Supporting Information: Bridging the Gap from Screening Assays to Estrogenic Effects in Fish: Potential Roles of Multiple Estrogen Receptor Subtypes

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Swine operation site description: The field site for this project is a commercial swine farrowing animal feeding operation (AFO) in North Carolina, centrally located in the major swine production region of the state. This operation utilizes a waste management scheme that is typical of swine AFOs in the southeastern United States. The anaerobic lagoon from which the lagoon slurry was collected receives waste from barns housing approximately 2500 sows, all of which are breeding, gestating, or lactating. Lagoon slurry is ultimately land applied to crop fields as a nutrient amendment, with no additional treatment performed on the waste prior to land application. A thorough discussion of the lagoon system, and the occurrence of estrogenic compounds in this lagoon, is provided in Yost et al.1

Lagoon slurry sample extraction: 30 liters of slurry was collected from the lagoon on April 14, 2010. Samples were centrifuged to remove suspended solids, and then sequentially filtered with 2.7 μm and 1.2 μm glass fiber filters. This yielded 26.65 liters of liquid, which was then extracted using solid phase extraction (SPE). SPE proceeded immediately after sample centrifugation and filtration. 500 mg Supelclean™ LC-18 SPE cartridges (Supelco, Bellefonte, PA) were pre-conditioned with 5 ml of pico-pure water followed by 5 ml of 5% methanol/95% pico-pure water. Aliquots of filtered lagoon slurry were then passed through Visiprep™ Large Volume Samplers (Supelco, Bellefonte, PA) and loaded onto individual cartridges at a rate of 10-15 ml per minute. After samples were loaded, the cartridges were washed with 1 ml of picopure water, and then dried by running the vacuum pump for 5-10 minutes. Extracts were eluted by passing two 4 ml aliquots of methanol through the cartridges. The eluents were evaporated to near dryness
using a gentle stream of N$_2$ and warming (40°C) in a water bath, and 10 µl of picopure water was added to each extract to prevent evaporation to complete dryness. Extracts were resuspended in two washings of ethanol to a final volume of 1ml. In order to prepare ample extract for use in this study, fifty 1 ml extracts were prepared, and then combined to make a single 50 ml composite in ethanol. This 50 ml compositred extract was used throughout the duration of this study, with storage at -20°C in order to preserve chemical integrity. As 26.65 liters of liquid were concentrated to 50 ml of extract, the concentration factor of the sample extract was determined to be 533.

**LC/MS-MS Analysis:** Concentrations of steroidal estrogens were quantified in all stock solutions using ultra pressure liquid chromatography (UPLC) / tandem mass spectrometry (MS-MS). For analysis, an aliquot of each stock solution was shipped on ice to the US Geological Survey Organic Geochemical Research Laboratory (OGRL) in Lawrence, KS. At OGRL, quantification of analytes was performed using Waters Acquity H-class Bio UPLC system with API 5500 triple quadrupole mass spectrometer. E1, E2β, E2α, and E3 were analyzed in negative ion mode and separated using a water/methanol gradient, with a post column infusion of a 10 mM ammonia hydroxide solution to enhance ionization. More details on the LC/MS-MS procedure can be found in Yost et al.\textsuperscript{1} Results of the LC/MS-MS analysis of the stock solutions can be found in Supplementary Table SI-1.

**Supporting Information: Yeast Estrogen Screen:** The YES utilizes a yeast cell line that has been stably transfected with the human estrogen receptor alpha (hERα), as well
as a β-galactosidase (lac-Z) reporter that is driven by estrogen responsive elements (ERE). The assay was performed using the protocol of Routledge and Sumpter, with modifications as described in Chen et al. All stock solutions were first diluted 1:200 in a solution of 90/10% water/ethanol to get within the working range of the assay. For each treatment, 200 µl of this diluted stock was added in duplicate to a 1 ml deep 96-well plate, and serially diluted 1:2 in a solution of 90/10% water/ethanol (12 dilutions total), leaving 100 µl of serially diluted stock solution in each well. E2β stock solution served as dose-response standard on each assay plate, and four wells with 90/10% water/ethanol served as the negative control. 300 µl of diluted yeast solution, at an optical density (OD) of 0.08 (620 nm), was added to each well. Plates were loosely covered with KimWipes (Kimberly Clark) and incubated at 37°C with gentle shaking. Following three days of incubation with yeast solution, an assay buffer containing ortho-nitrophenyl-β-galactoside (Calbiochem, EMD Chemicals, Inc; San Diego, CA) was added, producing a colorimetric response at 405 nm. After 20 minutes of incubation with the assay buffer (at 37°C, with gentle shaking), the reaction was stopped with addition of a 1 M sodium carbonate solution, and the plates were centrifuged at 3,000 rpm for 10 minutes. 100 µL of the resulting supernatant was taken from each well and transferred to a new 96-well microtiter plate to determine OD at 405 nm and 620 nm.

