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Site-specific effects of 17 β -estradiol in hornyhead turbot (*Pleuronichthys verticalis*) collected from a wastewater outfall and reference location

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Abstract

Studies throughout the southern California bight have indicated persistent estrogenic activity in male hornyhead turbot (*Pleuronichthys verticalis*). Plasma 17 β -estradiol (E2) concentrations correlated with gonadal DNA damage in fish collected near a wastewater treatment plant outfall, but not from fish collected at the reference location. When the same species was collected from the same reference location and treated with E2, no relationship between uptake and gonadal DNA damage was observed. To evaluate the site-specific effects of E2 in fish from a wastewater outfall and fish from a reference location, male hornyhead turbot from each location were exposed to 15 μ g/L aqueous E2 in a time-course experiment, with fish sampled every 12 h for 48 h. Concentrations of E2 were measured in the aqueous exposure and in plasma from the fish. Vitellogenin (vtg) was also measured in the plasma, and 8-oxo-7,8-dihydro-2'-deoxyguanosine levels in male gonads were measured as an indicator of DNA damage. Untreated fish from the outfall had significantly lower E2 in the plasma relative to the untreated reference fish, and this trend was consistent at each time point in the E2-treated fish. Vtg was significantly induced after 36 h of exposure in fish from both sites and no significant differences were observed between the sites. A significant increase of oxidative DNA damage was observed in E2-treated fish from the outfall population and the damage was significantly correlated with plasma E2 concentrations only in fish from the outfall after 48 h. These results indicated that there were significant differences in E2 disposition and gonadal genotoxicity between the hornyhead turbot populations following exposure to E2, suggesting that fish at wastewater outfalls may be more sensitive to DNA damage, which may be temporally related to concentrations of E2 in plasma.

Keywords

Fish; Estradiol; DNA Damage; Vitellogenin; 8-oxo-7; 8-dihydro-2'-deoxyguanosine

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1. Introduction

In 2003–2004 a field study was conducted off the coast of Orange County, California to determine effects of a wastewater treatment plant outfall on two resident flatfish populations, the English sole (*Parophrys vetulus*) and the hornyhead turbot (*Pleuronichthys verticalis*) (Rempel et al., 2006). There were some subtle effects on reproductive indices, including a skew towards males in the sex ratio, higher gonadosomatic index in males, and a higher number of male fish with elevated levels of the egg-yolk precursor protein vitellogenin (vtg) in the outfall versus a reference population. Male gonads of hornyhead turbot at the outfall had elevated DNA damage, which correlated with plasma 17 β -estradiol (E2) levels. However, no relationship was observed in fish from the reference location. E2 levels in sediments were found to be roughly three-fold higher at the outfall versus the far-field/reference station (Schlenk et al., 2005). Therefore it was hypothesized that the increase in sediment E2 levels was affecting the plasma E2 levels in fish living at the outfall, and this in turn was leading to an increase in gonadal DNA damage.

Estrogens (10 nM) have been shown to cause oxidative DNA damage in the testes of mammals (Wellejus et al., 2004; Wellejus and Loft, 2002). Metabolism of E2 can lead to the production of semiquinones and quinones, which through redox cycling produce reactive oxygen species (Cavalieri et al., 2000). Interaction of reactive oxygen species with DNA produces oxidized bases such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG). DNA damage has been recently observed in tissues of fish exposed to estrogenic wastewater and wastewater amended with ethinylestradiol (Filby et al., 2007). However, when hornyhead turbot collected from a reference site were exposed to E2-amended sediment in the laboratory for 7 days, no relationship between E2 uptake from sediments and gonadal DNA damage was observed (Rempel et al., 2008). One potential explanation for the inability to induce gonadal DNA damage in hornyhead turbot following 7 days of treatment may be that site-specific responses following laboratory exposure to E2 may occur between populations of fish residing at wastewater outfalls relative to reference locations. Site-specific adaptive responses have been observed in mummichog (*Fundulus heteroclitus*) collected from a polychlorinated biphenyl (PCB)- and polycyclic aromatic hydrocarbon (PAH)-contaminated site (Nacci et al., 2002). When exposed to benzo[*a*]pyrene in the laboratory, fish from the contaminated site had suppressed cytochrome P450 CYP1A inducibility and benzo[*a*]pyrene-DNA adducts compared to fish from a reference site. Offspring of mummichog from contaminated sites had decreased cardiac abnormalities on exposure to PAH-contaminated sediments than offspring of fish from cleaner sites (Ownby et al., 2002). The decrease in teratogenicity persisted even if the mummichog were raised in clean laboratory water, indicating some level of heritability of resistance (Meyer and Di Giulio, 2003).

