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Environmental Science & Technology

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# Bridging the Gap From Screening Assays to Estrogenic Effects in Fish: Potential Roles of Multiple Estrogen Receptor Subtypes

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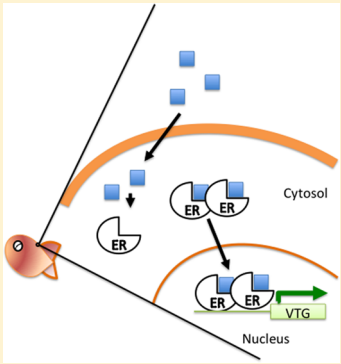
Supporting Information

**ABSTRACT:** This study seeks to delineate the ligand interactions that drive biomarker induction in fish exposed to estrogenic pollutants and provide a case study on the capacity of human (h) estrogen receptor (ER)-based *in vitro* screening assays to predict estrogenic effects in aquatic species. Adult male Japanese medaka (*Oryzias latipes*) were exposed to solutions of singular steroidal estrogens or to the estrogenic extract of an anaerobic swine waste lagoon. All exposure concentrations were calibrated to be equipotent based on the yeast estrogen screen (YES), which reports activation of hER$\alpha$. These exposures elicited significantly different magnitudes of hepatic vitellogenin and choriogenin gene induction in the male medaka. Effects of the same YES-calibrated solutions in the T47D-KBluc assay, which reports activation of hER$\alpha$ and hER$\beta$, generally recapitulated observations in medaka. Using competitive ligand binding assays, it was found that the magnitude of vitellogenin/choriogenin induction by different estrogenic ligands correlated positively with preferential binding affinity for medaka ER$\beta$ subtypes, which are highly expressed in male medaka liver prior to estrogen exposure. Results support emerging evidence that ER$\beta$ subtypes are critically involved in the teleost estrogenic response, with the ER$\alpha$:ER$\beta$ ratio being of particular importance. Accordingly, incorporation of multiple ER subtypes into estrogen screening protocols may increase predictive value for the risk assessment of aquatic systems, including complex estrogenic mixtures.




## INTRODUCTION

Estrogenic contaminants, including steroidal estrogens as well as a variety of anthropogenic chemicals, are commonly detected in aquatic environments due to inputs from wastewater[1] and have gained notoriety as endocrine disrupting compounds (EDCs).[2] Exposure of male fish to estrogenic pollutants is linked to numerous adverse reproductive effects, including the development of testicular oocytes, reduced sperm counts and sperm motility, testicular fibrosis, and reduced fecundity.[3−5] In addition to these apical end points, widely studied proximal biomarkers of estrogenic EDC exposure in male fish include hepatic induction of the egg precursor proteins vitellogenin (Vtg) and choriogenin (Chg), which are normally produced only by females in response to circulating serum estrogen.

Effects of estrogenic compounds are mediated in large part through nuclear estrogen receptors (ERs), which regulate genomic responses via action as ligand-activated transcription factors. Much interest has been garnered in determining the roles of multiple ER subtypes in modulating estrogenic responses in fish. While mammals have been found to have two nuclear ER subtypes (ER$\alpha$ and ER$\beta$), teleost fish have at least three (ER$\alpha$, ER$\beta$1, and ER$\beta$2), with the second ER$\beta$ subtype having arisen as a result of a genome duplication event in the teleost lineage.[6,7] These three ER subtypes have been shown in many cases to have distinctive tissue distribution patterns,[8−11] dissimilar ligand affinities,[12−14] and different patterns of gene regulation following ligand exposure.[8,11,15−17] Such differences offer evidence that these receptors have nonredundant physiological functions. A fourth ER subtype, ER$\alpha$2, has additionally been identified in rainbow trout (*Oncorhynchus mykiss*)[18] as well as some cyprinid species,[19,20] likely attributable to a single and more recent gene duplication event in these species.[18]

There remains significant debate surrounding the respective roles of piscine ER subtypes in regulating Vtg and Chg induction in response to estrogenic ligands. Studies in a variety of fish species have shown that Vtg induction is accompanied by a sharp increase in hepatic ER$\alpha$ expression and little change of hepatic ER$\beta$ subtype expression,[8,17,21] implying that ER$\alpha$ is the principle receptor mediating regulation of the Vtg gene. Furthermore, ER$\beta$1 and ER$\beta$2 of largemouth bass (*Micropterus salmoides*) have been found to have an inhibitory effect on recombinant ER$\alpha$-mediated transcription *in vitro*.[15] Conversely, recent studies using gene knockdown in goldfish (*Carassius auratus*) primary hepatocytes[22] and zebrafish (*Danio rerio*)

Received: September 13, 2013
Revised: January 10, 2014
Accepted: January 14, 2014
Published: January 14, 2014

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embryos[23] demonstrate that ER$\beta$1 and/or ER$\beta$2 subtypes are required for estrogen-mediated upregulation of hepatic ER$\alpha$ as well as Vtg induction. This emerging model suggests that ER$\beta$ subtypes play a critical role in vitellogenesis in the normal reproductive cycle of females, as well as in the estrogenic response of male fish exposed to EDCs.

