors test. Equal variance of the samples was assessed with plots of estimates versus studentized residuals and modified Levene's test. One-way ANOVA were performed on ΔCt values and allowed for analysis between all treatments for a given gene. When statistically significant differences were found between treatment groups, Tukey’s HSD post-hoc test was used to determine which treatments were significantly different from one another. To validate that exposure did not alter normalizing gene expression, Ct values were analyzed by one way nested ANOVA and p>0.8 was used as a threshold. DNA adducts were calculated based on regression analysis of the standard curve. Adduct data in exposed fish was analyzed by one way ANOVA with Tukey’s HSD post-hoc test to determine which treatments were significantly different from one another.
4.4 Results

4.4.1 CYP1A mRNA abundance

Adult male zebrafish exposed to 10ng/L or 100ng/L EE$_2$ exhibited 0.78 and 0.04 fold hepatic CYP1A mRNA abundance relative to unexposed controls of which the 100ng/L EE$_2$ was significantly decreased in comparison to controls (p<0.05) (Figure 4.1). Male zebrafish exposed to 1µg/L or 50µg/L BaP had 1.34 and 8.53 fold increases in hepatic CYP1A mRNA abundance in comparison to controls with the 50µg/L BaP exposure being statistically significant from controls (p<0.05). Adult males co-exposed to 10ng/L EE$_2$ and 1µg/L BaP or 50µg/L BaP showed no alteration of hepatic CYP1A transcript levels in comparison to unexposed controls. Adult males exposed to 10ng/L EE$_2$ and 50µg/L BaP exhibited significantly decreased CYP1A mRNA abundance in comparison to fish exposed to BaP alone (p<0.05). In contrast, fish co-exposed to 100ng/L EE$_2$ and 1µg/L BaP exhibited decreased hepatic CYP1A mRNA abundance 0.12 that of unexposed controls, which was statistically significant from both controls and fish exposed to 100ng/L EE$_2$ alone (p<0.05). Adult males exposed to 100ng/L EE$_2$ and 50µg/L BaP had hepatic CYP1A mRNA levels 1.58 fold higher than unexposed controls, which was statistically significant from fish exposed to EE$_2$ alone (p<0.05). Additionally, the adult males co-exposed to 100ng/L EE$_2$ and 50µg/L BaP did exhibit significantly lower CYP1A mRNA levels than those exposed to 50µg/L BaP alone (p<0.05).
Figure 4.1 Adult Male CYP1A mRNA abundance after co-exposure. Relative hepatic mRNA abundance of CYP1A in adult male zebrafish exposed to waterborne EE2 with or without BaP for 7 days with complete daily water renewal twice daily. a = statistically significant from unexposed controls, b = statistically significant from EE2 alone, c = statistically significant from BaP alone p<0.05

Adult female zebrafish exhibited similar patterns of hepatic CYP1A mRNA abundance after EE2 exposure, BaP exposure or co-exposure as their male counterparts. Females exposed to 10ng/L or 100ng/L EE2 showed CYP1A transcript levels of 0.5 and 0.04 that of controls respectively (Figure 4.2). The females CYP1A mRNA level after 100ng/L EE2 were statistically significant from unexposed controls (p<0.05). Adult females exposed to 1µg/L or 50µg/L BaP exhibited hepatic CYP1A mRNA levels similar to control and 3.5 fold higher respectively, with the 50µg/L BaP being statistically significant from unexposed controls (p<0.05). Fish exposed to 10ng/L EE2 with 1µg/L or
50µg/L BaP showed hepatic CYP1A mRNA abundance similar to that of unexposed controls. However, fish exposed to 10ng/L EE2 with 50µg/L BaP showed a significant dampening of CYP1A induction in comparison to fish exposed to 50µg/L BaP alone (p<0.05). Females exposed to 100ng/L EE2 with 1µg/L or 50µg/L BaP exhibited hepatic CYP1A transcript levels of 0.05 and 2.6 fold that of control, respectively, both of which were statistically significant from unexposed controls (p<0.05). Females exposed to 100ng/L EE2 and 50µg/L BaP showed transcript levels that were significantly higher than those of fish exposed to 100ng/L EE2 alone (p<0.05).

**Figure 4.2** Adult Female CYP1A mRNA abundance after co-exposure. Relative hepatic mRNA abundance of CYP1A in adult female zebrafish exposed to waterborne EE2 with or without BaP for 7 days with complete daily water renewal twice daily. a = statistically significant from unexposed controls, b = statistically significant from EE2 alone, c = statistically significant from BaP alone  p<0.05.
4.4.2 XPC mRNA abundance

Adult male zebrafish exposed to 10ng/L EE$_2$ exhibited similar hepatic transcript levels of XPC as control fish (Figure 4.3). However adult males exposed to 100ng/L EE$_2$ for seven days exhibited significantly decreased hepatic XPC mRNA levels 0.3 that of unexposed controls ($p<0.05$). Males exposed to 1µg/L BaP or 50µg/L BaP showed a 2 fold increase and hepatic XPC transcript levels similar to controls respectively, neither of which was statistically significant from controls. Adult fish co-exposed to 10ng/L EE$_2$ and either 1µg/L BaP or 50µg/L BaP showed no change in XPC mRNA levels in comparison to control fish. In contrast, adult males exposed to 100ng/L EE2 and 1µg/L BaP showed a 3.6 fold increased in hepatic XPC transcript levels that were significantly higher than those from unexposed controls and fish exposed to 100ng/L EE$_2$ alone ($p<0.05$). Males co-exposed to 100ng/L EE$_2$ and 50µg/L BaP had XPC mRNA levels similar to those of control fish.
Figure 4.3 Adult Male XPC mRNA abundance after co-exposure. Relative hepatic mRNA abundance of XPC in adult male zebrafish exposed to waterborne EE₂ with or without BaP for 7 days with complete daily water renewal twice daily. a = statistically significant from unexposed controls, b = statistically significant from EE₂ alone, c = statistically significant from BaP alone p<0.05

Adult female zebrafish exposed to 10 ng/L or 100ng/L EE₂ exhibited statistically significant decreases in hepatic XPC mRNA abundance 0.5 and 0.3 that of unexposed control females (Figure 4.4) (p<0.05). Fish exposed to 1µg/L BaP or 50µg/L BaP alone showed hepatic XPC transcript levels similar to those of control. Females exposed to 10ng/L EE₂ with 1µg/L BaP or 50µg/L BaP exhibited XPC mRNA levels 0.4 and 2.3 that of control. Co-exposure of 10ng/L EE₂ and 1µg/L BaP resulted in hepatic XPC transcript levels significantly lower than fish exposed to 1µg/L BaP alone (p<0.05). Females exposed to 10ng/L EE₂ and 50µg/L BaP had XPC mRNA abundance that was
significantly higher than fish exposed to either EE$_2$ or BaP alone (p<0.05). Adult females exposed to 100ng/L EE$_2$ with 1µg/L BaP or 50µg/L BaP both exhibited slight increases in hepatic XPC mRNA 1.5 fold higher than controls, both of which were significantly different than fish exposed to EE$_2$ alone (p<0.05).

