

17 α -Ethinylestradiol decreases expression of multiple hepatic nucleotide excision repair genes in zebrafish (*Danio rerio*)

Emily G. Notch, Danielle M. Miniutti, Gregory D. Mayer*

Department of Biochemistry, Microbiology and Molecular Biology, University of Maine, 5735 Hitchner Hall, Orono, ME 04469, United States

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Abstract

Waterborne 17 α -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 organisms 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 nucleotide excision repair (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 decreased 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.

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1. Introduction

Documentation of pharmaceuticals and personal care products in surface waters has stimulated diverse research examining their environmental impacts (Daughton and Ternes, 1999). 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 sew-

ers and landfills. However, pharmaceuticals and their metabolic products will remain a constant source of wastewater contamination as long as pharmaceuticals are consumed and excreted (Desbrow et al., 1998; Aguayo et al., 2004; Servos et al., 2005).

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 17 α -ethinylestradiol (EE₂), the semisynthetic hormone found in numerous oral contraceptives and hormone replacement therapies (Gutendorf and Westendorf, 2001). A recent survey of more than 100 streams in the U.S. revealed a median EE₂ concentration of 73 ng/L (Kolpin et al., 2002). Given that estrogens elicit response in aquatic organisms at concentrations in the low ng/L range, the survey suggests that EE₂ is present in suffi-

* Corresponding author. Tel.: +1 207 581 2852; fax: +1 207 581 2801.
E-mail address: gmayer@maine.edu (G.D. Mayer).

cient amounts in the aquatic environment to induce biological effects (Kolpin et al., 2002). Additionally, EE₂ is more resistant to degradation than natural steroids such as 17 β -estradiol (E₂) (Jurgens et al., 2002). Due to its greater stability and higher potency *in vivo*, EE₂ may be of disproportional toxicological importance despite being found at much lower concentrations in surface waters than natural steroids such as estrone (E₁) (Jurgens et al., 2002).

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 (Panter et al., 1998; Rodgers-Gray et al., 2001; Sohoni et al., 2001; Jobling et al., 2002; Van den Belt et al., 2002; Sole et al., 2003). A wide range of estrogens and estrogen mimics are known to increase plasma vitellogenin in male fish (Jones et al., 2000; Rose et al., 2002; Van den Belt et al., 2003; Versonnen and Janssen, 2004; Fenske et al., 2005). 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 (Jobling et al., 1998; Carballo et al., 2005; Gibson et al., 2005). 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 EE₂, are known to promote mutagen-induced hepatic neoplasia in medaka (*Oryzias latipes*) and rainbow trout (*Oncorhynchus mykiss*) (Cooke and Hinton, 1999; Tilton et al., 2006). However, the mechanism of action of estrogens in increased rates of mutagen-induced neoplastic transformation is not known (Cooke and Hinton, 1999; Tilton et al., 2006). Increased somatic mutations, regardless of resultant neoplastic transformation, can lead to genomic instability of individuals and decreased fitness of populations (Wirgin and Waldman, 1998). 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 (Klaunig et al., 2000; Evans et al., 2003).

Nucleotide excision repair (NER) is the primary DNA repair pathway responsible for removing a variety of lesions caused by bulky adduct forming mutagens (de Laat et al., 1999; Sancar et al., 2004). Bulky adduct forming mutagens, such as benzo(a)pyrene, are ubiquitous in the environment and are concentrated in areas impacted by anthropogenic pollution (Wirgin and Waldman, 1998; Kolpin et al., 2002; Ohe et al., 2004). NER removes small sections of adducted DNA via a multiple step process involving the assembly of numerous proteins at the site of DNA damage (Evans et al., 1997; de Laat et al., 1999; Sancar et al., 2004). 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 (Thoma and Vasquez, 2003). 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 (de Laat et al., 1999). 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 (Sugasawa et al., 2001). 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 (de Laat et al., 1999; Hanawalt, 2002; Sancar et al., 2004). 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 (de Laat et al., 1999; Volker et al., 2001). Two nucleases, XPG and XPF, then cleave 3' and 5' ends of the open complex (Sancar et al., 2004). 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. Materials and methods

2.1. 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 city water, with 7.5 mg/L dissolved oxygen and 42 mg/L hardness) and maintained at 27.6 °C. Aqueous 17 α -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 (Dlugos and Rabin, 2003). 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 17 α -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 17 α -ethinylestradiol.

