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Conversion of Estrone to 17 β -Estradiol: A Potential Confounding Factor in Assessing Risks of Environmental Estrogens to Fish

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Abstract

Feminization of male fish and the role of endocrine-active chemicals in this phenomenon has been an area of intense study for many years. Estrone (E1), a natural steroid, is found in aquatic environments sometimes at relatively high concentrations. However, E1 has been less thoroughly studied than 17 β -estradiol (E2) or 17 α -ethynylestradiol due in part to a relatively lower potency in metabolically-limited estrogen receptor (ER) binding/activation assays. Recent evidence suggests that *in vivo* biotransformation of E1 to E2 may occur in fathead minnows (*Pimephales promelas*) residing in environments with high concentrations of E1, such as near wastewater treatment plants. The enzymes likely responsible for this biotransformation, 17 β -hydroxysteroid dehydrogenases (17 β HSDs), have been well characterized in mammals but to a lesser extent in fish species. In the current study, a novel systematic analysis of amino acid sequence data from the National Center for Biotechnology Information database demonstrated that multiple 17 β HSD isoforms are conserved across different fish species. Experimentally, we showed that metabolically-active hepatic cytosolic preparations from two commercially important salmonid species, rainbow trout and lake trout, biotransformed E1 to E2 to a degree sufficient to alter results of competitive ER binding assays. These results from *in silico* and *in vitro* analyses indicate E1 and biotransformation may play a significant role in the feminization of a variety of fish species in contaminated aquatic environments.

Keywords

metabolism; estrogen receptor; fish; estrone; 17 β -estradiol; 17 β -hydroxysteroid dehydrogenases

INTRODUCTION

Endocrine-active chemicals (EACs) have dominated toxicological research and chemical regulatory activities for more than two decades (Hotchkiss et al. 2008). One of the most commonly cited examples of the negative effects of EACs has been feminization of male fish exposed to discharges associated with municipal wastewater treatment plant (WWTP) and animal feeding operations (Purdum et al. 1994; Leet et al. 2015). Feminization can be manifested several ways, including decreased expression of male secondary sexual characteristics, production of testis-ova in the gonad, and the induction of vitellogenin (VTG), an egg yolk protein precursor normally present only in reproductively-active females (Purdum et al. 1994; Sumpter and Jobling 1995; Harries et al. 1996, 1997). Due to their

potency in binding and activation of estrogen receptor- α (ER- α), and common occurrence in WWTP discharges, the natural and synthetic steroids 17 β -estradiol (E2) and 17 α -ethynylestradiol (EE2), respectively, have been hypothesized as major contributors to the feminization seen in fish from the field (Desbrow et al. 1998; Routledge et al. 1998; Thorpe et al. 2003). Estrone (E1) is another natural steroid commonly found in the environment, often at concentrations substantially greater than E2 or EE2 (Matthiessen et al. 2006; Sarmah et al. 2006; Chen et al. 2010; Alvarez et al. 2013; Blazer et al. 2014; Cavallin et al. 2016; Ma et al. 2016). However, E1 has been of lesser environmental concern than E2 or EE2 due to its lower potency relative to ER- α interactions in fish *in vitro* assays (e.g., Denny et al. 2005; Peterson and Tollefsen 2011; Lange et al. 2012).

Although reported E1 potencies tend to be lower than for other steroidal estrogens, the range of reported E1 relative binding affinity (RBA) values can be comparatively large. For instance, human ER α RBA values (expressed relative to E2) for E1 varied between 0.7 to 60% across laboratories, while much less variability was observed with other chemicals tested at the same time, e.g., variability in RBAs for estriol, a steroidal metabolite, ranged from 7 to 14% (Kuiper et al. 1997; Gutendorf and Westendorf 2001). Similarly, a wide range of E1 RBAs have been reported across mammalian species/tissue types (e.g., from 7.3% in rat uterine cytosol to 66% in rabbit uterine cytosol), compared to ranges of RBAs for EE2 (190 to 191%) and estriol (9.7 to 16%) from these same studies (Korenman 1969; Blair et al. 2000). These data suggest that one or more undefined factors may be contributing to variability in estimates of potency/binding of E1 in different assay systems.