The current study was designed to determine whether site-specific differences in estrogenic activity and gonadal DNA damage following E2 exposure occur in male hornyhead turbot collected from a wastewater outfall relative to a reference location. The temporality of DNA damage was also evaluated as the fish were exposed in a time-course experiment over 48 h with plasma E2 and vtg levels measured, and compared to 8-oxodG content in male gonads.

2. Materials and methods

2.1. Fish collection and holding

In contrast to earlier studies (Rempel et al., 2006, 2008), which used fish collected from the wastewater outfall of the Orange County Sanitation District, the populations studied in the current experiment were purposely collected at a different outfall (Los Angeles County Sanitation Districts—LACSDs) in southern California, and compared with fish residing at a

far-field monitoring location utilized for NPDES permit compliance for LACSD. The rationale for collecting fish from the LACSD outfall was to determine if an “outfall” effect could be repeated using a site other than the OCSD outfall. To see the treatment type and discharge rates for each treatment facility see Schlenk et al. (2005). Biological responses of fish from the far-field location were compared against untreated laboratory-reared fish (Rempel et al., 2008) as well as fish from another reference location in southern California (Dana Point, CA; Deng et al., 2007) to evaluate the suitability of the LACSD far-field site as a reference site. No significant differences were observed between the three groups of animals (data not shown). Thus, the far-field site was used as a reference for the current study. Hornyhead turbot were collected using an otter trawl along the 60m contour line at stations T4-61 (outfall) and T0-23 to T0-61 (reference) on February 22, 2007 (Fig. 1). The fish were brought back to the laboratory and held for 6 days in artificial seawater at pH 7.5 ± 0.3 , temperature 14.5 ± 0.5 °C, and salinity 33.5 ± 0.5 ppt. Water was renewed daily. Both populations of fish were held in the same recirculating holding chamber, separated by a mesh divider. The fish were fed earthworms every other day and prior to initiation of exposure.

2.2. Exposure regime

The fish were randomly assigned to four replicate chambers containing 25 L each of solvent control (0.0004% acetone) and a treatment of 15 µg/L E2 in artificial seawater. Dissolved oxygen was >95% saturation, pH 7.8 ± 0.3 , temperature 14.5 ± 0.5 °C, and salinity 34 ± 1 ppt. Since ammonia concentrations after 48 h of exposure were less than 0.2 mg/L in previous studies (Rempel et al., 2008), the water was not renewed. Water samples were collected in duplicate at each time period for analysis of E2 levels. Both populations of fish were treated simultaneously, and no mortalities occurred during exposure. Fish were sampled at time zero (baseline), then at 12, 24, 36, and 48 h by removing one fish from each of the replicates. The concentration of 15 µg/L E2 and the duration of exposure were based on earlier studies showing transient DNA damage from genotoxic agents (Rodriguez-Ariza et al., 1999) and previous studies in our laboratory that failed to show DNA damage after 7 days of E2 exposure (Rempel et al., 2008). The fish were anesthetized with buffered MS-222 (1 g/L), weighed, and measured. Blood was collected via a heparinized 22 g syringe from the dorsal aorta and immediately spun with a centrifuge at 7000 rpm for 3 min. The plasma was removed and immediately transferred to a -80 °C freezer and stored until analysis. Gonads were removed and also transferred to a -80 °C freezer and stored until analysis. Otoliths were collected for age verification. As absolute gender identity could not be made until dissection, female animals were not included in analysis.