2.2. 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 (Beaver et al., 2000). Liver and intestinal tissues were surgically removed, after which the liver was separated from intestinal tissue. Liver samples were lysed with 500 μ L 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 μ L 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 1 μ L of RNA ladder (RNA 6000 ladder, Ambion) with a known concentration of 150 ng/ μ L 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. 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/primer3.www.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.

2.4. 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 (0.2 mM each dNTP, MgCl₂, Taq polymerase, 10 nM fluorescein, SYBR green dye and stabilizers, BioRad), forward and reverse primers (30–150 nM 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° every cycle. Relative change in transcript abundance was normalized to 18S rRNA and calculated utilizing the 2^{– $\Delta\Delta$ Ct} analysis method (Livak and Schmittgen, 2001). Prior to analysis, amplification efficiency was examined using LinRegPCR software, which calculates efficiency based on raw real-time PCR data (Ramakers et al., 2003). Efficiencies for normalizing gene (18S) and all other transcripts were the same (1.8 \pm 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.

2.5. 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 $\Delta\Delta$ Ct values for a given gene for all treatments. When sta-

Table 1

Primers used for quantitative RT-PCR for specific gene amplification, NCBI accession numbers for sequences used in primer design and amplicon size of PCR product

Gene product	Accession number	Forward primer	Reverse primer	Amplicon size
CYP1A1	AF210727	CCTGGGCGGTTGTCTATCTA	TGAGGAATGGTGAAGGGAAG	183
Vitellogenin-1	BC094995	TTTGAACGAGCAACGAACAG	AGTTCCGTCTGGATTGATGG	155
XPC	XM694603	GCCAACATCCGTCTCAGAAT	GAACGGTTGGAAAAACCAAG	239
HR23B	BC056578	GAGGAGAAACCCAGCAGTGA	GGGATACCCGTGAGCAGATA	235
XPA	BC055179	GCTGGGGAGACATGAAACTC	TGCTGATGAATGCTGGTGTC	192
XPD	BC049410	AACGCCGACAATAGCAAATC	ATCATTCCCTCGACCAACAA	228
XPF	BC054895	AACTCAAAGAAACCCGGCAA	GGGTCAGACTGTAGGGGTCA	229
18S rRNA	AC139725	CATGGCCGTTCTTAGTTGGT	CGGACATCTAAGGGCATCAC	180

tistically 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 SYSTAT 11 software (SYSTAT Inc.).

3. Results

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

Exposure to 17 α -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 ($p < 0.05$) (Fig. 1A). The significant increase in male vtg expression after 10 ng/L and 100 ng/L EE₂ exposure