The subfunctionalization of ERs presents an interesting challenge to the use of *in vitro* estrogen screening assays as ecological risk assessment tools for aquatic environments. Classical estrogen screening assays, e.g., the yeast estrogen screen (YES),[24] the T47D-KBluc assay,[25] as well as the ER transcriptional assays used by the U.S. EPA Endocrine Disruptor Screening Program (EDSP),[26,27] report activation of the human (h) ER$\alpha$ and/or ER$\beta$ and thus are inherently anthropocentric in terms of their molecular targets. Nevertheless, results from these assays are commonly extrapolated to other species, including fish. These assays are also often used to assess the estrogenic potency of aquatic environmental samples, which is reported in terms of 17$\beta$-estradiol (E2$\beta$) equivalents (EEQ). Such standardized assays offer a rapid, sensitive, and cost-effective means of screening for the presence of estrogenic compounds and thus have great utility for hazard characterization. However, given the complex involvement of multiple ER subtypes in modulating estrogenic response in fish, the relationship between assay-derived EEQs and *in vivo* effects is unlikely to be straightforward.

In this study, the ability of assay-derived EEQs to recapitulate estrogenic effects in fish was examined, using Japanese medaka (*Oryzias latipes*) as a model. Stock solutions of estrogenic compounds were prepared at concentrations determined to be of equal potency in the YES, which reports activation of the hER$\alpha$. Test compounds included E2$\beta$, estrone (E1), 17$\alpha$-estradiol (E2$\alpha$), and estriol (E3), all steroidal estrogen species that are commonly detected in wastewater effluents.[28] Also tested was an extract from the anaerobic waste lagoon of a commercial swine operation, a potently estrogenic environmental matrix that contains a mixture of estrogen species, with E1 being the predominant estrogenic compound in the waste.[29] Effects of these YES-calibrated solutions in medaka following waterborne exposure were determined using quantitative real-time PCR (qPCR), and affinity of these solutions for medaka (m) ER$\alpha$, mER$\beta$1, and mER$\beta$2 was determined using competitive ligand binding assays. Additionally, in order to compare the YES to another classical screening assay that reports both hER$\alpha$ and hER$\beta$, the same YES-calibrated stock solutions were also tested in the T47D-KBluc assay.

## ■ MATERIALS AND METHODS

**Test Compounds.** The same stock solutions were used across all experiments in this study, with stocks stored at −20 °C in order to preserve chemical integrity. E2$\beta$, E1, E2$\alpha$, and E3 standards were purchased from Steraloids Inc. (Newport, Rhode Island). The E2$\beta$ standard was dissolved in ethanol and diluted to a concentration of 8.8 $\mu$M. All other estrogen standards were dissolved in ethanol and diluted to levels found to have an EEQ of 8.8 $\mu$M ± 5% in the YES assay, as described below. Swine lagoon extract was prepared from the anaerobic lagoon slurry of a commercial swine sow operation, which receives waste from approximately 2500 gestating sows.[29] Details on the field site and extraction procedure are provided in Supporting Information; see also Yost et al.[29] Estrogen concentrations in all stock solutions, determined using liquid chromatography/tandem mass spectrometry, are provided in Supplementary Table SI-1.

**YES Assay.** The YES utilizes a recombinant yeast line that expresses hER$\alpha$, as well as a $\beta$-galactosidase reporter driven by estrogen responsive elements (ERE).[24] For the assay, yeast cells were dosed with a serial dilution of E2$\beta$ stock solution alongside a serial dilution of E1, E2$\alpha$, E3, or lagoon extract stock solution; details are in Supporting Information. Sigmoid concentration−response curves were fit using GraphPad Prism version 6.0 for Mac OS X (GraphPad Software, La Jolla, California, USA, www.graphpad.com). EEQs were calculated as the ratio of the concentration of E2$\beta$ that evoked a half-maximal response ($EC_{50E2\beta}$) to the dilution factor of E1, E2$\alpha$, E3, or lagoon extract stock solution that evoked a half-maximal response ($DF_{50}$). All stock solutions were confirmed to have the same EEQ ± 5% in the YES assay, based on average values from 3 or 4 runs of the assay.

**T47D-KBluc Assay.** Stock solutions that had been calibrated to be equipotent in the YES were subsequently run in the T47D-KBluc estrogen screening assay, which utilizes a T47D human breast cancer cell line that maintains endogenous levels of hER$\alpha$ and hER$\beta$ and stably expresses a luciferase reporter driven by a triplet ERE.[25] For the assay, cells were dosed with a serial dilution of E2$\beta$ stock solution alongside a serial dilution of E1, E2$\alpha$, E3, or lagoon extract stock solution in RPMI 1640 media (Sigma Aldrich, St. Louis, MO; 5% dextran-coated charcoal-treated fetal bovine serum vol/vol); details are in Supporting Information. Sigmoid concentration−response curves were fit using GraphPad Prism software, and EEQ for each stock solution was calculated as a ratio of $EC_{50E2\beta}$ to $DF_{50}$. Final EEQs were calculated on the basis of average values from 2 or 3 runs of the assay.

**Medaka Exposures.** Adult male medaka, between six and eight months of age, were obtained from the breeding colony at NCSU Environmental and Molecular Toxicology (description in Supporting Information). Fish were exposed for 7 days to E2$\beta$ at 0.64 nM (174 ng/L; actual concentration); to E1, E2$\alpha$, E3, or swine lagoon extract, each at a YES-derived EEQ of 0.64 nM ± 5%; or to a negative (ethanol) control. Exposures were conducted in 2-L glass beakers containing 1 L of exposure media. For each treatment, 12 fish were randomly distributed between four replicate beakers, 3 fish per beaker. Exposure media were prepared daily by spiking 4 L of rearing media (5.1 mM NaCl, 0.12 mM KCl, 0.198 mM $MgSO_4 \cdot 7H_20$, and 0.081 mM $CaCl_2 \cdot 2H_20$ in picopure water) with estrogen stock solution or with ethanol, for a final ethanol concentration of >0.01% in all media. Freshly prepared batches of media were then aliquoted equally between quadruplicate beakers in each treatment. Treatments were maintained by static renewal, with 100% renewal of media every 24 h. Survival rate through the experiment was 75−91%, with no significant relationship between survival and treatment. At 7 days, all fish were euthanized with tricaine methanesulfonate in accordance with the IACUC-approved protocol. Livers were excised, transferred to cryovials, immediately frozen in liquid nitrogen, and then moved to storage at −80 °C.