**Figure 4.4** Adult Female XPC mRNA abundance after co-exposure. Relative hepatic mRNA abundance of XPC in adult female zebrafish exposed to waterborne EE$_2$ with or without BaP for 7 days with complete daily water renewal twice daily. a = statistically significant from unexposed controls, b = statistically significant from EE$_2$ alone, c = statistically significant from BaP alone  p<0.05

**4.4.3 XPA mRNA abundance**

Hepatic XPA mRNA abundance in adult male zebrafish exposed to EE$_2$, BaP or combinations of the two toxicants exhibited similar patterns as hepatic XPC (Figure 4.5). Males exposed to 10ng/L or 100ng/L EE$_2$ for seven days exhibited decreased XPA mRNA levels of 0.9 and 0.5 that of controls although only the 100ng/L EE$_2$ concentration
was statistically significant (p<0.05). Adult male zebrafish exposed to 1µg/L BaP exhibited a 2.57 fold increase (p<0.05) in hepatic XPA while fish exposed to 50µg/L BaP showed XPA transcript levels similar to control. Fish exposed to 10ng/L EE\textsubscript{2} with 1µg/L BaP or 50µg/L BaP showed transcript levels similar to control. While fish co-exposed to 100ng/L EE\textsubscript{2} with 1µg/L BaP or 50µg/L BaP showed a 2.8 and 2 fold increase in hepatic XPA mRNA levels respectively which were significantly different from unexposed controls (p<0.05).

**Figure 4.5** Adult Male XPA mRNA abundance after co-exposure. Relative hepatic mRNA abundance of XPA in adult male zebrafish exposed to waterborne EE\textsubscript{2} with or without BaP for 7 days with complete daily water renewal twice daily. a = statistically significant from unexposed controls, b = statistically significant from EE\textsubscript{2} alone, c = statistically significant from BaP alone  p<0.05
Similar to adult males, adult female zebrafish exhibited somewhat similar transcript patterns in both hepatic XPC and XPA after seven day exposure. Adult female zebrafish exposed 10ng/L or 100ng/L EE2 exhibited hepatic XPA mRNA levels that were 0.6 and 0.3 that of controls (Figure 4.6). Only the 100ng/L EE2 was statistically significant from controls (p<0.05). Fish exposed to 1µg/L BaP or 50µg/L BaP alone had hepatic XPA transcript levels similar to that of control. Fish exposed to 10ng/L EE2 with 1µg/L BaP or 50µg/L BaP had hepatic XPA mRNA abundance of 0.4 and 0.7 that of controls, with the 10ng/L EE2 and 1µg/L BaP being significantly different from unexposed controls (p<0.05). Females exposed to 100ng/L EE2 with 1µg/L BaP or 50µg/L BaP showed hepatic XPA mRNA levels 0.5 and 0.6 that of controls although they were not statistically different from unexposed controls.
Figure 4.6 Adult Female XPA mRNA abundance after co-exposure. Relative hepatic mRNA abundance of XPA in adult female zebrafish exposed to waterborne EE$_2$ with or without BaP for 7 days with complete daily water renewal twice daily. a = statistically significant from unexposed controls, b = statistically significant from EE$_2$ alone, c = statistically significant from BaP alone p<0.05

4.4.4 DNA Adducts

BPDE DNA adducts were only measured in control male fish and adult males exposed to BaP with and without 100ng/L EE$_2$. DNA adduct levels in all fish, control and exposed, were very low. All DNA adduct levels are expressed in fmol adducts per 200ng DNA. Control males had 0.001fmol DNA adducts (Figure 4.7). Adult males exposed to 1µg/L BaP or 50µg/L BaP alone exhibited significantly higher adduct levels of 0.096 fmol and 0.192 fmol respectively (p<0.05). Adult males co-exposed to 100ng/L EE$_2$ and 1µg/L BaP exhibited 0.190 fmol BPDE DNA adducts, which was significantly higher
than controls and fish exposed to 1μg/L BaP alone (p<0.05). Males exposed to 100ng/L EE₂ and 50μg/L BaP exhibited 0.365 fmol DNA adducts, which was also significantly higher than controls and fish exposed to BaP alone (p<0.05).

Figure 4.7 DNA Adduct levels after EE₂ and BaP co-exposure. BPDE DNA adducts in genomic DNA from the liver of adult male zebrafish exposed to waterborne EE₂ with or without BaP for 7 days with complete daily water renewal twice daily. a = statistically significant from unexposed controls, b = statistically significant from EE₂ alone, c = statistically significant from BaP alone  p<0.05

4.5 Discussion

This is the first study to examine effects of co-exposure of 17α-ethinylestradiol (EE₂) and benzo(a)pyrene (BaP) on hepatic gene expression in adult zebrafish, particularly as it relates to nucleotide excision repair (NER). Previous research has
indicated that environmentally relevant concentrations of EE\textsubscript{2} alter hepatic NER processes in adult zebrafish [167]. This raised the question of whether EE\textsubscript{2} potentiates the effects of bulky adduct forming mutagens, such as BaP.

In contrast to the original hypothesis and fish exposed to EE\textsubscript{2} alone, fish co-exposed to EE\textsubscript{2} and BaP did not exhibit decreased mRNA levels of NER genes. In fact, in most exposures, XPC and XPA mRNA abundance was higher than those of fish exposed to EE\textsubscript{2} alone, and unexposed controls. Regardless of these increased NER transcript levels, DNA adducts corroborated the original hypothesis that EE\textsubscript{2} potentiates genotoxicity of BaP. However, the question remains whether this increase in DNA adducts is due to alteration of DNA repair, or BaP detoxification via CYP1A.

Cytochrome p4501A (CYP1A) is responsible for both detoxification and metabolic activation of BaP. Which function, metabolism or detoxification, is dominant depends on the concentration of both CYP1A and BaP in the cell [188]. It is also well established that estrogen exposure decreases both mRNA and function of CYP1A [192-194]. However, co-exposure of estrogen and aryl hydrocarbon receptor (AhR) agonists are rarely studied. In this study we show that adult male and female zebrafish co-exposed to EE\textsubscript{2} and BaP exhibit decreased CYP1A mRNA levels in comparison to fish exposed to BaP alone. This presents the possibility BaP is not being as readily detoxified from the cell. This longer retention time may allow for metabolic activation by other enzymes, resulting in increased DNA adduct levels.