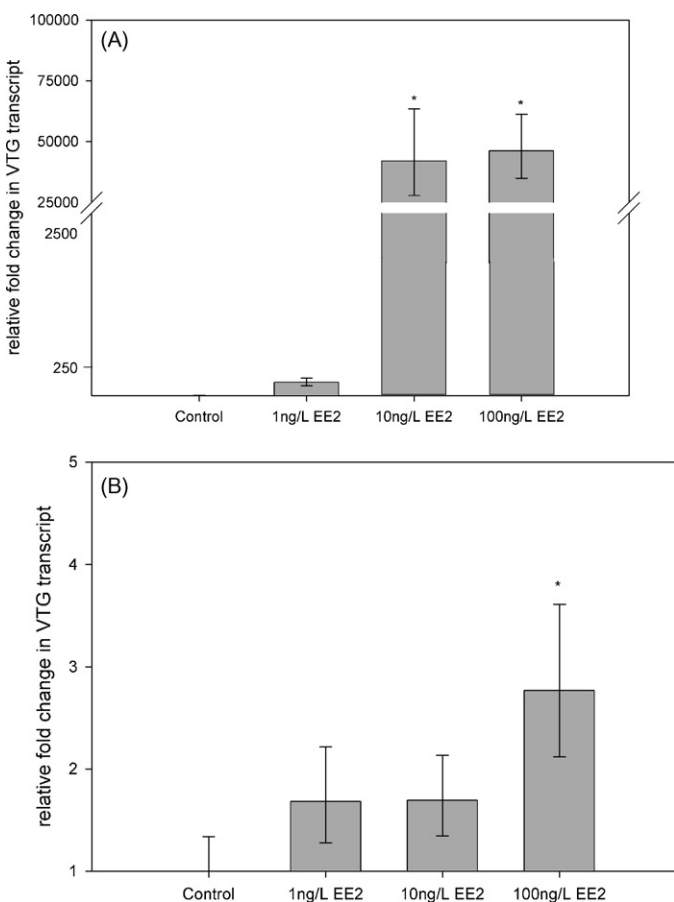


Fig. 1. Relative levels of hepatic vitellogenin-1 transcript abundance \pm standard error (S.E.M.) in (A) 1-year-old male and (B) 1-year-old female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE₂ for 7 days ($n = 4$). *Statistically significant ($p < 0.05$).

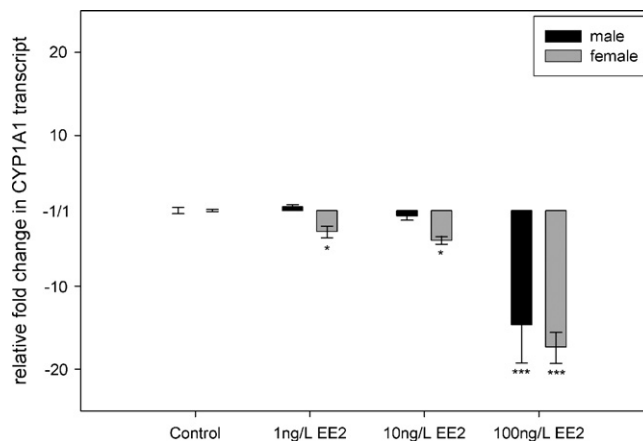


Fig. 2. Relative levels of hepatic CYP1A1 transcript abundance \pm S.E.M. in 1-year-old male and female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE₂ for 7 days ($n = 4$). *Statistically significant ($p < 0.05$), ***Statistically significant ($p < 0.001$).

represents similar transcript levels to those of female control fish. Adult female zebrafish exposed to 17 α -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 ($p < 0.05$) (Fig. 1B). Adult male zebrafish exposed to 1 ng/L or 10 ng/L of 17 α -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 ($p < 0.001$) (Fig. 2). Exposure of females to 1 ng/L, 10 ng/L or 100 ng/L 17 α -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 $p < 0.001$).

3.2. 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 (Fig. 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 ($p < 0.05$).

3.3. 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

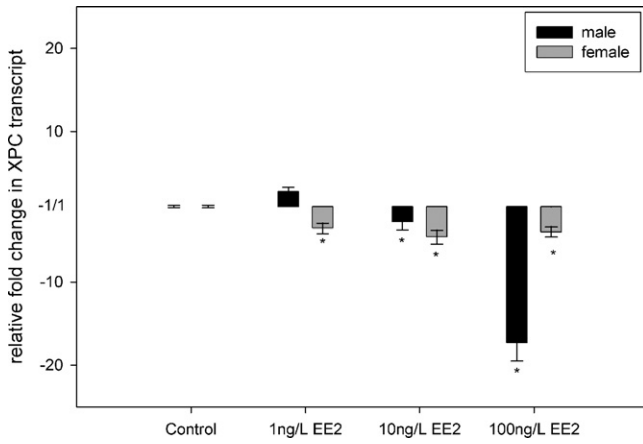


Fig. 3. Relative levels of hepatic XPC transcript abundance \pm S.E.M. in 1-year-old male and female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE₂ for 7 days ($n=4$). *Statistically significant ($p < 0.05$).

EE₂ exhibited no change in hepatic HR23B expression at any of the exposure concentrations (Fig. 4).

3.4. 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 (Fig. 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

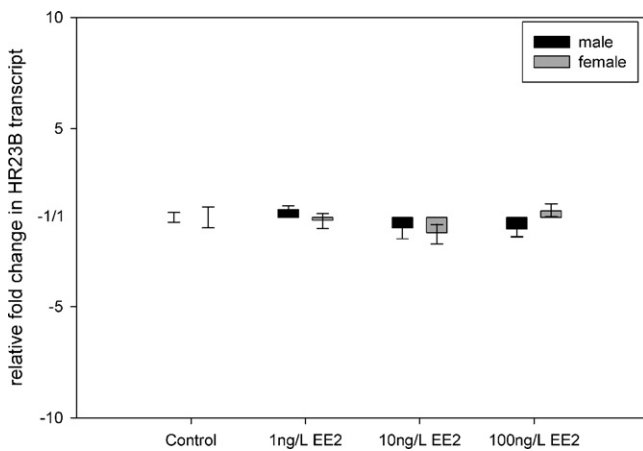


Fig. 4. Relative levels of hepatic HR23B transcript abundance \pm S.E.M. in 1-year-old male and female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE₂ for 7 days ($n=4$). *Statistically significant ($p < 0.05$).