For data analysis, OD620 (yeast cells) was subtracted from OD405 (β-galactosidase) in each well, and values in all wells were then normalized to the negative control. The sigmoid concentration-response curve of the E2β standard was fit to a symmetric logistic function using GraphPad Prism software (GraphPad, San Diego, CA), and the response of each stock solution was expressed as a percentage of the maximum response evoked.
by E2β. The concentration of E2β that evoked a half-maximal response (EC₅₀E2β), and the dilution factor of E1, E2α, E3, or lagoon extract stock solution that evoked a half-maximal response (DF₅₀), were fit using the software. EEQ for each stock solution was then calculated as a ratio of EC₅₀E2β to DF₅₀. Final EEQs were calculated based on 3-4 replicate runs of the assay.

Supporting Information: T47D-KBluc assay: The T47D-KBluc assay utilizes a human breast cancer cell line that has been stably transfected with a luciferase reporter driven by a triplet ERE. This assay reports activation of endogenous hERα and hERβ. Cell maintenance and assays were performed as described by Wilson et al.⁴ Stock cells were maintained in RPMI 1640 media (without phenol red, with glutamine; Sigma-Aldrich, St. Louis, MO) supplemented with 2.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate, 10% vol/vol fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Gibco; purchased as a 100x mixture of antibiotics/antimycotics). One week prior to use in the assay, cells were moved to a modified media containing 10% vol/vol dextran-coated charcoal-treated (DCC) FBS with no antibiotics/antimycotics. Modified media containing 5% vol/vol DCC-FBS and no antibiotics/antimycotics was used when plating cells for assays.

For the assay, cells were seeded at 10,000 cells/well in 96-well luminometer plates (Corning Inc, Corning, NY), with overnight incubation to allow for cell attachment. Cells were dosed with a serial dilution of E2β stock solution in RPMI 1640 media (5% vol/vol DCC-FBS), with final E2β concentrations ranging from 10 fM to 300 pM; and, on the
same plate, a serial dilution of E1, E2α, E3, or lagoon extract in RPMI 1640 media, at YES-derived EEQs ranging from 10 fM to 300 pM. Final ethanol concentration in all doses was 0.1%. Also included on each plate was a negative (ethanol) control, which was used for sample normalization; an ICI 182,780 estrogen receptor antagonist (1 μM; in ethanol), which was used as a qualitative control for background; and ICI 182,780 plus E2β, which was used as a qualitative control for reporter specificity. All doses were performed in quadruplicate wells on each plate, with 100 μl of dosing solution in each well. Following overnight incubation (18 – 24 hours), cells were rinsed with phosphate buffered saline (pH 7.4) at room temperature, and 25 μl reporter lysis buffer (Promega, Madison, WI) was added to each well to harvest cells. Luciferase activity was quantified using a FLUOStar Omega microplate reader (BMG Labtech) with the automated injection of 25 μl reaction buffer (25 mM glycylglycine, 15 mM MgCl₂, 5 mM ATP, and 0.1 mg/ml bovine serum albumin, pH 7.8) followed by 25 μl 1 mM D-luciferin (Promega, Madison, WI) into each well, as described by Wilson et al.⁴