The argument could also be made that lack of CYP1A activity would result in decreased adducts due to the inability of the cell to metabolize BaP to BPDE, the ultimate carcinogenic metabolite that is capable of adducting DNA. However, CYP1A null mice
exhibit higher adduct levels than their normal CYP1A expression counterparts when exposed to benzo(a)pyrene [185, 198]. Other research has shown that hepatic cytochrome p450 reductase null mice that lack cytochrome p450 oxidoreductase, the unique electron donor to CYP enzymes that essentially results in loss of hepatic function of all CYP enzymes, exhibit higher BaP-DNA adduct levels than wild type mice [188]. BaP is not only a substrate for CYP1A, but also CYP1B and cyclooxygenase (COX, or prostaglandin H synthase PGHS) [198-200]. It seems likely that in light of decreased CYP1A mRNA levels in zebrafish co-exposed to EE₂ and BaP, other CYP or COX enzymes metabolize BaP to BPDE in the liver [198, 200]. This may explain why increased mRNA levels of both XPC and XPA were observed in both male and female zebrafish, but mainly in the samples that also exhibited decreased CYP1A mRNA levels. Higher DNA adduct levels were observed in adult males exposed to EE₂ and BaP in comparison to both controls and males exposed to BaP alone.

DNA adduct levels in all fish were extremely low, which was expected due to low exposure concentrations utilized in this study. Control fish exhibited virtually no BPDE DNA adducts. Exposure to benzo(a)pyrene resulted in increased BPDE DNA adducts. However, increased adduct levels were observed regardless of CYP1A mRNA levels. This is in contrast to previous research in mouse hepatoma cells where BPDE DNA adduct formation was correlated with induction of CYP1A1 [188, 201].

The increased adducts in adult males exposed to EE₂ and BaP do correspond with increased mRNA abundance of XPA, however XPC mRNA levels were only increased in males exposed to 100ng/L EE₂ and 1µg/L BaP. This may indicate that hepatic XPA mRNA abundance is more predictive of DNA adduct levels than hepatic XPC transcripts.
XPA functions in both subpathways of NER and has previously been shown to be a rate limiting step in the NER process, which may, in part, explain why it is a better predictor of adduct levels [125]. In light of this, it will be interesting to examine adduct levels in female fish co-exposed to EE₂ and BaP, as XPC, but not XPA hepatic mRNA levels are increased. Examining mRNA levels of proteins involved in transcription coupled repair may provide additional insight.

These findings have significant implications for both aquatic organisms in contaminated environments and human health. Zebrafish are gaining wide acceptance as a cancer model, in particular for hepatocellular carcinoma (HCC) [65]. The ability of estrogen to potentiate effects of bulky adduct forming mutagens has potential indications for human HCC, since estrogens are highly implicated in HCC and the mostly highly correlated causative agent of HCC is aflatoxin, a bulky adduct forming mutagen [126, 130]. In addition, these findings may provide explanation for research showing gender specific incidence of neoplasia in medaka and the ability of estrogen to promote mutagen induced tumorigenesis in rainbow trout and medaka [99, 100, 202]. For aquatic organisms living in contaminated environments, this research indicates the potential for higher mutation rates and increased neoplastic transformation with estrogen co-exposure than what would be expected with mutagens alone.
Chapter 5

WASTEWATER TREATMENT EFFLUENT ALTERS NUCLEOTIDE
EXCISION REPAIR IN ZEBRAFISH (Danio rerio)

5.1 Abstract

Wastewater treatment effluent is a complex mixture of anthropogenic pollutants including estrogenic substances and chemicals, such as polyaromatic hydrocarbons, that cause bulky DNA adducts. Significant research focuses on reproductive effects of aquatic estrogens from wastewater treatment plants. However, other studies suggest genotoxic and immunological effects occur at lower concentrations of wastewater treatment effluent than reproductive endpoints. Recently, effects of estrogen on DNA repair processes in zebrafish have been suggested as a possible mechanism by which estrogen can modulate incidence of DNA mutations. Because wastewater treatment facilities are a significant source of estrogenic compounds, we hypothesized that exposure to whole effluents would also affect DNA repair in zebrafish (Danio rerio). Exposure to effluent from multiple treatment facilities in northern Maine decreased repair of DNA adducts in zebrafish liver cells. Expression of two nucleotide excision repair genes, XPC and XPA, were quantified and showed varied response after exposure in adult male zebrafish. Evidence of estrogen and aryl hydrocarbon receptor activation after exposure varied between treatment plants and temporally within treatment plants when evaluated using a traditional biomarker, vitellogenin-1 (VTG) and, cytochrome p450 1A1 (CYP1A1) mRNA abundance. This research highlights the continuing importance of examining non-reproductive effects of wastewater treatment effluent.
5.2 Introduction

Wastewater treatment plants represent a major point source of many classes of anthropogenic pollutants to the aquatic environment [71, 182, 203]. Contaminants found in wastewater effluents comprise a complex mixture of chemicals including pharmaceuticals, industrial contaminants, polyaromatic hydrocarbons, dioxins and mutagenic compounds [67, 96, 97, 182, 204]. In complex mixtures such as wastewater effluent, biological interaction of two or more compounds can result in toxic effects not observed in single-chemical exposures.

Wastewater treatment plants constitute a significant entry source of estrogens, particularly of pharmaceutical origin, to the aquatic environment [76, 79]. Synthetic and natural hormones are excreted as glucuronide or sulfate conjugates that can be cleaved by bacteria present in sewers and treatment facilities to yield active parent compounds [205-207]. Bisphenol A, nonylphenol ethoxylates, pesticides, and other industrial compounds are also known endocrine disruptors that may enter the aquatic environment via wastewater treatment plants [79, 140, 208].

The majority of research related to effects of xenoestrogens on aquatic organisms has focused on reproductive effects. These include increased proportions of intersex fish, decreased egg and sperm production, reduced gamete quality and complete feminization of male fish [73, 142, 144, 209]. Increased vitellogenin protein or mRNA in male or juvenile fish has been well established as biomarker of exposure to estrogenic compounds [29]. Increased plasma vitellogenin, altered bile estrogen levels, and increased intersex proportions have been found in fish downstream of sewage treatment outflows with known estrogenicity [84, 88, 96].
In addition to reproductive effects on aquatic organisms, recent research has shown that 17α-ethinylestradiol can alter mRNA abundance of nucleotide excision repair (NER) genes in adult zebrafish (*Danio rerio*) and the rate and magnitude of NER processes in zebrafish liver (ZFL) cells [167, 191]. Nucleotide excision repair is the primary DNA repair pathway responsible for removing bulky DNA adducts, such as those caused by environmental mutagens including benzo(a)pyrene [115]. In particular, significant decreases, ranging from 2 to 17 fold, were seen in Xeroderma pigmentosum, complementation groups C and A (XPC and XPA) mRNA abundance in adult zebrafish after laboratory exposure to 17α-ethinylestradiol [167]. XPC is responsible for initial damage recognition in the global genome repair subpathway of NER [171]. XPA functions as damage verification in both transcription-coupled repair and global genome repair [113]. In addition, other compounds that may be found in wastewater treatment effluent including arsenic, cadmium, nickel and other chemicals have been shown to alter NER processes [210, 211]. Altered DNA repair capacity has significant implications for accumulation of DNA mutations and carcinogenesis.