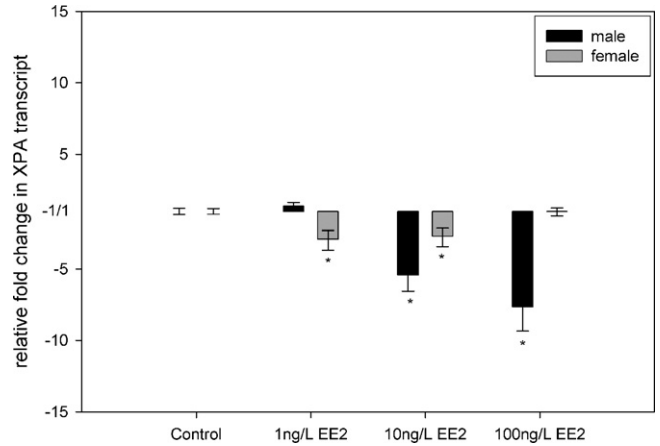


Fig. 5. Relative levels of hepatic XPA transcript abundance \pm S.E.M. in 1-year-old male and female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE₂ for 7 days ($n=4$). *Statistically significant ($p < 0.05$).

no change in XPA expression from unexposed control fish.

3.5. 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 (Fig. 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.

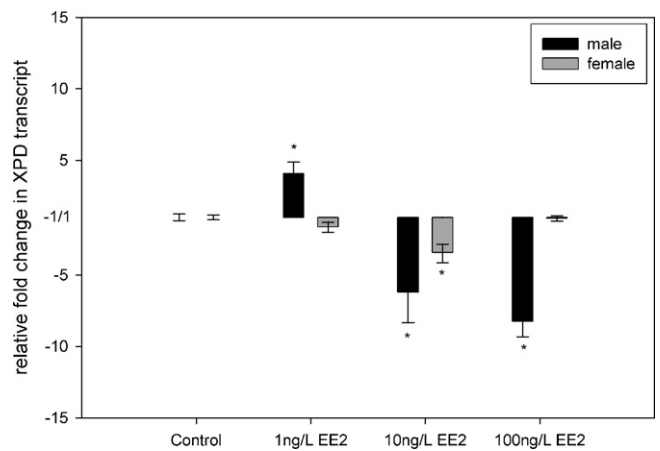


Fig. 6. Relative levels of hepatic XPD transcript abundance \pm S.E.M. in 1-year-old male and female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE₂ for 7 days ($n=4$). *Statistically significant ($p < 0.05$).

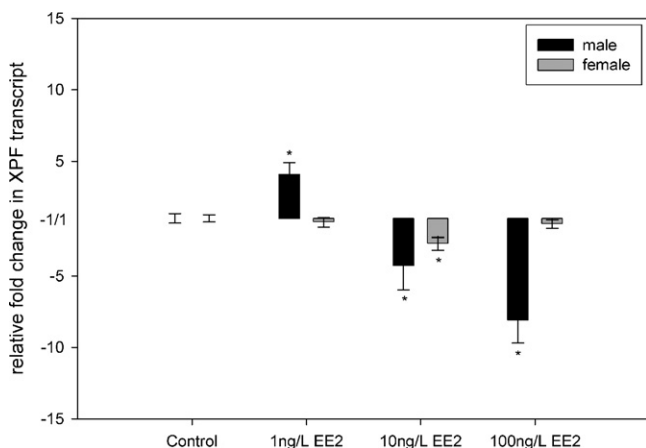


Fig. 7. Relative levels of hepatic XPF transcript abundance \pm S.E.M. in 1-year-old male and female fish exposed to 1 ng/L, 10 ng/L and 100 ng/L EE₂ for 7 days ($n = 4$). *Statistically significant ($p < 0.05$).