2.3. Vitellogenin analysis

Plasma was analyzed for vtg by a competitive-binding enzyme linked-immunosorbant assay (ELISA) using purified California halibut (*Paralichthys californicus*) vtg as a standard (Rempel et al., 2006). Briefly, wells were coated with purified vtg standard. Either standard or plasma diluted in buffer was then added in triplicate to the wells with primary antibody (rabbit anti-turbot vtg from Cayman Chemical, Ann Arbor, MI) and incubated for 2 h at 37 °C. After washing a secondary antibody (goat anti-rabbit IgG [H+L] labeled with alkaline phosphatase (from Biorad, Hercules, CA) was then added and a second incubation step was performed for 45 min at 37 °C. After washing *p*-nitrophenylphosphate (from Biorad) was added and the plate was developed at room temperature in the dark. The absorbance was measured with a microplate reader at a wavelength of 405 nm. The limit of quantification was 0.1 ng/µg.

2.4. Water and plasma estradiol analysis

Concentrations of E2 in the exposure water and the plasma were quantified using a commercially available enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). Briefly, either standard, plasma, or water sample diluted in buffer was added to the wells in duplicate. Samples were not extracted. E2 acetylcho-linesterase tracer was then added to each, along with E2 antiserum. The plate was incubated for 1 h at room temperature. Ellman's Reagent was then added, the plate developed for 1 h, then the absorbance was read with a microplate reader at a wavelength of 405 nm.

2.5. 8-Oxo-7,8-dihydro-2'-deoxyguanosine analysis

Techniques for isolation and analysis of 8-oxodG followed those outlined in Hong et al. (2006) and Rempel et al. (2008). Briefly, thawed gonads were rinsed with 0.9% NaCl, and then transferred into 4 °C lysis buffer (30mM Tris-HCl [pH 8], 0.1M NaCl, 1mM desferoxamine mesylate [DFAM], 10mM 2-mercaptoethanol, 0.5% Triton X-100). The gonads were then homogenized on ice and then centrifuged at 1300g for 10 min at 4 °C. DNA was then extracted from the resulting nuclear pellet using a Wako Chemicals (Richmond, VA) DNA Extractor Kit. The pellet was vacuum dried, then reconstituted in 100 µl of ddH₂O prior to DNA digestion. All enzymes used in digestion of the DNA were purchased from US Biological (Swampscott, MA); 10 µl of buffer (300mM sodium acetate buffer, 10mM zinc acetate, pH 5.0), 10 units nuclease P1, and 0.01 units phosphodiesterase II were added to the DNA and the solution was incubated at 37 °C for 2 h; 20 µl of a second buffer (500mM Tris-HCl, pH 8.9), together with 0.05 units of phosphodiesterase I and 95 units of alkaline phosphatase, was then added and the resulting solution was incubated for an additional 2 h at 37 °C. After enzymatic digestion the proteins were removed using a Millipore (Billerica, MA) Microcon® YM-10 centrifugal filter with 10 kDa molecular weight cut off. The filtrate was then vacuum dried and stored at -80 °C until enrichment.

8-OxodG in the nucleoside mixture was enriched using a Shimadzu (Columbia, MD) HPLC equipped with a Waters (Milford, MA) Atlantis® dC18 column (5 µm in particle size, 4.6×250mm²). The nucleoside mixture was reconstituted in ddH₂O, 5 pmol of ¹⁵N-labeled standard was added, and the solution injected into the HPLC. The flow rate was 0.5 ml/min. A gradient of 0–2% acetonitrile in 10mM ammonium formate in 5 min, followed by 2–5% acetonitrile in 55 min, was employed. The fraction eluted between 58 and 64 min was collected and vacuum dried.

The enriched 8-oxodG was then reconstituted in ddH₂O and analyzed via LC-MS/MS. A Zorbax SB-C18 column (Agilent, Santa Clara, CA), 5 µm in particle size, 0.5×150mm², was used for separation of the enriched 8-oxodG solution. The column was connected to an Agilent 1100 series LC, coupled to a Thermo Finnigan (San Jose, CA) LCQ Deca XP ion-trap mass spectrometer set up for monitoring the fragmentation of the [M+H]⁺ ions of the labeled and unlabeled 8-oxodG. A 60-min gradient of 0–25% acetonitrile in 20mM ammonium acetate was employed, at a flow rate of 8.0 µl/min. The estimated limit of detection was 0.125 pmol. The quantity of 2'-deoxyguanosine calculated from the peak area on the HPLC chromatogram during enrichment was used for normalization of 8-oxodG values.