The vertebrate steroidogenesis pathway converts cholesterol to different steroidal structures through reactions catalyzed by an assortment of enzymes, including several cytosolic 17 β -hydroxysteroid dehydrogenase (17 β HSD) isoforms (Peltoketo et al. 1999a, 1999b; Lukacik et al. 2006). A potentially important 17 β HSD catalyzed reaction from the standpoint of environmental estrogens and their potential risk to fish is the conversion of E1 to E2 (Ryan and Engel 1953a, 1953b; Moeller and Adamski 2006). Ankley et al. (2017) reported that caged fathead minnow (*Pimephales promelas*) males held in the vicinity of WWTP discharges had increased plasma concentrations of E2. In subsequent controlled lab studies with E1 water concentrations similar to those measured near the WWTPs, E2 levels in the plasma of exposed male fathead minnow males were found to be comparable to those in reproductively-active females (e.g., Jensen et al. 2001; Ankley et al. 2017). Furthermore, there was a significant induction of VTG in the E1-exposed males, to a magnitude comparable to that observed in fish treated with E2 as a positive control (Ankley 2017 et al.). Based on these fathead minnow studies, Ankley et al. (2017) postulated that failure to consider metabolic conversion of E1 to E2 could result in a substantial underestimation of the environmental risks of the former steroid to fish. Accordingly, knowledge concerning the taxonomic conservation of this reaction is critical to assessing possible E1 risks to different fish species.

Steroidogenic 17 β HSDs have been well studied in several mammalian species, but comparatively less is known concerning the occurrence and attributes of these enzymes in fish. In studies with one model species, zebrafish (*Danio rerio*), there was evidence of structural and functional similarities with rodent 17 β HSDs, including several cytosolic

isoforms (1, 3, 7, 12A) that catalyze the conversion of E1 to E2 (Mindnich et al. 2004, 2005, 2009). However, the degree to which this extends to other ecologically- and commercially-important fish species is uncertain. The overall goal of the present work was to better define structural and functional characteristics of 17 β HSDs in different fish species. One aspect of our studies involved an *in silico* analysis of cross-species protein sequence conservation using the US Environmental Protection Agency (USEPA) Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS, seqapass.epa.gov/seqapass) tool, a publicly accessible web-based tool that allows for evaluation of protein sequence similarity for extrapolation of toxicity knowledge across species (LaLone et al. 2013, 2016, 2018). This tool “mines” the extensive National Center for Biotechnology Information (NCBI) protein structure database. In addition, we report the results of studies with two model salmonid species native to North America, rainbow trout (*Oncorhynchus mykiss*) and lake trout (*Salvelinus namaycush*), evaluating the hepatic conversion of E1 to E2, and the consequences of this conversion relative to determination of putative competitive binding of E1 to ERs.

MATERIALS AND METHODS

Evaluating conservation of 17 β HSDs across bony fish species using SeqAPASS

The publicly-accessible SeqAPASS tool (v3.0, Data Version 3) allows for cross species comparisons of protein sequence and structure to evaluate protein conservation (LaLone et al. 2016, 2018). Four 17 β HSDs, 1, 3, 7, and 12A, previously characterized in zebrafish as able to catalyze the conversion of E1 to E2 (Mindnich and Adamski 2009) were used as query sequences in SeqAPASS. The NCBI protein accessions corresponding to the four zebrafish 17 β HSDs 1, 3, 7, 12A were NP_991147.1, AAS58451.1, NP_001070796.1, and AAS58452.1, respectively. Level 1 SeqAPASS comparisons of primary amino acid sequences were conducted for all four proteins across bony fish species (Class Actinopteryi). Protein conservation was determined based on a default susceptibility cut-off, derived as described by LaLone et al. (2016, 2018). Briefly, SeqAPASS uses a reciprocal best hit BLAST (Basic Local Alignment Search Tool) to identify ortholog candidate sequences (i.e., those more likely to maintain similar function) from all sequences that align to the query sequence. The tool then identifies the lowest percent similarity (based on primary amino acid sequence alignments) where ortholog candidates are detected and sets this value as a cut-off. Those sequences above the cut-off, or those that lie below the cut-off but found within a taxonomic group of species above the cut-off, are considered conserved. Those below the cut-off are considered less likely to be conserved.