**RNA Isolation and cDNA Production.** Total RNA was isolated from individual medaka livers using RNA-Bee reagent (IsoTex Diagnostics, Friendswood, TX) according to the manufacturer's protocol. RNA integrity was assessed using the Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA). All samples were found to have RNA integrity numbers of 9 or greater, indicating high-

quality RNA. cDNA was then synthesized using 2 $\mu$g RNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY).

**qPCR.** Hepatic expression of 8 target genes was quantified using qPCR. Vtg-1 (AB064320) and Vtg-2 (AB074891) are two distinct polypeptides that are precursors for the Vtg phospholipoproteins in egg yolk. Chg-H (D89609), Chg-H minor (Chg-Hm) (AB025967), and Chg-L (AF500194) are the three glycoproteins that comprise the zona pellucida (ZP), which is the thick inner layer that makes up the bulk of the egg envelope; Chg-H and Chg-Hm are identified as being in the ZPB protein family, while Chg-L is in the ZPC protein family.[30] mER$\alpha$ (AB033491.1), mER$\beta$1 (NM_001104702.1), and mER$\beta$2 (NM_001128512.1) are the three medaka nuclear estrogen receptor subtypes. 18S rRNA was quantified as an internal control. Vtg and Chg primers were designed according to Zhang et al,[31] and 18S primers were designed according Zhang et al.[32] Primers for mERs were designed using the Primer3 program (http://frodo.wi.mit.edu/). See Supporting Information for primer sequences, validation procedures, and qPCR reaction conditions.

To quantify relative gene expression, the threshold cycle (Ct) for 18S amplification was first subtracted from Ct for target gene amplification to yield ΔCt. 18S expression did not vary significantly with treatment. To determine fold change in each target gene following estrogen exposure, mean ΔCt of the negative control group was subtracted from ΔCt of each sample to yield ΔΔCt. Fold change in target gene expression was then calculated using $2^{-\Delta\Delta Ct}$.[33] As an additional comparison, the relative levels of the three mERs were compared within the negative control treatment and within the E2$\beta$ treatment, using mER$\alpha$ expression as a calibrator. Mean mER$\alpha$ ΔCt within a treatment was subtracted from the ΔCt of each mER in the same treatment, and fold expression relative to mER$\alpha$ was then calculated using $2^{-\Delta\Delta Ct}$.

**Statistical Analysis.** Using GraphPad Prism, target gene expression in each estrogen treatment was compared to gene expression in the negative control and E2$\beta$ treatments using unpaired $t$ test ($\alpha = 0.05$). Correlation ($R^2$) between expression of each mER subtype and other target genes was then determined using linear regression. To compare relative expression of mERs within the control and E2$\beta$ treatments, the expression of each mER subtype was compared to the expression of mER$\alpha$ within the same treatment using unpaired $t$ test ($\alpha = 0.05$).

**Ligand Binding Assay.** Bacterial lysates containing full-length mER$\alpha$, mER$\beta$1, or mER$\beta$2 proteins were produced using bacterial expression systems, and assays were carried out as described by Hawkins et al.[14] (details in Supporting Information). For each mER subtype, saturation binding analysis was first performed by incubating lysate with a range of [$_3$H]E2$\beta$ concentrations between 0.5−19 nM, and $K_d$ values for specific binding to each receptor were determined using GraphPad Prism. Competition analysis was then performed on all mER subtypes by incubating lysate with a saturating concentration of [$_3$H]E2$\beta$ (2−3 nM, determined from saturation analysis) and a range of competing analyte concentrations. Sigmoidal competition curves were fit to specific binding data, and the concentration of each steroidal estrogen competitor that inhibited 50% of [$_3$H]E2$\beta$ binding ($IC_{50}$) was determined for each mER using GraphPad Prism, using the steps outlined in Supporting Information. Relative binding affinity (RBA) was calculated as the ratio of the $IC_{50}$ of E2$\beta$ to the $IC_{50}$ of other steroidal estrogen competitors. For lagoon extract, the concentration factor of sample extract that inhibited 50% of [$_3$H]E2$\beta$ binding ($CF_{50}$) was determined for each mER. Each assay was performed at least twice in order to calculate final $K_d$, $IC_{50}$, and $CF_{50}$ values.

## RESULTS

**Calibration of Stock Solutions in YES Assay.** Estrogen and lagoon extract stock solutions used in this study were all found to have YES-derived EEQs of 8.8 $\mu$M ± 5% (Figure 1; values in Supplementary Table SI-1).

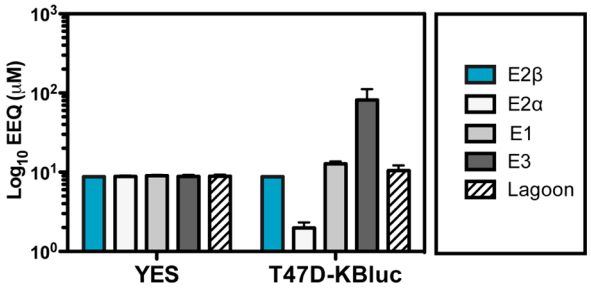


**Figure 1.** Estrogenic potency (EEQ) of the steroidal estrogen and lagoon extract stock solutions in the YES and T47D-KBluc estrogen screening assays. Mean ± standard error of the mean (SEM) is shown ($n$ = 3 or 4 for YES assay; $n$ = 2 or 3 for T47D-KBluc assay).