2.6. Statistics

Data were analyzed using STATISTICA Version 6.0 (StatSoft, Inc. Tulsa, OK). For temporal comparisons, Bartlett's homogeneity test was utilized. If data were homogeneous, analysis of variance with Dunnett's post-hoc test was performed to determine significance ($p \leq 0.05$). If data were heterogenous, the Shapiro-Wilk's test was used to evaluate normality. If data were considered normal, Kruskal-Wallis non-parametric ANOVA was

carried out with the distribution free multiple comparison test employed as a post-hoc evaluation. For comparison between the two populations at each time point, or between untreated and treated groups at a given time point, the Shapiro-Wilk test was used for normality evaluations. If data were considered normal, equality of variance was assessed by an *F*-test and homoscedastic *t*-test was performed. A linear regression was used to calculate r^2 and *p*-values to determine the relationship between plasma concentrations of E2 and gonadal 8-oxodG at each time point when allowed.

3. Results

Age, length, and weight for all treatments are presented in Table 1. Hornyhead turbot are believed to reach sexual maturity above 150mm. Therefore it is likely that all of these fish were sexually immature (Cooper, 1996; Deng et al., 2007). Since hornyhead turbot are not sexually dimorphic, final replicates per time point were determined following termination of E2 exposure and subsequent gonadal dissection. Removal of females from the analysis led to final replicates per treatment per time period varying between three and four individuals. With the exception of water measurements, the 24 h time point was not utilized due to limited N caused by multiple females at this time point after sampling.

3.1. Estradiol levels in water and plasma

Concentrations of E2 in untreated exposure chambers were below detection limits (15.6 ng/L). The initial concentration in the treatment was 15.1 µg/L. The concentration of E2 diminished over the first 24 h of exposure, then appeared to reach equilibrium at about 6 µg/L (data not shown). Plasma concentrations of E2 reached maximum values at 12 h (Fig. 2). At $t = 0$ h, E2 concentrations in plasma from untreated fish collected from the outfall were significantly lower than in fish from the reference location ($p \leq 0.05$). Throughout the 48 h exposure, there was a trend towards suppressed E2 levels in outfall fish, but the difference was not significant. With the exception of the 12 h time point, where significant increases were observed in both populations relative to $t = 0$, significant differences between time points were not observed in any group.

3.2. Vitellogenin levels in plasma

Levels of vtg were detected but below quantification (0.1 ng/µg plasma protein) at all time points for untreated animals from the reference location (Fig. 3). A trend towards higher concentrations of vtg was observed in untreated fish collected from the outfall relative to the reference location at $t = 0$ and 12 h, but the difference was not statistically significant. Significant elevations of plasma vtg were not observed in either population until 36 h after treatment. Levels of vtg at initial induction in E2-treated fish (36 h) were unchanged after 48 h and no significant differences were observed between fish from the outfall or reference locations.

3.3. 8-Oxo-7,8-dihydro-2'-deoxyguanosine levels in gonads

Levels of 8-oxodG formation in gonads of untreated male fish were at or below 0.06 mol/ 10^5 dG (Fig. 4). Significant differences were not observed until 48 h of E2 treatment in fish collected from the outfall, where a 94% increase in 8-oxodG was observed. Correlation analyses between plasma E2 and 8-oxodG were statistically significant ($p \leq 0.05$) with an r^2 of 0.5056 in fish collected at the outfall. Correlation analyses of plasma E2 concentrations and 8-oxodG in fish collected from the reference location failed to show a significant relationship (Fig. 5).