Wastewater treatment effluent does not only contain endocrine disruptors, but other contaminants that may also cause DNA damage. Increased single-strand DNA breaks were seen in fathead minnow (*Pimephales promelas*) and roach (*Rutilus rutilus*) after wastewater treatment effluent exposure [183, 212]. Studies have also shown other non-reproductive effects of wastewater treatment effluent including; immune suppression, and altered hepatic biotransformation enzyme expression and activity [183, 212]. Many of these non-reproductive effects, particularly genotoxic endpoints, have been observed at concentrations lower than those that produce measurable reproductive
effects [183]. These adverse non-reproductive effects may have greater significance for overall health of aquatic organisms than previously believed.

While it is difficult to attribute specific biological effects to individual components of mixtures, hypotheses regarding wastewater toxicity can be derived based upon endpoints generated from tests utilizing individual chemicals. If components of wastewater effluent such as estrogens are able to hinder NER processes, this increases risk of DNA damage in exposed organisms. The goal of this study was to examine biological effects of wastewater treatment effluent from three treatment plants in northern Maine to test the hypothesis that effluent affects DNA repair. If wastewater does alter DNA repair this may explain in part, the increased DNA damage previously reported with wastewater treatment effluent exposure [183, 212].

5.3 Materials and Methods

5.3.1 Sample Collection

Grab sample effluent was collected monthly from three treatment plants near the greater Bangor, ME area. Samples were stored in the dark in high-density polyethylene plastic containers with airtight lids at 4°C throughout exposures. Effluent for cell culture assays was filtered with 0.22 µm Durapore Membrane Filters (Millipore) and stored at 4°C. Effluent for zebrafish exposures was not filtered. Zebrafish exposures were started on the day of water collection. Cell culture assay exposures were started one day after water collection.

5.3.2 Cell Culture

MVLN cells were maintained at 37°C with 5% CO₂. MVLN culture media consists of 50% DMEM and 50% Ham's F-12 nutrient mix supplemented with 10% FBS.
For all luminescence assays with MVLN cells, phenol red-free DMEM with 5% charcoal-dextran stripped FBS was used to minimize external estrogenic content. Zebrafish liver cells (ZFL) were purchased from ATCC. ZFL cells were maintained at 28°C with ambient CO₂. Normal culture media (LDF media) for ZFL cells was comprised of 50% L-15, 35% DMEM, 15% Ham's F-12 nutrient mix with 50 ng/mL murine epidermal growth factor, insulin-transferrin selenium supplement, 0.5% trout serum and 5% fetal bovine serum (FBS). All cell culture reagents for both MVLN and ZFL cells were purchased from Invitrogen. ZFL and MVLN cells were passed when they reached 90–95% confluence at a ratio of 1:5 into T25 tissue culture flasks approximately every five days. Cells were subcultured by first washing the flask with sterile phosphate-buffered saline (PBS) two times and then adding 1 mL of 0.05% trypsin. Once cells had detached, 4 mL of serum containing media was added and each new flask was seeded with 1 mL of cell suspension.

5.3.3 MVLN Luminescence

MVLN cells were plated in 96-well plates at $3 \times 10^4$ cells per well in phenol red free DMEM with 5% charcoal-dextran stripped FBS. Cells were allowed to adhere for 24h prior to exposure. Cells were exposed to media only, filtered wastewater treatment effluent, zebrafish facility water or 10 nM 17β-estradiol as a positive control. All exposures were replicated with the estrogen receptor antagonist ICI 182,780. A minimum of 3 wells per exposure regime were measured. Cells were exposed for 72 h prior to measurement of luciferase. Luciferase was measured using the Steady Glo Luciferase assay system (Promega). Briefly, exposure media were aspirated from the wells and replaced with 50 µL of PBS. 50 µL of Steady-Glo buffer was added to each well.
Luciferase signal was allowed to develop for 5 min prior to reading luminescence on a GloMax luminometer (Promega). Luminescence was normalized to total protein content in each well.

5.3.4 Fish Exposures

One-year-old zebrafish (*Danio rerio*, AB strain) were maintained at the University of Maine zebrafish facility with a light:dark cycle of 14:10 h. Prior to effluent exposure, 5 male fish were placed in 3.5 L tanks with water from the University of Maine zebrafish facility (carbon filtered and UV-treated Orono, ME city water, with 7.5 mg/L dissolved oxygen and 42 mg/L hardness) and maintained at 27.6°C. Zebrafish facility water was then replaced with 1.5 L of effluent from one of three treatment plants. Effluent was warmed from 4°C to 28°C prior to addition to each tank. Control fish were exposed to zebrafish facility water with no addition. Experimental and control fish were exposed for 7 days in static water with complete renewal once per day. During daily water renewal, fish were visually inspected for overall health. Fish were fed commercially available fish food daily, 2h prior to water renewal.

5.3.5 RNA Isolation

After 7-day effluent exposure zebrafish were sacrificed and livers were surgically removed. Total RNA was isolated using phenol free RNA isolation procedures from single adult zebrafish livers (RNAqueous kit, Ambion/ABI). Liver tissue was manually homogenized in 500 µL of cold guanidinium thiocyanate lysis/binding solution, and diluted with equal volume ethanol. Samples were then bound to a glass fiber filter and washed three times with ethanol. RNA was eluted with 60 µL of 75°C DNAse/RNAse free water and stored at −80°C. Five distinct samples were collected per exposure. RNA
integrity and concentration was assessed utilizing micro-capillary electrophoresis with the Agilent 2100 bioanalyzer. One microliter total RNA from each sample was compared to 1 µL RNA ladder with 6 RNA transcripts of varying sizes and known concentration of 150 ng/µL. RNA quality was verified by observation of corresponding 18S and 28S peaks on electropherograms for each sample tested. Only intact RNA was used for further analysis.

5.3.6 qRT-PCR

Fluorescence based quantitative RT-PCR was performed using primer sets and methodology previously reported [167]. Relative change in transcript abundance was normalized to 18S rRNA and calculated utilizing the $2^{-\Delta\Delta Ct}$ analysis method [153]. Prior to analysis, amplification efficiency was examined using LinRegPCR software, which calculates efficiency based on raw real-time PCR data [154]. Efficiencies for normalizing gene (18S) and all other transcripts were the same (1.8±0.1). Control expression levels were normalized to a value of 1. Each RNA sample was run in triplicate with three to five distinct RNA samples per exposure. A single peak in all dissociation curves verified production of a single amplicon per primer pair.