**T47D-KBluc Assay.** Stock solutions that had been calibrated to be equipotent in the YES were subsequently tested in the T47D-KBluc assay. Results indicate that the YES-calibrated stock solutions were not equipotent in the T47D-KBluc (Figure 1; values in Supplementary Table SI-1). E1 and lagoon slurry extract were both slightly more potent in the T47D-KBluc versus the YES, and E3 was approximately 10-fold more potent in the T47D-KBluc versus the YES. Conversely, the potency of E2$\alpha$ was 3.3-fold less in the T47D-KBluc versus the YES. As with the YES, the same E2$\beta$ stock solution at a concentration of 8.8 $\mu$M was used as the calibration standard in this assay.

**Medaka Gene Expression (qPCR).** Fold change in hepatic gene expression in male Japanese medaka following estrogen exposure is shown in Figure 2. As expected, the expression of Vtg and Chg was upregulated with estrogen exposure (Figure 2A); however, magnitude of gene induction was strikingly and often significantly different between exposures. Gene induction by E2$\beta$ exposure averaged 27,004-fold (Vtg-1), 73,735-fold (Vtg-2), 12,730-fold (Chg-H), 3,796-fold (Chg-Hm), and 324-fold (Chg-L). Comparatively, induction of these genes in E1 exposures was 36−82% of that induced by E2$\beta$ and in lagoon extract exposures was 16−59% of that induced by E2$\beta$. As shown in Figure 2A, the difference between these exposures and E2$\beta$ was often statistically significant; this was especially true for lagoon extract exposure. Meanwhile, gene induction by E2$\alpha$ was only 2−15% of that evoked by E2$\beta$, making E2$\alpha$ the least potent of these estrogen treatments in the medaka. Expression of Vtg/Chg was always significantly lower in E2$\alpha$ exposures relative to E2$\beta$ and was not significantly different from the negative control for Vtg-1 and Vtg-2 induction. Conversely, E3 exposure almost always elicited the greatest magnitude of response of all the estrogens, often significantly greater than E2$\beta$, with upregulation in Vtg and Chg that was 91−241% of that observed in E2$\beta$-exposed fish.

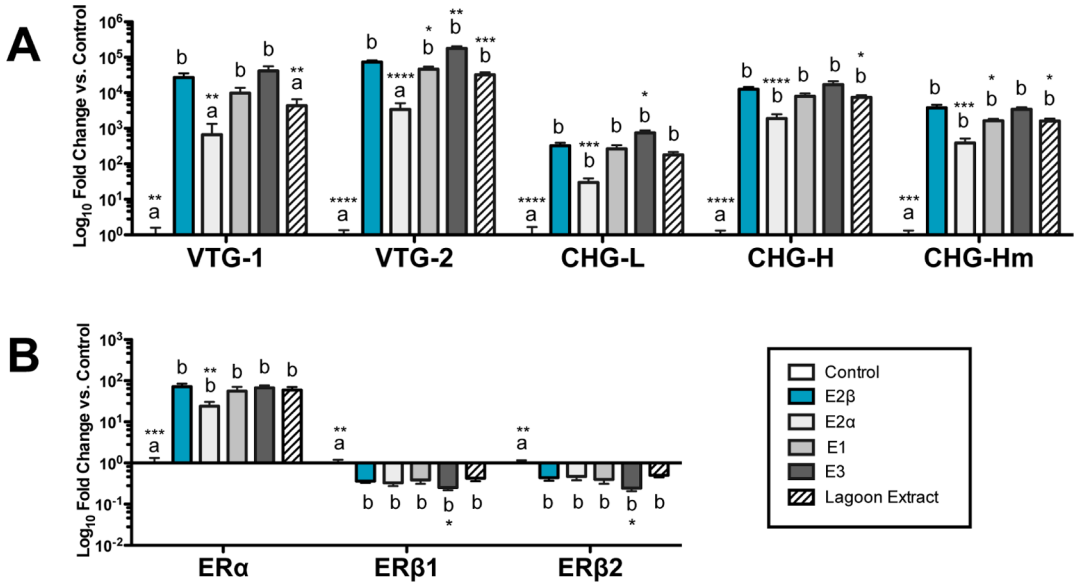


**Figure 2.** Fold change (log scale) in hepatic expression of (A) Vtg and Chg genes and (B) ER genes in medaka exposed to E2$\beta$, E1, E2$\alpha$, E3, or swine lagoon extract at a YES-derived EEQ of 0.64 nM. Mean ± SEM is shown ($n$ = 9−11). Significant difference between each exposure and the negative control is given by the letters "a" (not significantly different from control) or "b" (significantly different from control) ($p$ < 0.05). Additionally, significant differences between each exposure and the E2$\beta$ exposure group are indicated by asterisks. *$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001, and ****$p$ < 0.0001).

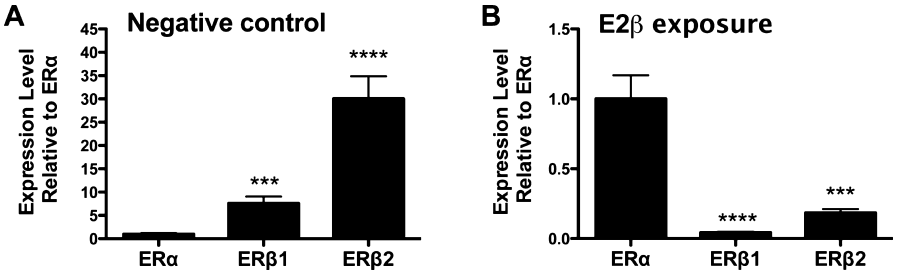


**Figure 3.** Relative hepatic expression of the three mER subtypes, normalized to mER$\alpha$ expression, in (A) male medaka from the negative control group and (B) male medaka exposed to E2$\beta$. Mean ± SEM ($n$ = 9) is shown. Asterisks indicate significant difference relative to mER$\alpha$ expression ($p$ > 0.05). *$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001, and ****$p$ < 0.0001.