4. Discussion

Male hornyhead turbot collected at wastewater outfalls in southern California have repeatedly demonstrated induction of vtg and possessed elevated plasma E2 concentrations (Rempel et al., 2006; Deng et al., 2007). Correlations between plasma concentrations of E2 and gonadal DNA damage have also been observed, but only within animals collected from outfalls (Rempel et al., 2006). Since earlier laboratory studies using fish from a reference location failed to show a relationship between plasma E2 and gonadal DNA damage, the current study was carried out to explore potential site-specific differences in sensitivity of E2 uptake, estrogenic activity, and gonadal DNA damage following aqueous E2 exposure.

Concentrations of E2 in the exposure water decreased over the first 24 h, and then reached equilibrium at approximately 40% of the original concentration for the remainder of the exposure. These values are consistent with rapid uptake and measurement of E2 in plasma from equilibrium and after 36 h. A drop in E2 levels in water was seen in a time-course experiment with gilthead seabream (*Sparus aurata*) as well, but a much more rapid change occurred over the first 4 h to less than 1% of the original concentration; then the E2 levels diminished slowly for the remaining 12 h of the study to less than 0.5% of the original concentration (Teles et al., 2005). The differences of E2 uptake between the current study and the seabream study could be due to either differences in the loading of fish in the water (i.e. experimental design), differences in uptake between the species, or loss to the walls of the test chambers and/or microbial degradation.

Plasma E2 levels were suppressed in untreated fish collected at the outfall compared to fish collected from the reference location, and a general trend of lower concentrations was consistently observed in the fish from the outfall even after E2 treatment. Previous studies in fish from the LACSD outfall also observed diminished E2 in females (Spies and Thomas, 1997). These data indicate the potential for disruption of either E2 biosynthesis or an augmentation of its clearance. Alkylphenol and alkylphenol ethoxylate concentrations in sediments at the outfall have been shown to be relatively high ($\mu\text{g/g}$) at the LACSD outfall (Schlenk et al., 2005). In several fish studies, alkylphenols have been shown to upregulate CYP1A and CYP3A, which are responsible for biotransformation and eventual clearance of E2 (Baldwin et al., 2005; Hasselberg et al., 2004). However, nonylphenol has also been shown to induce CYP19 (aromatase) expression in Atlantic salmon (*Salmo salar*), which would elevate plasma E2 (Meucci and Arukwe, 2006). In contrast, Lavado et al. (2004) reported that nonylphenol inhibited catalytic activity of aromatase as well as several Phase II enzymes such as UDP-glucuronosyl transferases, which conjugate E2 (Lavado et al., 2004). If conjugation is impaired, clearance would theoretically be reduced and would lead to higher plasma concentrations. Conversely if aromatase is inhibited, plasma E2 should be lower.

Aryl-hydrocarbon receptor (AhR) agonists such as PCBs and PAHs within outfall sediments may also induce CYP1A (Spies et al., 1982) or impair gonadotropin release, which has been proposed as a mechanism for lower E2 concentrations observed in fish from the LACSD outfall and Palos Verdes area (Spies and Thomas, 1997). Additional studies focused on site-specific differences in steroidogenic and steroid catabolic enzymes as well as other endocrine endpoints may help identify reasons for the differences in plasma concentrations of E2 in fish from both sites.

Although plasma concentrations of E2 tended to be lower in untreated and E2-treated fish collected from the outfall, vtg responses were not significantly different, suggesting that the ER-mediated expression of vtg from fish collected at the outfall may be more sensitive and may provide a higher magnitude of response than fish from the reference location. Similar

results have been observed in animals “sensitized” to E2 pretreatment, leading to either an increase in the number of estrogen receptors present in liver tissue from the pre-exposure or sensitization of the receptor, which enhances efficacy of the response (Barton and Shapiro, 1988; Nimrod and Benson, 1997; Sabo-Attwood et al., 2004). An alternative mechanism may be persistent structural modification of the vtg gene, the presence of additional transcription factors (Pakdel et al., 1991), or ER subtype differences between populations (Greytak and Callard, 2007).