5.3.7 Host Cell Reactivation

5.3.7.1 UV Damage. pHRL-CMV (Promega) was UV damaged with a Stratalinker UV Crosslinker (Stratagene). Plasmid was diluted to a final concentration of 50 ng/µl in DNAse/RNAse free water and placed in a 6 well cell culture plate without the lid. pHRL-CMV was subjected to 1000 J/m² UV light, aliquoted and stored at −80°C until use. Undamaged pHRL-CMV was also diluted to 50 ng/µl and stored at −80°C until time of transfection.
5.3.7.2 Transient Transfections. ZFL cells were plated in 6 well plates at $5 \times 10^5$ cells/well in 2mL of LDF media for 40% confluence at time of transfection. After plating, cells were allowed to adhere for 24 h and then exposed to filtered effluent from each site in normal LDF medium (1:10 dilution) for 24 h in the presence of 100 units/mL penicillin and streptomycin (Invitrogen). Control wells were exposed to solvent control (1:10 water). Transient transfections were performed as previously described [195].

5.3.7.3 Luciferase Assay. Wells were assayed for luciferase after 24h of incubation. To measure both firefly and renilla luciferase activity the Dual-Glo Luciferase Assay system (Promega) was utilized as previously described. Plates were read with a GLOMAX 96 well plate luminometer. pGL3 was used to normalize for transfection efficiency. The ratio of pHRL to pGL3 was first normalized to total protein in each well. Percent repair was calculated as normalized damaged luciferase expression divided by normalized undamaged luciferase expression.

5.3.8 Statistics

Quantitative RT-PCR data were analyzed using one-way analysis of variance (ANOVA). Both equal variance and normality were validated on the raw Ct values prior to ANOVA. Normality of error was assessed with Lillifors test. Equal variance of the samples was assessed with plots of estimates versus studentized residuals and modified Levene's test. One-way ANOVA were performed on raw Ct values and allowed for analysis between all treatments for a given gene. When statistically significant differences were found between treatment groups, Dunnett's test was used to determine which treatments were significantly different from unexposed controls. To validate that effluent exposure did not alter normalizing gene expression, Ct values were analyzed by
one way nested ANOVA and p>0.8 was used as a threshold. Host cell reactivation and MVLN luminescence data were analyzed using t-test. Results were deemed statistically significant at p<0.05. All statistical analyses were done using SigmaStat 3.0 (SYSTAT Inc.) or SYSTAT 11 software (SYSTAT Inc.).

5.4 Results

5.4.1 Treatment Types

Wastewater treatment effluent was collected from the outflow or last step of three wastewater treatment plants. Each plant utilizes different secondary treatment technologies and serves varied sized communities. Site A serves a community of approximately 9000 people including residential, commercial and university. Site A has an average flow of 0.99 million gallons per day and utilizes activated sludge as secondary treatment. Site B serves a community of approximately 9500 people including residential and commercial. Site B uses rotating biological contactors with aeration as secondary treatment and has an average flow of 1.7 million gallons per day. Site C serves a community of approximately 35,000 people including residential, commercial and industrial. Site C has an average flow of 8.8 million gallons per day and utilizes trickling filter and activated sludge as secondary treatment.

5.4.2 MVLN Luminescence

MLVN cells are a stably transfected construct of MCF-7 cells containing the vitellogenin promoter region from *Xenopus laevis* upstream of the firefly luciferase gene [213]. MVLN cells were exposed to filtered wastewater treatment effluent collected each month from November through February. MVLN cells exposed to 10 nM 17β-estradiol as a positive control showed a significant increase in luminescence, validating this in
*vitro* model system (Figure 5.1). Effluent samples also exhibited significantly increased luminescence in the MVLN bioassay in comparison to media alone, indicating estrogenic compounds in the effluent. All increases in luminescence by 17β-estradiol or wastewater treatment effluent were blocked with co-exposure to ICI 182,780, a complete estrogen receptor antagonist (data not shown), indicating these responses were estrogen receptor mediated. Zebrafish facility water exhibited no estrogenic activity in this bioassay at any time.

![Figure 5.1. Estrogen activity of wastewater treatment effluent. Estrogenic activity of wastewater treatment effluent measured using *in vitro* assay in MVLN cells with luciferase controlled by three estrogen response elements. Luminescence was measured after 72 h exposure of MVLN cells. *Significantly different from media only control (p<0.05).](image-url)
5.4.3 Vitellogenin mRNA abundance

After seven-day effluent exposure adult male zebrafish showed increased hepatic vitellogenin-1 (VTG) mRNA abundance in almost all exposures (Figure 5.2). The magnitude of increased hepatic VTG mRNA varied greatly with treatment plant, and sample. Effluent collected in February caused the greatest increases in VTG transcript for all three plants. Adult male zebrafish exposed to effluent from site A exhibited a 3.4, 199.1, 2.7 and 139.0 fold increases in hepatic VTG in November, December, January and February respectively, all of which were statistically significant from controls. Adult male zebrafish exposed to effluent from site B showed 20.0, 12.8, and 10,945.1 fold increase in VTG mRNA in November, December, and February respectively. All of the increases over control levels for site B were statistically significant. Males exposed to effluent from site B in January showed no change in VTG expression in comparison to controls. The VTG transcript levels seen in February at Site B were the highest in any exposure. Exposure to effluent from site C caused a 1.8, 2.8 and 123.2 fold increase in hepatic VTG in adult male zebrafish for December, January and February respectively. All increases in hepatic VTG from site C were significantly greater than controls. Fish exposed to effluent from site C in November exhibited no change in VTG expression in comparison to control males.
Figure 5.2. Hepatic VTG mRNA after wastewater exposure. Relative hepatic expression of vitellogenin-1 mRNA ± standard error of the mean (S.E.M.) in adult male zebrafish exposed to wastewater treatment effluent for seven days. Measured using fluorescence based quantitative RT-PCR. *Significantly different from control (p<0.05).

5.4.4 CYP1A1 mRNA abundance

Adult male zebrafish exposed to wastewater treatment effluent exhibited varied response in cytochrome p450-1A1 (CYP1A1) mRNA abundance, although most common was increased transcript level relative to controls (Figure 5.3). Males exposed to effluent from November from site A exhibited significantly lower CYP1A1 transcript levels 0.4 relative to control. Males exposed to site A effluent from December showed no alteration in CYP1A1 mRNA abundance. Males exposed to effluent from site A showed 3.0 and 1.8 fold increase in CYP1A1 transcript in January and February respectively, both of which were significantly different from controls. Male fish exposed to effluent from site
B showed no difference in CYP1A1 mRNA abundance in November. Site B effluent caused 2.5, 3.1 and 3.2 fold increases in CYP1A1 transcript in December, January and February respectively, all of which were significantly different from control. A similar trend was seen with effluent from site C. CYP1A1 transcript levels were the same as controls for male zebrafish exposed to effluent from site C in November and December. Adult males exposed to effluent from site C in January and February exhibited 2.2 and 4.4 fold increase in CYP1A1 mRNA abundance respectively, which were significantly different from control.