3.6. 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 (Fig. 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 ($p < 0.05$).

4. 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 (Wang et al., 2005). We found that vitellogenin mRNA levels were greatly increased after estrogen exposures, similar to previous studies (Rose et al., 2002; Van den Belt et al., 2003; Wang et al., 2005). CYP1A1 is known to be repressed after estrogen exposure as evidenced by reduced EROD activity (Sole et al., 2000; Teles et al., 2005). In addition, CYP1A1 mRNA abundance and activity has been shown to decrease after estrogen exposure and our results were consistent with this trend (Navas and Segner, 2001). We feel the response of these two genes in our studies validates normal estrogen response for zebrafish exposed to EE₂. 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 EE₂ exposures. This gives rise to many questions regarding estrogen exposure in environmental carcinogenesis.

Induction of hepatic vitellogenin-1 mRNA validated our 17 α -ethinylestradiol exposures in adult zebrafish (Panter et al., 1998; Jones et al., 2000; Sole et al., 2000; Rose et al., 2002; Van den Belt et al., 2003; Fenske et al., 2005). As in previously published studies, exposure to a range of EE₂ concentrations caused significant increases in vitellogenin-1 transcript levels in adult male fish (Rose et al., 2002; Van den Belt et al., 2003; Fenske et al., 2005; Wang et al., 2005). At 10 ng/L and 100 ng/L EE₂ male fish exhibited vitellogenin-1 expression levels similar to those seen in female control fish. In contrast, female fish exposed to EE₂ exhibited a mild vitellogenin induction, which is also consistent with other data (Van den Belt et al., 2003; Wang et al., 2005). Mild responses in vitellogenin-1 induction in female fish after EE₂ exposure has been attributed to higher levels of endogenous estrogens, and normally fluctuating vitellogenin production during the reproductive cycle (Jones et al., 2000).

Previous studies have shown decreased CYP1A1 activity after estrogen exposure in rainbow trout hepatocytes, carp (*Cyprinus carpio*) and seabream (*Sparus aurata*) (Navas and Segner, 2001; Sole et al., 2000; Teles et al., 2005). 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 (Navas and Segner, 2001). 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 (Stegeman and Lech, 1991; Wirgin and Waldman, 1998; Ohe et al., 2004). Decreased transcript levels of CYP1A1 seen in this study after EE₂ exposure may suggest that carcinogenic PAH metabolites are 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 -naphthoflavone (Navas and Segner, 2001). 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 (Cooke and Hinton, 1999; Tilton et al., 2006). 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 (Cooke and Hinton, 1999; Tilton et al., 2006). 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 (de Laat et al., 1999). Normal intracellular XPC protein levels are significantly lower than those of XPA, making decreases in XPC more likely to impact repair capacity (Koberle et al., 2006). 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 (Koberle et al., 2006). 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 (de Laat et al., 1999; Sancar et al., 2004). 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 (de Laat et al., 1999; Sancar et al., 2004). Studies have shown that XPA protein levels must be decreased to <10% of normal concentrations in order to impact NER function (Koberle et al., 2006). 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 (Koberle et al., 2006).

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 (de Laat et al., 1999). XPF is a 5' nuclease which also plays a role in cross-link repair and homologous recombination. The mecha-

nisms 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 (Benhamou and Sarasin, 2000; Benhamou and Sarasin, 2002). Additionally, Kang et al. showed increased benzo(a)pyrene-induced DNA adduct formation with co-exposure of BAP and 17 β -estradiol in human breast cancer cells (Kang and Lee, 2005). They hypothesized that the increased adduct formation was due to increased radical generation with estradiol metabolism (Kang and Lee, 2005). 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) (Amatruda et al., 2002; Spitsbergen and Kent, 2003; Stern and Zon, 2003). Zebrafish hepatic neoplasias are similar to humans (Amatruda et al., 2002; Spitsbergen and Kent, 2003; Stern and Zon, 2003). Further studies have shown zebrafish gene expression to be very similar to humans in liver tumors and tumor progression (Lam et al., 2006). 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 (Farazi and DePinho, 2006).

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 (Baumann and Harshbarger, 1985; Wirgin and Waldman, 1998; Rose et al., 2000; Vogelbein and Unger, 2006). 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 (Kolpin et al., 2002; Hemming et al., 2004; Servos et al., 2005). 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.

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