In addition to APEs, estrone and E2 have been measured in sediments from the LACSD with concentrations less than 2 ng/g for E2 and estrone (Schlenk et al., 2005). Sediment exposures to E2 in hornyhead turbot failed to show estrogenic activity at these concentrations after 7 days of exposure, suggesting limited impact by steroids (Rempel et al., 2008). In contrast, 2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1,1-trichloroethane (*o,p'*-DDT) and 2,2-(2-chlorophenyl-4'-chlorophenyl)-1,1-dichloroethene (*o,p'*-DDE) concentrations at the LACSD outfall are historically very high. In 2004 total DDT concentrations in sediment at the outfall were 11.3 µg/g dry weight, 15-fold higher than at the reference location (LACSD, 2005); *o,p'*-DDT has been implicated in sensitization of medaka to E2 (Metcalfe et al., 2000). Maternal transfer of *o,p'*-DDT to offspring led to increases in vtg on exposure of offspring to E2, 10 months after hatching. The whole body *o,p'*-DDT concentration in the exposed progeny ranged from 0.02 to 102 µg/g wet weight, depending on age. Since total DDT concentrations in Dover sole (*Microstomus pacificus*) muscle tissue at the outfall were 7.87 µg/g wet weight in 2004 (LACSD, 2005), the whole body residue in fish at the outfall might be high enough to elicit enhanced magnitude of vtg observed in the turbot. Further studies would be necessary to determine if this is the case.

Induction of vtg occurred in both turbot populations after 36 h, and no significant site-specific differences were observed in the relative concentrations of vtg in plasma. A similar result was found in a study treating flounder (*Platichthys flesus*) from contaminated and clean areas with 50 ng/L of ethinylestradiol for 6 days (Kirby et al., 2006). The authors suggested that the concentrations of ethinylestradiol may have been too high and saturated the response with a short-term exposure. A similar possibility may exist in the current study and perhaps exposures with lower environmentally relevant concentrations over a longer duration may provide different results.

Elevated 8-oxodG levels correlated with plasma E2 levels only at the 48 h time point and only within fish collected from the LACSD outfall. Significant increases of 8-oxodG in turbot testes were observed only in fish collected from the outfall after 48 h of E2 treatment. There was a 36 h difference between maximum plasma concentrations of E2 (12 h) and oxidative DNA damage. In seabream exposed to 4 µg/L E2 there was a transient, non-significant peak in erythrocytic nuclear anomalies at 8 h, at least 4 h after the plasma E2 peak (Teles et al., 2005). The delay between the peak in plasma E2 levels and DNA damage response may be due to the time necessary to biotransform E2 to its reactive metabolites and the subsequent distribution to the target (gonad). Estrogens have been linked with oxidative DNA damage and DNA adduct formation in a number of studies in mammals presumably through the production of catechol metabolites via hydroxylation at the 2 or 4 position (Cavalieri et al., 2002; Wellejus et al., 2004; Wellejus and Loft, 2002). Concentrations of 6 µM/100 g body weight of the 2,4 catechol were observed to cause DNA damage in the gonads of rats (Cavalieri et al., 2002). Further metabolism of the catechols to semiquinones and quinones can initiate redox cycling, producing hydrogen peroxide and hydroxyl radicals that can oxidize DNA bases. Tissues and cell types that are low in detoxification enzymes such as catechol-*O*-methyltransferase, quinone reductase, or P450 reductase are believed to be more susceptible to DNA damage (Cavalieri et al., 2002). The activity of catechol-*O*-methyltransferase may be relatively low in fish (Mishra and Joy, 2006). The primary

enzyme believed to generate 4-catechol E2 is CYP1B, and this enzyme has been shown to be expressed in several fish tissues, including the gonad (Willett et al., 2006). The expression of CYP1B has also been shown to be upregulated by benzo[a]pyrene, indicating that exposure to PAHs or other AhR agonists that are present in outfall sediments (LACSD, 2005) may contribute to the increase of oxidative DNA damage caused by estrogen exposure in the outfall fish (Butala et al., 2004; Willett et al., 2006).