**Figure 5.3.** Hepatic CYP1A1 mRNA after wastewater exposure. Relative hepatic expression of CYP1A1 mRNA ± S.E.M. in adult male zebrafish exposed to wastewater treatment effluent for seven days. Measured using fluorescence based quantitative RT-PCR. *Significantly different from control (p<0.05).
5.4.5 XPC mRNA abundance

Adult male zebrafish exhibited varied hepatic XPC mRNA abundance after wastewater treatment effluent exposure (Figure 5.4). Males exposed to effluent from site A showed significantly lower hepatic XPC transcript levels 0.08 relative to control in November. In contrast, males exposed to effluent from site A exhibited no change from controls in December. In January and February males exposed to effluent from site A exhibited 3.3 and 3.2 fold increases in hepatic XPC expression, which were significantly different from control. Male zebrafish exposed to effluent from site B also showed variable XPC mRNA abundance. Fish exposed to effluent from site B in November exhibited significantly lower XPC mRNA abundance 0.3 that of control. Males exposed to site B effluent from December had a significant 4.2 fold increase in XPC transcript. There was no difference in XPC mRNA abundance in comparison to controls after exposure to site B effluent from January or February. XPC mRNA abundance after exposure to effluent from site C was the most consistent, exhibiting 5.0, 3.8 and 2.7 fold increases in December, January and February respectively. All of these increases in XPC transcript after site C effluent exposure were significantly different from controls. XPC expression from site C effluent exposure in November was 0.1 that of control, which was significantly different.
Figure 5.4. Hepatic XPC mRNA after wastewater exposure. Relative hepatic expression of XPC mRNA± S.E.M. in adult male zebrafish exposed to wastewater treatment effluent for seven days. Measured using fluorescence based quantitative RT-PCR. *Significantly different from control (p<0.05).

5.4.6 XPA mRNA abundance

XPA mRNA abundance after exposure to wastewater treatment effluent showed similar patterns of response as XPC transcript levels (Figure 5.5). Males exposed to effluent from site A in November had significantly different XPA mRNA abundance 0.1 that of control. Males exposed to site A effluent exhibited 4.1, 2.1 and 3.1 fold increase in XPA transcript in December, January and February respectively. Increases seen from site A exposures in December and February were significantly different from control. Adult male zebrafish exposed to effluent from site B in November and February had
significantly decreased XPA expression 0.2 and 0.5 that of control respectively. In contrast, fish exposed to effluent from site B in December showed a significant 5.4 fold increase in XPA transcript. There was no change in XPA expression in males exposed to site B effluent from January. Adult male fish exposed to effluent from site C in November had significantly decreased XPA transcript 0.05 that of control. Fish exposed to effluent from site C in December also exhibited no alteration in XPA expression. However, males exposed to effluent from January and February had 3.8 and 2.7 fold increase in hepatic XPA expression respectively, which was significant from control.

![Hepatic XPA mRNA after wastewater exposure.](image)

**Figure 5.5.** Hepatic XPA mRNA after wastewater exposure. Relative hepatic expression of XPA mRNA± S.E.M. in adult male zebrafish exposed to wastewater treatment effluent for seven days. Measured using fluorescence based quantitative RT-PCR. *Significantly different from control (p<0.05).
5.4.7 Nucleotide excision repair capacity

To examine whether wastewater treatment effluent had any effect on repair of bulky DNA lesions an in vitro host cell reactivation assay was used. Zebrafish liver cells were pre-exposed to wastewater treatment effluent, or control water and then transfected with UV damaged plasmid. After 24 h, unexposed control ZFL cells had 39.6 and 40.9% repair (Figure 5.6). Site A exposure caused a consistent decrease in nucleotide excision repair capacity. Cells exposed to site A effluent from November had 31.4% repair that was significantly lower than control. ZFL cells exposed to site A effluent from February had 36.1% repair, which was also significantly different from control. ZFL cells exposed to effluent from site B showed slightly increased NER capacity in comparison to control, although it was not statistically significant. ZFL cells exposed to November and February effluent from site B had 45.8 and 41.5% repair, respectively. ZFL cells exposed to effluent from site C in November had NER capacity similar to control cells with 36.3% repair. Cells exposed to effluent from site C in February exhibited decreased NER capacity with 33.1% repair, which was significantly different from control.
Figure 5.6. Repair capacity of wastewater exposed ZFL cells. Nucleotide excision repair capacity of zebrafish liver cells ± S.E.M. at 24 h after pre-exposure to wastewater treatment effluent from one of three treatment plants. Measured using a host cell reactivation assay with 1000 J/m² UV damage. *Significantly different from control (p<0.05).

5.5 Discussion

This study examined biological effects of wastewater treatment effluent from three plants in Northern Maine to test the hypothesis that wastewater treatment effluent affects DNA repair. Expression of two well established biomarkers; vitellogenin (VTG) and cytochrome p450 1A1 (CYP1A1) were examined in adult male zebrafish exposed to effluent from all three plants to determine if estrogenic and/or AhR mediated pathways were activated by the effluent. Vitellogenin has been validated in the literature as a
biomarker for estrogen exposure [29] and CYP1A1 is used as a marker of exposure to AHR agonists including polyaromatic hydrocarbons (PAH), dioxin and others [214-216].

Induction of VTG protein and mRNA in zebrafish occurs after exposure to a variety of estrogenic compounds [93, 95, 167]. In the present study, increased hepatic VTG mRNA abundance was observed in adult male zebrafish exposed to effluent from all three plants. The magnitude of response varied considerably between treatment plants and even temporally at the same treatment plant. This likely represents differing compositions of effluents based on temporal changes including precipitation, snowmelt and other changes in initial influent constituents. Other studies have also shown variations in VTG responses after exposure of aquatic organisms to differing effluent samples [69, 84, 91]. The increase in VTG mRNA abundance in response to wastewater treatment effluent exposure was very mild in comparison to laboratory exposures to low concentrations of synthetic estrogens and more consistent with the response seen with weaker estrogens [95, 143, 167].

In addition to examining VTG mRNA upregulation, an in vitro reporter assay was used to determine estrogenic activity of wastewater treatment effluents. Similarly to adult male zebrafish, MVLN cells exposed to wastewater treatment effluent exhibited increased luminescence in comparison to controls, although not as great an induction as cells treated with estradiol. Taken collectively these data suggest the presence of estrogenic compounds in effluent from all three plants but at variable levels throughout the sampling.

Many PAHs that activate CYP1A1 are ubiquitous in the environment and are known to cause DNA damage that is preferentially repaired by nucleotide excision repair
Wastewater treatment effluent has been shown to increase hepatic CYP1A activity and message levels [182]. In contrast, studies have shown that estrogens alone decrease basal levels of both mRNA and enzyme activity of CYP1A1 in aquatic organisms [167, 192, 194]. In this study, both decreased and increased transcript levels of CYP1A1 were observed after exposure to effluent. Decreased CYP1A1 mRNA levels were only observed in a few select samples. The most consistent response in adult male zebrafish exposed to effluent was significantly increased CYP1A1 mRNA abundance, potentially indicating the presence of genotoxic, Ah receptor agonists in effluent samples. Studies have suggested that increased DNA damage results in juvenile roach after effluent exposure, which may be the result of genotoxic compounds present in the effluent [182, 183].