The SeqAPASS tool also enables comparative cross-species evaluation of specific amino acid residues that have been identified as particularly critical to activity of a given protein. Based on existing information concerning critical amino acid residues in human 17 β HSD1 (He et al. 2016), SeqAPASS Level 3 comparisons of individual amino acids were made across fish species. For this evaluation, NCBI human protein accession P14061.3 was used as the template sequence aligning amino acid residues arginine (R) 38, asparagine (N) 115, serine (S) 143, tyrosine (Y) 156, lysine (K) 160, histidine (H) 222, and glutamic acid (E) 283 across sequences. Conservation of selected amino acid residues is determined

automatically by the SeqAPASS tool according to exact amino acid residue match, and/or similar protein classification (e.g., basic, acidic, aromatic) and size (molecular weight) (Doering et al. 2018). Both the protein classification and the size of an amino acid must be different from the template amino acid for it to be considered an important alteration in the Level 3 evaluation.

In vitro evaluation of E1 metabolism and binding affinity

In these studies, we utilized metabolically-active hepatic cytosol from two salmonid species to assess the conversion of E1 to E2, and the consequences of this conversion to measures of apparent RBA values for the steroid.

Chemicals.—Acetonitrile (ACN) was procured from Burdick and Jackson, Muskegon, MI. Ethanol, 200 proof, was obtained from Aaper Alcohol and Chemical Co., Shelbyville, KY. Tritiated 17 β -E2 [2, 4, 6, 7, 16, 17-³H] ([³H]-E2), 99.9% pure, specific activity 120 to 180 Ci/mmol, was from Amersham Pharmacia Biotech, Inc., Piscataway, NJ. Hydroxyapatite (HAP) was purchased from Bio-Rad Lab., Hercules CA. Ultima Gold Scintillation cocktail was purchased from Packard Bioscience Co., Meriden, CT.

Tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA), glycerol, dithiothreitol (DTT), 17 β -estradiol (E2, 98% pure, CASRN [50-28-2]), estrone (E1, 99% pure, CASRN [53-16-7]) and all other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Fish attributes and tissue collection.—Adult, mixed-sex rainbow trout were obtained from the Seven Pines Fish Hatchery (Lewis, WI). Lake trout were from Crystal Springs State Fish Hatchery (Altura, MN). Fish were acclimated to the on-site USEPA holding facilities (Lake Superior water, 2 μ m filtered, ultraviolet light (UV) treated, 11°C, pH = 7.7, hardness = 45 mg/L) for at least 2 weeks prior to use. They were fed Silver Cup Fish Food (Nelson and Sons, Murray, UT) and kept under a constant photoperiod of 16-h light: 8-h dark. Livers were collected from 12 rainbow trout and two lake trout after anesthetizing them with a buffered Tricaine (MS-222) solution. The livers were held in 1.15% potassium chloride on ice until used for cytosol preparation as described below. All procedures involving the fish were performed in accordance with an approved Animal Care and Use Plan for the Duluth USEPA facility.

Cytosol and nuclear extract preparation.—Cytosol preparations were conducted using previously described methods (Denny et al. 2005). Nuclear fractions were also prepared from the livers of two of the 12 rainbow trout, RT-1 and RT-12. Briefly, livers were homogenized in TEDG buffer (50 mM Tris, 1.5mM EDTA, 1 mM DTT, 30% glycerol, pH 7.4, 4°C) and separated into nuclear fraction (820 x g) and cytosol (103,000 x g) by centrifugation (Loomis and Thomas 1999). The pelleted nuclear fraction was extracted in TEDG with the addition of 600 mM KCl. Cytosol and nuclear fraction protein concentrations were determined utilizing the Bradford method (Bradford 1976), then diluted to 4 mg protein/mL with TEDG. All preparations were subsequently stored at -80°C until analyzed.

Estrone metabolism.—Conversion of E1 to E2 concentrations were determined in 12 rainbow trout cytosols, the two lake trout cytosols, and RT-1 and RT-12 nuclear fractions dosed with 10 μM E1. A more extensive biotransformation analysis was performed on the RT-9 cytosol preparation, where analytes were measured after dosing with 0.1, 0.5, 1, 5, 7.5, 10, 50, and 100 μM E1.

Concentrations of E1 and E2 were measured in E1 dosed cytosol and nuclear preparations after 20 hours of incubation at 4°C. The E1 reaction mixtures were conducted in glass incubation tubes in duplicate for each sample type. Reaction mixtures contained a total of 4 mg/mL of cytosolic protein in a total volume of 257 μL , comprised of 255 μL of the TEDG buffer and 2 μL of ethanol. The ethanol was used to mimic the addition of [^3H]-estradiol in the competitive binding assays described below. After addition of the E1 (dissolved in the ethanol), sample tubes were capped and incubated for 20 h at 4°C on a cell culture roller drum.