Transient increases in DNA damage caused by oxidative agents have been demonstrated in other fish species. In gills of paraquat-exposed seabream, there was a significant increase in 8-oxodG levels at 8 and 24 h, but the level dropped until it was below control levels at 48 h. (Rodriguez-Ariza et al., 1999). 4-Nitroquinoline-*N*-oxide caused a spike in DNA damage in a trout gonadal cell line after 2 h of exposure but DNA damage returned to control values after 16 h of exposure (Nehls and Segner, 2005). The addition of DNA polymerase α inhibitor counteracted the decrease in toxicity over time, indicating that DNA repair was in part responsible for the decrease in toxicity and that fish in general have sufficient repair capacity to counteract oxidative insult on DNA after initial base modification.

If gonadal DNA damage of outfall fish is sustained, it may have implications on reproduction. Duroquinone increased reactive oxygen species in carp (*Cyprinus carpio*) sperm, causing DNA damage and lipid peroxidation, which were linked with decreases in sperm motility and hatching success (Zhou et al., 2006). Hydrogen peroxide increased sperm DNA damage in sea lamprey (*Petromyzon marinus*), and also decreased sperm motility and caused a moderate decrease in embryo survival (Ciereszko et al., 2005). Exposure of male rainbow trout to ethinylestradiol, which undergoes similar metabolism as that of E2, led to a decrease in survival of eggs fertilized with sperm from exposed males, perhaps through similar mechanisms (Brown et al., 2006). Thus, the ramifications of this effect are uncertain and deserve additional study.

In conclusion, there appear to be differential responses to estrogen exposure in juvenile male hornyhead turbot from a wastewater treatment plant outfall versus a far-field location. Outfall turbot had decreased levels of plasma E2, but vtg induction was not different in animals from either site. Oxidative DNA damage in gonads was significantly higher in outfall fish treated with E2 after 48 h and correlated with plasma concentrations of E2, indicating a unique temporal sensitivity in the outfall populations of hornyhead turbot. Additional study is necessary to identify the mechanistic basis for the observed differences.

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Fig. 1. Collection locations for hornyhead turbot. Outfall collection location is T4-61 (black circle). Far-field collection location is T0-23 to T0-61 (gray circle). Adapted from LACSD (2005).

Fig. 2.

Concentrations of E2 in plasma from fish collected from outfall and reference locations exposed to 15 µg/L of E2 for 48 h. Each value represents the mean of three to four replicates $\pm$ SE). ^aStatistically significant relative to untreated control ($p \leq 0.05$). ^bStatistically significant relative to $t = 0$ ($p \leq 0.05$). ^cStatistically significant difference relative to population ($p \leq 0.05$).

Fig. 3. Concentrations of vtg in plasma from fish collected from outfall and reference locations exposed to 15 µg/L of E2 for 48 h. Each value represents the mean of three to four replicates $\pm$ SE). ^aStatistically significant relative to untreated control ($p \leq 0.05$). ^bStatistically significant relative to $t = 0$ ($p \leq 0.05$).

Fig. 4.

Concentrations of 8-oxo-7,8-dihydro-2'-deoxyguanosine in testes from fish collected from outfall and reference locations exposed to 15 µg/L of E2 for 48 h. Each value represents the mean of three to four replicates $\pm$ SE). ^aStatistically significant relative to untreated control ($p \leq 0.05$). ^bStatistically significant relative to $t = 0$ ($p \leq 0.05$). ^cStatistically significant difference relative to population ($p \leq 0.05$).

Fig. 5.

Linear regression of 8-oxo-7,8-dihydro-2'-deoxyguanosine in gonad and plasma estradiol concentrations in fish collected from the outfall and reference populations exposed to 15 µg/L of E2 for 48 h (outfall r^2 of 0.5056).

Table 1

Average age, length, and weight ($\pm$ SD) for hornyhead turbot in all treatment groups.

Treatment group	Age (year)	Length (mm)	Weight (g)
Reference control	2.8 ($\pm$ 0.7)	119 ($\pm$ 14)	46 ($\pm$ 15)
Outfall control	2.9 ($\pm$ 0.6)	122 ($\pm$ 15)	48 ($\pm$ 17)
Reference treated	3.1 ($\pm$ 0.7)	123 ($\pm$ 14)	51 ($\pm$ 16)
Outfall treated	3.3 ($\pm$ 0.7)	135 ($\pm$ 11)	58 ($\pm$ 16)

Age determined by otolith ring counts.