Relative to the negative control, expression of mER$\alpha$ was significantly upregulated by all estrogen exposures (Figure 2B). Magnitude of mER$\alpha$ upregulation was not significantly different between E2$\beta$ (72-fold induction), E1 (56-fold induction), lagoon extract (59-fold induction), and E3 (67-fold induction) exposures; however, induction by E2$\alpha$ exposures (24-fold induction) was significantly lower than that induced by E2$\beta$. Meanwhile, all estrogen exposures resulted in significant downregulation (2- to 5-fold) of hepatic mER$\beta$1 and mER$\beta$2 expression relative to negative control. The greatest magnitude of mER$\beta$ downregulation was by E3 exposure.

A significant correlation was observed between mER$\alpha$ expression and expression of Vtg/Chg genes ($p$ < 0.0001 for all), with linear regression analysis indicating $R^2$ of 0.5405 for correlation of mER$\alpha$ and Vtg-1, and $R^2$ ranging from 0.8065 to 0.8551 for correlation of mER$\alpha$ and Vtg-2/Chg genes. No linear correlation was observed between expression of mER$\alpha$ and mER$\beta$1 ($R^2 = 1.83 \times 10^{-6}$; $p$ = 0.9913) or mER$\beta$2 ($R^2 = 8.86 \times 10^{-4}$; $p$ = 0.8109). Similarly, linear correlation was poor between mER$\beta$1 and Vtg-1 ($R^2$ = 0.002872, $p$ = 0.6667), Vtg-2 ($R^2$ = 0.05925; $p$ = 0.0472), Chg-L ($R^2$ = 0.04323; $p$ = 0.0914), Chg-H ($R^2$ = 0.05959; $p$ = 0.0465), and Chg-Hm ($R^2$ = 0.06816; $p$ = 0.0328); and mER$\beta$2 and Vtg-1 ($R^2$ = 0.002861; $p$ = 0.6673), Vtg-2 ($R^2$ = 0.04610; $p$ = 0.08100), Chg-L ($R^2$ = 0.03588; $p$ = 0.3588), Chg-H ($R^2$ = 0.04187; $p$ = 0.0967), and Chg-Hm ($R^2$ = 0.04876; $p$ = 0.0725). Expression of the two mER$\beta$ subtypes, however, was highly correlated ($R^2$ = 0.7713; $p$ < 0.0001).

Using mER$\alpha$ expression as a calibrator, it was estimated that expression of mER$\beta$2 in control fish was significantly greater (av 30-fold) than mER$\alpha$ (Figure 3A), making mER$\beta$2 the most highly expressed ER subtype in control male medaka liver. Average mER$\beta$1 expression in control fish was also significantly greater than that of ER$\alpha$ (av 8-fold). Following estrogen exposure, the receptor population shifted dramatically. mER$\alpha$ became the most highly expressed hepatic ER subtype in E2$\beta$-exposed fish, with expression significantly elevated an average of 23-fold above mER$\beta$1 and 5-fold above mER$\beta$2 (Figure 3B).

**Ligand Binding Assay.** Saturation binding curves for the three mERs are shown in Supplementary Figure SI-1, and competitive binding curves are shown in Supplementary Figure SI-2. Results of competitive ligand binding assays are provided in Table 1. Saturation binding analysis with [$_3$H]E2$\beta$ demonstrated that $K_d$ values were similar for mER$\beta$1 (1.017 nM) and mER$\beta$2 (1.107 nM), while the $K_d$ for mER$\alpha$ was higher (1.654 nM). This indicates that the [$_3$H]E2$\beta$ ligand has

**Table 1. $IC_{50}$ (nM) and Relative Binding Affinity (RBA) of Steroidal Estrogen Competitors for mER$\alpha$, mER$\beta$1, and mER$\beta$2, Determined Using the Competitive Ligand Binding Assay**

| test compounds | mER$\alpha$ | | mER$\beta$1 | | mER$\beta$2 | |
|---|---|---|---|---|---|---|
| | $IC_{50}$ (nM) | RBA (%) | $IC_{50}$ (nM) | RBA (%) | $IC_{50}$ (nM) | RBA (%) |
| E2$\beta$ | 3.1 | 100.0 | 1.8 | 100.0 | 2.1 | 100.0 |
| E1 | 8.4 | 36.7 | 14.4 | 12.6 | 14.4 | 14.6 |
| E2$\alpha$ | 13.8 | 22.4 | 18.8 | 9.6 | 31.1 | 6.8 |
| E3 | 78.7 | 3.9 | 18.1 | 10.0 | 34.2 | 6.1 |
| lagoon extract | 0.14[a] | N/A | 0.27[a] | N/A | 0.24[a] | N/A |

[a]For the swine lagoon extract competitor, $CF_{50}$ is indicated rather than $IC_{50}$.

a greater affinity for mER$\beta$1 and mER$\beta$2 than for mER$\alpha$ and that the affinity of this ligand is similar between the two mER$\beta$ subtypes. In competitive ligand binding assays, $IC_{50}$'s for E2$\beta$ with each receptor followed the rank order of mER$\beta$1 ≈ mER$\beta$2 > mER$\alpha$ (Table 1). Overall, both saturation binding analysis and competitive binding results indicate that E2$\beta$ has approximately 1.5- to 1.7-fold greater affinity for mER$\beta$ subtypes versus mER$\alpha$.