Previous research has shown that estrogen exposure decreases mRNA abundance of several NER genes in adult zebrafish, particularly XPC and XPA [167]. XPC and XPA are responsible for initiation and assembly of the open repair complex around damaged DNA [113, 115]. NER is exceedingly important for removal of DNA damage caused by many environmental mutagens including PAH's. Other studies have shown increased DNA strand breaks in fathead minnows after wastewater treatment effluent exposure [182]. This is the first study to examine the effects of wastewater treatment effluent on NER processes. While not consistent for all treatment plants and samples, the most significant finding of this study is that wastewater treatment effluent can decrease overall NER capacity in zebrafish liver cells.

Wastewater effluent exposure elicited varied responses in mRNA abundance of both XPC and XPA in adult male zebrafish in this study. Decreased XPC and XPA
transcript levels were only observed in fish exposed to samples collected in November. Decreased CYP1A1 mRNA expression was also observed in fish exposed to effluent from site A in November. This may indicate lower concentrations of genotoxic compounds or possibly higher estrogen concentrations in that particular effluent sample, although those samples did not cause the greatest induction in VTG mRNA. It would be advantageous to use chemical analysis of effluent in future studies to further test this hypothesis. Similar to CYP1A1, wastewater treatment effluent exposure primarily resulted in increased mRNA abundance of both XPC and XPA. This may indicate higher levels of genotoxic compounds including PAH's, in those particular samples.

Based on current knowledge little can be gained by solely examining NER gene expression as it has not been established whether gene expression is indicative of repair capacity [125, 217]. Therefore, in subsequent effluent samples we examined the effect of effluent on overall NER capacity. Effluent from site A was the only sample to significantly decrease NER capacity in ZFL cells with both effluent samples tested. Effluent from site C also decreased NER capacity in ZFL cells in one of the two samples tested. Site B effluent did not have any effect on overall NER capacity. These varied responses in both mRNA abundance of NER genes and overall NER capacity likely reflect the varied compositions of effluents. No correlations between mRNA abundance in adult zebrafish and NER capacity in ZFL cells can be directly inferred due to the different effluent samples used and the fact than an in vitro model is necessary for the host cell reactivation assay. However, it is worth noting that the treatment plant that elicited significant decreases in mRNA of NER genes was also the only plant to decreased NER capacity in both samples tested. In future studies it will also be
interesting to note whether specific chemical constituents or secondary treatment type has any influence on the affect of effluent on NER capacity.

Regardless of the varied response, decreased NER capacity at any time may potentiate the effects of environmental mutagens resulting in greater levels of DNA damage. Studies of fish living in contaminated sites have shown high levels of hepatic DNA damage [148]. Uncorrected DNA damaged can lead to higher mutation frequency and ultimately neoplasia. The finding that wastewater treatment effluent can alter DNA repair capacity of zebrafish liver cells has not been previously reported in the literature although increased DNA damage after effluent exposure has been observed [183]. This finding coupled with the knowledge that other non-reproductive effects of wastewater treatment effluent including genotoxic and immune end points occur at concentrations lower than those necessary to cause reproductive effects highlights the need to further examine non-reproductive impacts of effluents on aquatic organisms [183].
Chapter 6

CONCLUSIONS AND FUTURE DIRECTIONS

The research presented in the previous chapters indicates that in addition to a variety of reproductive effects, aquatic estrogens alter DNA repair processes in adult zebrafish. In particular, 17α-ethinylestradiol (EE₂) suppresses hepatic nucleotide excision repair (NER) processes at both the transcript and functional levels. Several key NER genes are decreased in adult male and female zebrafish exposed to environmentally relevant concentrations of EE₂ for seven days including XPC, XPA, XPD and XPF. Zebrafish liver (ZFL) cells are hindered in their ability to repair UV light induced DNA adducts after pre-exposure to a variety of physiologically relevant EE₂ concentrations. The decrease in XPC mRNA abundance and DNA repair capacity of ZFL cells after EE₂ exposure occurs in a seemingly esr1 independent manner. This conclusion is based upon the observation that XPC mRNA and NER function remain decreased in the presence of the complete estrogen receptor antagonist ICI 182,780.

In co-exposures with a prototypical polycyclic aromatic hydrocarbon and bulky adduct forming carcinogen, benzo(a)pyrene (BaP), EE₂ caused a dampening of cytochrome p4501A (CYP1A) induction. CYP1A is important for both detoxification and metabolic activation of BaP to its ultimate carcinogenic metabolite, benzo(a)pyrene 7,8 dihyrdodiol-9,10 epoxide (BPDE). However CYP1A is not the only enzyme that can metabolize BaP to BPDE [188, 216, 218]. Interestingly, adult fish that had decreased CYP1A mRNA abundance after co-exposure to EE₂ and BaP also exhibit increased hepatic XPC and XPA transcript levels. This may indicate that lower CYP1A levels result in residual BaP remaining in the cell longer, resulting in higher DNA adducts that
must be repaired by NER machinery. Adult males co-exposed to 100ng/L EE$_2$ exhibited higher hepatic BPDE DNA adducts than fish exposed to BaP alone, indicating that EE$_2$ does potentiate genotoxicity of BaP. This has significant implications for aquatic organisms living in contaminated environments and indicates the potential for higher mutation rates and increased neoplastic transformation with estrogen co-exposure than would be expected with mutagens alone.

To examine whether laboratory based exposures had real world implications, similar studies were done with wastewater treatment effluent from three facilities with differing treatment technologies. As was expected with the complex mixture of toxicants found in wastewater treatment effluent the results varied greatly seasonally, within treatment plants, and between plants. However, adult zebrafish exposed to certain samples of wastewater treatment effluent exhibited decreased XPC and XPA mRNA abundance, while the majority of exposures resulted in increased hepatic expression of both genes. Additionally, some samples from multiple treatment plants also hindered the ability of ZFL cells to repair bulky DNA adducts indicating that while not all wastewater treatment effluent will have impacts on exposed fauna the possibility of effluent altering DNA repair processes clearly exists.

This research has interesting implications for exposed aquatic fauna as well as the etiology of human cancer, particularly hepatocellular carcinoma (HCC). Human HCC is characterized by several phenomena that relate to this research. Nucleotide excision repair plays a key role in human hepatocellular carcinoma (HCC) as one of the most causative agents worldwide involved in HCC is aflatoxin, which creates bulky DNA adducts that are preferentially repaired by the NER pathway [126]. Several other factors
are commonly associated with human HCC including alcohol-induced cirrhosis, hepatitis B and C infection and other chronic inflammation inducing conditions [127]. Additionally, core proteins from both hepatitis B virus and hepatitis C virus impair DNA repair, in particular NER [128, 129]. HCC is also marked by a very long preneoplastic period, where accumulation of DNA damage and mutations may represent key events for initiation of neoplastic transformation [130].