For data analysis, luciferase activity in all wells was first normalized to the negative (ethanol) control. The sigmoid concentration-response curve of E2β was then fit to a symmetric logistic function using GraphPad Prism software, and the response of each stock solution (E1, E2α, E3, or lagoon extract) was expressed as a percentage of the maximum response evoked by E2β. As with the YES assay, EEQ was then calculated as the ratio of EC₅₀E₂β to DF₅₀. Final EEQs were calculated based on average values from 2-3 runs of the assay.
**Medaka culture:** Adult male Japanese medaka used in this study were obtained from the breeding colony at NCSU Environmental and Molecular Toxicology. Fish care and maintenance in this colony was provided daily in accordance with NCSU IACUC-approved animal protocol (NCSU# 07-183-B). Broodstock were housed in a charcoal filtrated and UV-treated recirculating aquatic system. Water temperature and pH were monitored daily and maintained at ~25°C +/- 2°C and ~7.4, respectively, and broodstock were maintained under a strict light:dark cycle of 16:8 hours. Dry food (Otohime B1, Reed Mariculture, Campbell, CA) was fed several times per day via automated feeders with daily supplementation of newly-hatched *Artemia nauplii*.

**Supporting Information:** qPCR: Primer sequences, sources, and amplicon sizes are listed in Supplementary Table SI-2. All primers were verified to have approximately equal efficiencies (90-100%) using a serial dilution of cDNA. A subset of cDNA was amplified using a primer set that spanned a single intron (mERβ2), separated using gel electrophoresis, and verified to have a single amplicon, indicating no significant genomic DNA contamination.

qPCR was performed in 96-well Optical Reaction Plates (Applied Biosystems, Grand Island, NY) using the ABI 7300 Real-Time PCR system. Each 25 μL qPCR reaction mixture consisted of 12.5 μL SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY); 1 μL forward primer (10 μM); 1 μL reverse primer (10 μM); 8.5 μL of sterile, nuclease-free water; and 2 μL of cDNA template. cDNA concentrations used were 0.75 ng/μL for Vtg genes, Chg genes, and 18S, and 7.5 ng/μl for mER genes. Each reaction was performed in triplicate. qPCR conditions were as follows: 50°C for 2
minutes and 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

**Supporting Information: Ligand Binding Assay:** *Bacterial expression systems:* Plasmid constructs containing full-length medaka (m) ERα, mERβ1, and mERβ2 coding sequences were provided to our laboratory by the laboratory of Dr. Taisen Iguchi. To produce heterologous proteins for use in ligand binding assays, these cDNA were subcloned into pET-32a(+) vectors for expression in BL21(DE3)PLyss chemically competent *E. coli* cells. Transformed cells were grown in LB media (50 mg/L ampicillin) at 37°C with shaking at 200 rpm until the cells reached an approximate OD of 0.5 – 0.8 at 600 nm. Translation was then induced through addition of 1 mM isopropyl-b-d-thiogalactopyranoside, with 20 μM ZnCl₂ added to aid in ER protein folding, and cells were moved to incubation at 25°C with shaking at 200 rpm for 3 hours to allow protein expression to occur. Cells were then centrifuged at 4600 rpm for 30 minutes at 4°C, supernatant was discarded, and pellets were stored at -80°C.

From frozen pellets, cell lysate was prepared for use in the assays. Cells were weighed and then resuspended in ice-cold assay buffer (20 mM HEPES, 150 mM NaCl, 10% wt/vol glycerol, 1.5 mM EDTA, 6 mM monothioglycerol, and 10 mM NaMoO₄ in pico-pure water) at a volume of 3.5 ml buffer per gram pellet. Chicken egg white lysozyme (Calbiochem, EMD Chemicals, Inc; San Diego, CA) was added to final concentration of 1 mg/ml, and protease inhibitor cocktail III (Calbiochem, EMD Chemicals, Inc) was added at a volume of 14 μl per ml of buffer. This mixture was incubated on ice for 5 minutes, and then sonicated (twelve 1-second bursts at 30% power). The crude lysate was
centrifuged at 4600 rpm for 30 minutes at 4°C. Resulting supernatant was aliquoted into fresh microtubes, and stored at -80°C prior to use in the assays.