In competitive ligand binding assays, all test compounds including lagoon extract were able to displace [$_3$H]E2$\beta$ binding to all three mERs (Supplementary Figure SI-2). RBAs for the five estrogen treatments indicate that E2$\beta$ is the strongest competitor and E2$\alpha$ or E3 are the weakest competitors for all three mER subtypes (Table 1). However, when $IC_{50}$'s of each compound are compared across the three receptors, it is evident that each ligand has a unique pattern of binding preferences. In contrast to E2$\beta$, $IC_{50}$'s for E1 followed the rank order of mER$\alpha$ > mE2$\beta$1 ≈ mER$\beta$2 and indicate that E1 has an approximately 1.7-fold greater affinity for mER$\alpha$ than for either of the mER$\beta$ subtypes. Similar to E1, E2$\alpha$ had $IC_{50}$'s following a rank order of mER$\alpha$ > mER$\beta$1> mER$\beta$2. E3 displayed $IC_{50}$'s following the rank order of mER$\beta$1 > mER$\beta$2 > mER$\alpha$, indicating that, like E2$\beta$, this ligand also has greater binding affinity for the mER$\beta$ subtypes compared to mER$\alpha$; however, while E2$\beta$ had a similar affinity for the two mER$\beta$ subtypes, E3 had a 1.9-fold higher affinity for mER$\beta$1 versus mER$\beta$2.

Finally, $CF_{50}$'s of swine lagoon extract followed the rank order of mER$\alpha$ > mER$\beta$1 ≈ mER$\beta$2 and indicate that the mixture of compounds in this extract has approximately 1.7- to 1.9-fold greater binding affinity for the mER$\alpha$ versus the mER$\beta$ subtypes (Table 1). Notably, this is the same rank order of binding affinity observed for E1, which is the predominant species of steroidal estrogen found in the lagoon extract (Supplementary Table SI-1).

## ■ DISCUSSION

Piscine responses to estrogenic EDC exposure are driven by the complex interplay of ligand interactions with multiple ER subtypes, which may be difficult to predict using classical hER-based transactivation assays. In this study, qPCR results clearly indicate a discrepancy between the activity of various steroidal estrogens in the YES assay and effects on exposed male medaka. This discordance is not unexpected, given the inherent challenges of extrapolating not only from an *in vitro* system to a living organism but also between human and medaka molecular targets. While ER ligand binding domains are well conserved evolutionarily, several key amino acid changes have been identified in ER ligand binding pockets of teleost fish relative to humans, which may suggest functional differences.[14] Nevertheless, RBAs of steroidal estrogens for hER$\alpha$ have been reported to follow the rank order of E2$\beta$ (100%) > E1 (60%) > E2$\alpha$ (58%) > E3 (14%),[34] which is similar to those reported here for mER$\alpha$. Likewise, $EC_{50}$'s for the *in vitro* transactivation of recombinant mER$\alpha$ have been reported to follow the rank order of E2$\beta$ > E1 > E3.[35] Our laboratory has determined that the relative potencies of steroidal estrogens in the YES follow the same rank order of E2$\beta$ (100%) > E1 (47%) > E2$\alpha$ (2.9%) > E3 (0.76%).[29] These similarities suggest that sequence differences between hER$\alpha$ and mER$\alpha$ are not likely a predominant factor contributing to discordance between YES-derived EEQs and Vtg/Chg induction in medaka for the suite of compounds examined in this study. Indeed, other studies have indicated that ligand specificity of ER$\alpha$ for common xenoextrogens is often well conserved across species,[36] but it should be noted that this might not be the case for all compounds. For instance, a recent study demonstrated that several subtype-specific ligands of mammalian ERs did not maintain the same selectivity in Mozambique tilapia (*Oreochromis mossambicus*), indicating that agonistic characteristics cannot always be extrapolated between species.[37] The YES assay is also notably unable to detect the estrogenic activity of chlorinated chemicals, which has led to its exclusion from the U.S. EPA EDSP.[38] However, this limitation is not applicable to the suite of estrogens used in this study.

Although numerous other variables could potentially come into play, such as metabolic differences or variations in nuclear receptor coactivators between yeast cells and medaka hepatocytes, ligand binding data suggest that lack of recapitulation of EEQs *in vivo* may be due in part to ligand interactions with ER$\beta$ subtypes, which are not accounted for by the YES. Of note, ligand interactions with plasma membrane-bound ERs (e.g., GPR30) *in vivo* may also contribute to our observed differences, but this mechanism is not considered here. The potential involvement of such receptors in this response should not be conclusively discounted, although it has been demonstrated in rainbow trout that the synthetic estrogen 17$\alpha$-ethynylestradiol (EE2) does not stimulate Vtg induction via membrane-bound ERs.[39] In medaka, the magnitude of Vtg and Chg mRNA induction elicited by estrogen exposure followed the consistent rank order of E3 > E2$\beta$ > E1 ≈ lagoon extract > E2$\alpha$. Expression of Vtg and Chg genes was highly correlated with mER$\alpha$ expression and poorly correlated with mER$\beta$ subtype expression, which is consistent with observations in other studies.[8,17,21] However, when data from competitive ligand binding assays are compared with medaka gene expression data, it is evident that the compounds that elicited the most robust biomarker induction in medaka, i.e., E2$\beta$ and E3, both exhibited preferential binding affinity for the two mER$\beta$ subtypes over mER$\alpha$. In contrast, E2$\alpha$, E1, and swine lagoon extract, all of which elicited comparatively weak responses in the exposed medaka, exhibited greater affinity for mER$\alpha$ than for mER$\beta$ subtypes. These binding preferences are similar to those reported in a closely related teleost species, Atlantic croaker (*Micropogonias undulates*).[14]