Estrogen plays a key role in human HCC despite being a male dominated disease. Prolonged used of oral contraceptives results in increased risk of HCC [131]. Patients with alcohol induced cirrhosis and alcoholic hepatitis have increased hepatic ERα levels and show patterns of liver feminization [133, 134]. Alcohol induced cirrhosis also results in a hyper-estrogenic state in the liver due to unusually high levels of aromatase, which converts testosterone to estradiol [219, 220]. If estrogen does alter NER processes in the human liver, this hyper-estrogenic state could result in accumulation of unrepaired DNA damage resulting in mutations that could initiate neoplastic transformation.

In addition to cancer etiology, this research presents possible therapeutic uses for estrogens in conjunction with bulky adduct forming compounds. It is already known that estrogen co-exposure results in increased sensitivity of cells to cisplatin, a widely used antitumor drug used to treat a variety of cancers [174]. Cisplatin forms a variety of DNA adducts including crosslinks and bulky adducts that distort the normal B DNA helix [221-223]. Cell lines that lack functional NER processes through mutations in all of the Xeroderma pigmentosum complementation groups (A-G) are unable to repair cisplatin induced DNA damage [224]. Loss of NER function increases sensitivity to cisplatin, and
therefore may in part explain the efficacy of estrogen as a therapeutic co-treatment for a variety of cancers.

This research also raises questions for further examination. One of the most prominent questions to arise from this research is whether this decrease in NER transcript and function results in increased mutations ultimately leading to neoplastic transformation and tumor formation. This is a very important piece of the puzzle, but also an exceedingly difficult question to answer using the zebrafish model for several reasons. Current tumor initiation protocols in zebrafish require embryo or juvenile immersion in a mutagenic compound such as diethylnitrosamine (DEN) or 7,12-dimethylbenz(a)anthracene (DMBA) followed by examination of mature fish for tumor formation [66, 225]. While this is a viable strategy for tumor initiation, zebrafish embryos do not exhibit the same alteration in NER processes after estrogen exposure as their adult counterparts (Notch unpublished data). These facts combined with the long wait for tumor progression, make examining this question difficult until a more reliable mechanism for adult tumor initiation is discovered. However the findings showing increased adduct levels in adult fish co-exposed to EE$_2$ and BaP indicate increased mutations and tumor formation are likely.

Beyond tumors and mutations, which are the ultimate carcinogenic endpoints of concern, the specific mechanisms by which estrogens alter NER processes is of great interest. Results from chapter 3 of this dissertation indicate that esr1 is not involved in alteration of NER after EE$_2$ exposure. However, these studies were done using pharmacological antagonists designed for human estrogen receptors. While ICI 182,780 is known to block zebrafish esr1, it is not as effective on either esr2a or esr2b [36]. All
three isoforms of estrogen receptor are expressed in the zebrafish liver, and therefore could be involved in the alteration on NER processes after estrogen exposure [36, 226]. There are no consensus estrogen response elements (ERE) in the promoter of any of the known NER genes in zebrafish. However, it has been established that estrogen receptors can initiate or block transcription of genes that do not contain EREs but include other cis-acting promoter elements such as AP-1 or SP-1 [44, 45]. So while there are no canonical EREs it is still possible that one or more of the distinct estrogen receptors may be involved in the decreased mRNA levels of NER genes and loss of DNA repair capacity. Further work to clarify the role of each specific nuclear estrogen receptor is necessary.

In addition to nuclear estrogen receptors, there are newly discovered plasma membrane bound estrogen receptors that function as g-protein coupled receptors [227]. GPR30 is the membrane bound estrogen receptor that causes release of a variety of secondary signaling molecules including cAMP and epidermal growth factor (EGF) after ligand activation [49, 50]. Ligand bound GPR30 can also activate a variety of kinases and directly influence transcription [227]. It is possible that GPR30 is involved in alteration of NER after EE2 exposure, as the pharmacological antagonist used to examine estrogen receptor involvement does not antagonize membrane bound receptors [50, 52]. However the role of GPR30 in NER processes remains to be examined.

Alteration of NER by estrogen may be via a more indirect mechanism rather than direct estrogen receptor regulation of specific NER genes. Previous research has shown that in breast cancer cells, estrogen exposure results in cellular redistribution of tp53, which causes functional inactivation [168]. The cytosolic sequestration of tp53 instead of nuclear location results in the inability of tp53 to function as a transcription factor [168].
As tp53 is a known transcription factor for several key NER genes including DDB2, XPC and XPB, this may explain the alteration of NER mRNA abundance seen in the liver of adult zebrafish after EE₂ exposure [177, 228-230]. However, whether tp53 has any role in alteration of NER by estrogens still requires validation by further experimentation.

There are other potential mechanisms by which estrogens may have elicited decreased NER capacity that do not directly involve nuclear estrogen receptors. Estrogens have been shown to increase expression of high mobility group proteins (HMG), particularly HMG1 [174]. Overexpression of HMGA1 protein, either naturally or induced, in human breast cancer cells results in decreased transcription coupled-repair (TCR) and global genome repair (GGR), suggesting that HMGA1 overexpression inhibits one or more steps common to both NER pathways [175]. Interestingly, mammalian cells that lack HMGB1 exhibit significantly decrease NER capacity [231]. While they may function via different mechanisms, high mobility group proteins are important for DNA repair processes and any alteration by estrogen would impact DNA repair. This is an additional hypothesis for a non-estrogen receptor mediated mechanism by which estrogens could alter NER that remains to be tested.

Finally, one of the most important questions raised from this research that remains to be tested is whether or not this phenomenon of altered NER after EE₂ exposure can be recapitulated in a human liver model. While zebrafish are an excellent model for human HCC in both neoplastic transformation as well as primary tumors, the question must be posed in a normal, non-cancer derived human model [65]. Other confounding factors in human HCC development point to the importance of NER [126, 232]. Combined with a typically hyper-estrogenic state in the liver with chronic inflammation, these alterations
present a plausible carcinogenic mode of action for human liver [220]. However, until these results are recapitulated in a human model they remain only a hypothesis.

In closing, it is important to recognize that cancer is a heterologous disease of diverse origins. As such, it is very difficult to generalize mechanisms of carcinogenesis. So while the findings of these studies represent an additional risk for estrogen induced carcinogenesis in both aquatic organism and humans, it is exceedingly important to realize that they will not hold true for every individual due to genetic differences. However, these results do contribute to our overall understanding of potential mechanisms of estrogen to function as a co-carcinogen in both neoplastic transformation and neoplasia.
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Appendix A

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