*Saturation binding assays:* Assays were carried out according to the protocol by Hawkins et al.\(^5\) For each lysate preparation, the dilution factor of lysate resulting in a saturating concentration of 2-3 nM E2β was first determined through preliminary testing (data not shown). This dilution factor of lysate was then used for both saturation and competition binding assays. A dilution series of \(\text{[^3]H]E2}\) was prepared in assay buffer. 350 µl lysate, diluted in ice-cold assay buffer, was combined with 50 µl of each prepared dosage of \(\text{[^3]H]E2}\) in disposable glass culture tubes (Fisher Scientific, Waltham, MA), resulting in final \(\text{[^3]H]E2}\) concentrations ranging between 0.5–19 nM. For each concentration of \(\text{[^3]H]E2}\) tested, 4 µl of ethanol or 100 mM diethylstilbestrol (DES) (in ethanol; final concentration of 1 mM) were added to duplicate tubes in order to determine total binding and non-specific binding, respectively. Tubes were shaken vigorously to mix, and then were incubated overnight in ice baths at 4°C. The following day, an equal volume (400 µl) of charcoal buffer (assay buffer with 0.1% dextran and 0.5% charcoal) was added to each tube. Tubes were incubated in an ice bath for 10 minutes, then centrifuged at 4600 rpm for 10 minutes at 4°C in order to precipitate charcoal. The resulting supernatant, containing bound \(\text{[^3]H]E2}\), was transferred to scintillation vials (Fisher Scientific, Waltham, MA), and 5 ml of ScintiSafe Econo scintillation fluid (Fisher Scientific, Waltham, MA) was added. \(\text{[^3]H}\) was then measured using a Beckman LS 6500 scintillation counter. Specific binding was calculated as the difference between total and non-specific binding [in disintegrations per minute (DPMs)] for each concentration of \(\text{[^3]H]E2}\). One-site hyperbolic binding curves were fit, and \(K_d\) for specific binding to each
receptor was then determined using GraphPad Prism software. Saturation binding curves are provided in Supplementary Figure SI-1. Assays were performed at least twice in order to calculate final $K_d$ values.

**Competitive ligand binding assays**: Assays were carried out according to the protocol by Hawkins et al.\(^5\) In disposable glass culture tubes, 350 µl of diluted lysate was spiked with 50 µl of a saturating concentration of $[^3H]E2\beta$ (final concentration of 2-3 nM, determined from saturation analysis) and a range of competing analyte concentrations. 4 µl of competitor was added to duplicate tubes; final concentrations of steroidal estrogen competitors ranged between 100 fM to 10 µM depending upon the compound, while the final concentration factor of swine lagoon extract competitor ranged from 5.33 to 5.33e-6. Ethanol and DES controls were included for each curve in order to determine total binding and non-specific binding, respectively. Tubes were incubated in ice baths overnight at 4°C, and $[^3H]$ was measured in the bound fraction as described above.

For data analysis, specific binding was determined for each concentration of competitor by subtracting the value for non-specific binding. The specific bound level for each concentration of competitor was then expressed as a percentage of maximal specific bound. Sigmoidal curves were fit, and the concentration of each steroidal estrogen competitor that inhibited 50% of $[^3H]E2\beta$ binding ($IC_{50}$), or the concentration factor of lagoon extract which inhibited 50% of $[^3H]E2\beta$ binding ($CF_{50}$), was determined for each mER using GraphPad Prism software, following the steps outlined below. For each steroidal estrogen competitor, relative binding affinity (RBA) was also calculated for each receptor as the ratio of the $IC_{50}$ of $E2\beta$ to the $IC_{50}$ of each steroidal estrogen.
Competition binding curves are provided in Supplementary Figure SI-2. Each assay was performed at least twice in order to calculate final IC$_{50}$ and CF$_{50}$ values.