The correlation between ER$\beta$ affinity and Vtg/Chg induction is particularly interesting in light of recent findings indicating a primary role for ER$\beta$ subtypes in the initiation of vitellogenesis. Nelson and Habibi[22] used selective gene knockdown on goldfish (gf) primary hepatocytes to examine the functional

roles of ER subtypes on Vtg and gfER$\alpha$ mRNA expression. gfER$\beta$1 was found necessary for maintaining baseline expression of gfER$\alpha$, and both gfER$\beta$ subtypes contributed to upregulation of gfER$\alpha$ and Vtg following estrogen exposure. The authors speculated that gfER$\beta$-mediated upregulation of gfER$\alpha$ primes hepatocytes for further stimulation by estrogen, switching the liver into the mode for Vtg production. A more recent study by Griffin et al.[23] took a similar approach using gene knockdown in zebrafish (zf) embryos and determined that both zfER$\alpha$ and zfER$\beta$b (formerly known as zfER$\beta$1) were needed to induce zfER$\alpha$ and Vtg, while the role of zfER$\beta$a (formerly zfER$\beta$2) was unclear. These authors envisioned a scenario in which zfER$\alpha$ and zfER$\beta$b act cooperatively to upregulate zfER$\alpha$ and Vtg upon estrogen stimulation. Furthermore, knockdown of zfER$\beta$b also blocked induction of brain aromatase, an enzyme critically involved in teleost sexual differentiation.

Nelson and Habibi reported ER$\beta$1 to be the most highly expressed hepatic ER subtype in male and early recrudescent female goldfish and ER$\alpha$ to be the most highly expressed of these receptors in females approaching sexual maturity. In this study, we found mER$\beta$2 to be the most highly expressed ER subtype in adult male medaka liver, which is consistent with previous reports in medaka.[40] Following 7-day exposure to estrogens, mER$\alpha$ was significantly upregulated and mER$\beta$ subtypes were significantly downregulated relative to negative controls, shifting the receptor population so that mER$\alpha$ was the most highly expressed hepatic ER subtype. Interestingly, these expression patterns are similar to that observed in female fish in response to natural fluctuations in circulating estradiol levels. In seasonal spawning species such as largemouth bass[8] and rainbow trout,[41] females have been demonstrated to have elevated hepatic expression of ER$\beta$2 during the early vitellogenic stages of the reproductive cycle, while hepatic ER$\alpha$ reaches peak expression during the later stages of Vtg production and oocyte maturation. Hepatic expression of ER$\beta$1 is reported to be relatively static throughout the reproductive cycle of females in these species; however, slight but significant changes in ER$\beta$1 expression are positively correlated with ER$\alpha$ expression,[8,41] supporting the hypothesis that ER$\beta$1 regulates baseline expression of ER$\alpha$. While one study reported largemouth bass ER$\beta$ subtypes to be less sensitive than ER$\alpha$ to E2$\beta$-mediated transactivation,[15] studies in a variety of other fish species find that ER$\beta$1 and/or ER$\beta$2 have greater binding affinity for E2$\beta$[10,14,42] and greater sensitivity to E2$\beta$-mediated transactivation[7,10,12,13,43] relative to ER$\alpha$, which is consistent with binding results in our study. This heightened responsiveness to the endogenous ER ligand perhaps also supports a role for ER$\beta$ subtypes in the generation of the estrogenic response.

The hypothesis regarding ER$\beta$ as an inducer of hepatic ER$\alpha$ contrasts with reports that ER$\beta$ can oppose ER$\alpha$-mediated transcription.[44] Using human[45] and largemouth bass[15] ER transactivation assays, it has been shown that the addition or coexpression of ER$\beta$ attenuates the transcriptional activity of ER$\alpha$. This has been attributed in part to the formation of ER$\alpha$/$\beta$ heterodimers that possess limited transactivational capacity and indicates that the ratio of these subtypes within cells is a critical determinant of transcriptional activity.[45] ER$\alpha$ and ER$\beta$ have been found to have opposing actions in a number of scenarios, including regulation of the cyclin D promoter,[46] and transcriptional activation at activating protein 1 (AP1)[47] and stimulating protein 1 (SP1) response elements.[48] These trends may represent tissue- and/or species-specific differences in functional relationships between nuclear ER subtypes. Notwithstanding, it is noted that E3 exposure in our study generated both the greatest magnitude of Vtg/Chg upregulation and greatest magnitude of ER$\beta$1/ER$\beta$2 downregulation. The inverse relationship between these genes potentially indicates oppression of ER$\alpha$-mediated Vtg/Chg expression by ER$\beta$ subtypes. However, such a relationship was observed only for E3 and not for the other estrogen exposures. Although no firm conclusions can be drawn, it is possible that either or both of these hypotheses regarding the functional relationship of ER$\alpha$/ER$\beta$ played into the results observed in our study. The contrasting nature of these hypotheses begs the question of whether the functional roles of multiple ER subtypes differ throughout the chronology of the piscine estrogenic response, perhaps with ER$\beta$ subtypes playing a supporting role of ER$\alpha$ following initial exposure to estrogen, but a different role in primed hepatocytes.