It was observed that the top and bottom of the competition curves did not always correspond exactly with 100% and 0%, respectively. This was particularly evident for the lagoon extract, for which the competition curve leveled out somewhat above 0% (see Supplementary Figure SI-2); perhaps due in part to interference from unknown compounds in the lagoon matrix, or due to the presence of weak binders such as phytoestrogens. As a result, IC$_{50}$ calculated directly from these curves would not necessarily correspond to 50% bound, and thus would not be correct for comparison between receptors and between competitors. Therefore, an alternative approach was taken in order to more accurately calculate the IC$_{50}$ and CF$_{50}$ values, without artificially constraining the curves. The concentration (or concentration factor) of competitor that inhibited 50% of [$_3^H$]E$_2$ binding was first determined by constraining the top of the curve to 100 and the bottom of the curve to 0. The top/bottom constraints were then removed from the curve, and it was determined which value “F” on the y-axis of the unconstrained curve corresponded with the concentration that inhibited 50% of binding. IC$_F$ was then calculated from the unconstrained curve using the GraphPad Prism “Find ECanything” equation. This value is reported as IC$_{50}$ or CF$_{50}$ in the main text. Through this approach, the IC$_{50}$ and CF$_{50}$ values more accurately represent the concentration that inhibited 50% of [$_3^H$]E$_2$ binding, rather than simply the value halfway between the top and bottom of the unconstrained curve.
Table SI-1: Actual concentrations of steroidal estrogens in each of the stock solutions, determined using LC/MS-MS; and estrogenic potency (EEQ, in µM) of each stock solution in the YES and T47D-KBluc bioassays.

| Test Compound | Actual concentrations (µM) | YES assay | T47D-KBluc assay |
|---------------|-----------------------------|-----------|------------------|
|               | E2β | E1  | E2α | E3 | EEQ (µM) | Lower 95% CL | Upper 95% CL | EEQ (µM) | Lower 95% CL | Upper 95% CL |
| E2β           | 8.80| -   | -   | -  | 8.80    | -            | -            | 8.80     | -            | -            |
| E1            | -   | 19.30| -   | -  | 9.07    | 8.56         | 9.60         | 13.69    | 10.07        | 18.65        |
| E2α           | -   | -   | 293.70| -  | 8.81    | 8.38         | 9.34         | 2.63     | 1.88         | 3.72         |
| E3            | -   | -   | -   | 1213.70| 9.25 | 8.77         | 9.78         | 92.66    | 65.10        | 131.91       |
| Lagoon extract| 0.44| 66.00| 1.46| 0.22| 8.81    | 8.46         | 9.25         | 15.86    | 11.86        | 21.27        |
Table SI-2: Primer sequences used for qPCR amplification, amplicon sizes, and literature references for the source of the primer sequence (if applicable).

| Gene | Forward Sequence (5’-3’) | Reverse Sequence (5’-3’) | Amplicon Size (bp) | Reference |
|------|--------------------------|--------------------------|-------------------|-----------|
| Vtg-1 | ACTCTGCTGCTGCTGGCTGTAG | AAGGCGTGGGAGAGGAAAGTC | 85     | Zhang et al.⁵ |
| Vtg-2 | TCGCCGCAAGAGCAAGAC  | CTGGAGGAGCTGGAAGAAGCTG | 150     | Zhang et al.⁵ |
| Chg-L | TCCCTGTCTCTGACTCTGAATGG | GCTTGCTCCTCCTCACC | 137     | Zhang et al.⁵ |
| Chg-H | TGGCAAGGCACTGGAGTATCAG | CTGAGGCTCTGGCTGTGGATAG | 143     | Zhang et al.⁵ |
| Chg-Hm | GGAGCCATTACCAGGGACAG | AAGTTCCACACGCAAGATTCC | 143     | Zhang et al.⁵ |
| mERα | ATCGCTCCCGGTTCTATATCAG | AAGCATCACCTTGTCCCAAC | 188     |          |
| mERβ1 | ACCACCTGCACTGCATGAAGA | TGCTGCTAGCCAGGGCTTT | 195     |          |
| mERβ2 | TGACAGCTTCTCTGACCAGCA | CACTGATCTGGCGAGATGTAAC | 222     |          |
| 18S  | CGTTCAGCCACACGAGATTG | CCGGACATCTAAGGGCATCA | 56      | Zhang et al.⁷ |
**Figure SI-1:** Saturation binding of $[^3]H]$E2β to (A) mERα, (B) mERβ1, and (C) mERβ2. Specific ligand binding mean ± standard error of the mean ($n = 2$ or 3) is shown.

![Graph](image1.png)

**Figure SI-2:** Competitive ligand binding of steroidal estrogens and swine lagoon extract to (A, D) mERα, (B, E) mERβ1, and (C, F) mERβ2. Specific ligand binding mean ± standard error of the mean ($n = 2$ or 3) is shown.

![Graph](image2.png)
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