Since the swine lagoon extract employed in our study is representative of a suite of compounds that could reasonably be encountered in surface waters adjacent to livestock operations, it was of particular interest that the medaka response to this exposure often differed significantly from the response to E2$\beta$. E1 is the most abundant steroidal estrogen in the lagoon extract, and comparison of gene expression results demonstrates that E1 and the lagoon extract elicited similar magnitudes of Vtg and Chg mRNA induction. Likewise, E1 and swine lagoon extract exhibited nearly identical binding behavior, with both treatments having slightly less than 2-fold greater affinity for mER$\alpha$ versus the mER$\beta$ subtypes. Given this correlation, E1 seems to be the principle compound driving Vtg and Chg induction in medaka exposed to this estrogenic mixture. The hormone composition in this swine lagoon extract is typical of many livestock waste facilities, with E1 being by far the predominant estrogen species present in the waste.[49] E1 is also often found to be the most abundant steroidal estrogen in municipal wastewater effluents and impacted surface waters[50] and thus is arguably one of the most widespread estrogenic EDCs in aquatic environments. Our results suggest that caution should be taken when representing the estrogenic potency of these effluents and surface waters using E2$\beta$ as a calibration reference (i.e., assay-derived EEQs).

While a direct comparison was not made between the T47D-KBluc assay and medaka gene expression, EEQs derived using the two screening assays suggest that the T47D-KBluc may be more predictive than the YES of Vtg/Chg induction by this suite of compounds. E2$\alpha$ was less potent and E3 was more potent in the T47D-KBluc versus in the YES, mirroring the Vtg and Chg gene expression responses observed in medaka. T47D cells have been shown using Western blot to express slightly higher endogenous levels of ER$\beta$ relative to ER$\alpha$.[51] As male medaka also possess higher baseline hepatic levels of ER$\beta$ relative to ER$\alpha$, the relative levels of these receptors in T47D cells may enhance the translational capacity of this assay to Vtg/Chg induction in these fish. Other cellular factors might also be expected to play a role, such as different suites of endogenous nuclear receptor coregulators in human cells versus yeast cells. Conversely, E1 and lagoon slurry extract were slightly more potent in the T47D-KBluc versus in the YES, which does not reflect the effects of these compounds in medaka. This may be due to the cellular expression of E2$\beta$ dehydrogenase, an enzyme highly expressed in T47D cells[52] that may increase estrogenic potency via the formation of E2$\beta$ from E1. Notably, a recent study found that municipal

wastewater effluent elicited far greater effects in fathead minnows (*Pimephales promelas*) than predicted on the basis of the T47D-KBluc assay; the authors speculated that this was likely due to enterohepatic recirculation of EE2 in fish.[53] Such effects are less likely for swine effluent, as synthetic hormones are not used in U.S. swine production.[29]

In sum, results herein indicate that significant discrepancies exist between the YES assay and the induction of estrogenic biomarkers in a well-characterized model species, Japanese medaka. These discrepancies may be influenced by ligand interactions with piscine ER$\beta$ subtypes. Vtg and Chg are widely used biomarkers of endocrine disruption, with the Vtg biomarker demonstrated via meta analysis to have a significant quantitative relationship with fecundity in both female and male fish.[54] Results of our study should not discount the advantages of the YES assay: the YES is arguably easier and less expensive than vertebrate cell-based assays such as the T47D-KBluc and fits into the framework of the adverse outcome pathway[55] by identifying ER activation as an anchoring mode of action. However, results suggest direct extrapolation between YES-derived EEQs and effects in fish may be problematic, particularly if results observed for Vtg/Chg are potentially emblematic of apical level effects. The T47D-KBluc may offer better predictive capacity for effects in fish, although direct comparison between T47D-KBluc and *in vivo* effects would be necessary in order to substantiate this observation. Perhaps future studies could examine variables including the ER$\alpha$:ER$\beta$ ratio on the translational capacities of *in vitro* screening assays to effects in living organisms. Another observation in our study is the utility of recombinant protein binding assays to highlight the interactions between ligand and specific receptor subtypes, potentially helping to "bridge the gap" between screening assays and effects in whole organisms. Recombinant proteins could provide a favorable alternative to the cytosolic preparations that are often used for ER binding assays, for instance, the rat uterine cytosol that is used to assess ER binding in the U.S. EPA EDSP Tier 1 screening battery.[56] Another example is the use of trout liver cytosol to assess ER binding, which is performed in conjunction with trout liver slice Vtg induction to prioritize estrogenic compounds in another tiered approach by the U.S. EPA.[57] The use of recombinant proteins could potentially enhance these assays by providing specificity and allowing ligand interactions with individual receptor subtypes to be observed; these ligand interactions could then be linked to apical effects via further testing in higher tiered assays. Given the apparent complex involvement of ER$\alpha$ and ER$\beta$ subtypes in generating the estrogenic response, the inclusion of multiple ER subtypes in screening batteries could provide insight into the mechanisms of estrogenic activity, as well as enhance the translational capacities of *in vitro* assays for risk assessment. This includes the assessment of complex and environmentally relevant mixtures, such as livestock waste effluents.

## ■ ASSOCIATED CONTENT

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This work was supported by the Environmental Protection Agency (EPA) Science to Achieve Results (STAR) grant (R833420) awarded to S.W.K., as well as a National Institute of Environmental Health Sciences training grant (T32-ES007046) and EPA STAR Graduate Fellowship (FP917151) awarded to E.E.Y. We thank Dr. Michael T. Meyer and the US Geological Survey Organic Geochemistry Research Laboratory in Lawrence, KS for analytical chemistry support on this project; the laboratory of Dr. Taisen Iguchi for supplying the medaka ER cDNA used in this study; and Dr. Vickie Wilson and Mary Cardon of the US EPA for assistance with the T47D-KBluc assay. This publication has not been formally reviewed by the EPA, and the views expressed in this publication are solely those of the authors.

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