Estrone and estradiol measurement.—After the 20 h incubation, nuclear or cytosol samples (240 μL) were transferred to 1.5 mL microcentrifuge tubes. Acetonitrile (100 μL), ZnSO_4 25% (w/v) (25 μL) and saturated $\text{Ba}(\text{OH})_2$ solution (25 μL) were added to precipitate proteins. Samples were vortexed, incubated on ice for 5 min then centrifuged 5 min at 20,800 $\times g$ at 4°C. The supernatant was transferred to HPLC vials equipped with inserts, maintained at 4°C, and analyzed immediately. Analysis of samples was performed on a Beckman System Gold High-Pressure Liquid Chromatograph, equipped with a refrigerated autosampler, UV detector (280 nm), and a Coulochem II electrochemical detector (DC mode; high sensitivity analytical cell 5011 in series at $E^0 = -150 \text{ mV}$ and $E^0 = 800 \text{ mV}$ referenced to an internal platinum electrode). Injections (50 μL) were made onto a Shandon Hypersil ODS (C18) 5 μm 4.6 $\times$ 250mm column (Phenomenex, Torrance, CA). An isocratic mobile phase (1.5 mL/min) consisting of 50% ACN and 0.05 M sodium acetate (pH 4.2) was employed. Concentrations of E1 and E2 in samples were quantified by comparing sample response to E1 and E2 9-point standard curves. The E1 and E2 quantitation limits for the system were 0.05 μM . Blank samples from incubations with no added E1 did not have detectable E1 or E2.

Estrogen receptor binding assays.—Estrogen receptor saturation experiments were performed with aliquots of each of the rainbow trout cytosol samples to determine equilibrium dissociation constant (K_d) and maximum binding capacity (B_{max}) values before conducting the competitive binding assays as described by Laws et al. (1996) and Denny et al. (2005). Saturation curves were generated by incubating the cytosol preparations with [^3H]-E2 concentrations of 0.1, 0.5, 1, 5, 10, 25 nM for 20 h at 4°C. Nonspecific binding was determined by incubating the cytosol samples with an excess of unlabeled E2, at 100 times the [^3H]-E2 concentration. The mean (SD) K_d and B_{max} values for the nine rainbow trout cytosolic fractions used in these experiments were 1.9 nM (± 0.5) and 18.0 fmol/mg protein (± 8.8), respectively. Nuclear fraction K_d and B_{max} values were not generated due to the small amount of material available.

Nine of the 12 rainbow trout cytosol preparations were used for the ER competitive binding assay. In addition, the RT-1 and RT-12 nuclear fractions were evaluated in competitive binding assays. The lake trout samples were not evaluated using binding assays.

The rainbow trout hepatic cytosol and nuclear fractions contain both ER α and ER β (Pakdel et al. 1989, 2000; Denny et al. 2005), so the competitive binding assay results reflect the contribution from both receptor subtypes. Except for the addition of [3 H]-E2, the binding assay reaction mixtures were prepared as described for the E1 metabolism reaction mixtures. Radiolabeled [3 H]-E2 was added to cytosol or nuclear fractions to a final concentration of 5 nM. Unlabelled E1 competitor was added to the reaction mixture to achieve final concentrations of 0.001, 0.01, 0.1, 1, and 10 μ M. Unlabelled E2 competitor treatments at 0.0001, 0.001, 0.01, 0.1, and 1 μ M served as the positive control. Ethanol was used as a carrier solvent for the E1 and E2 additions (maximum final concentration of 1.5%) and ethanol-only treatments served as a negative control. All treatments were conducted in duplicate. The binding assays were performed under the same conditions as the metabolism studies, with an incubation of, 20 h at 4°C on a cell culture roller drum. Following the incubation, HAP was used to separate free [3 H]-E2 from [3 H]-E2 bound to the ER(s). The bound [3 H]-E2 was extracted from HAP with ethanol, added to 10 mL of Ultima Gold Scintillation cocktail and the radioactivity was counted using a Packard Tri Carb 2550TR/LL liquid scintillation analyser (Packard Instrument Co., Meriden, CT).

The radioactivity data were used to generate nonlinear binding curves from which IC₅₀s were calculated using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). The ER relative binding affinity (RBA) values were calculated as (E2 IC₅₀/chemical IC₅₀) x 100. The GraphPad software also was used to calculate the K_d and B_{max} values from the ER saturation experiments with the ER saturation experiments.

RESULTS

Cross-species conservation of 17 β HSD: SeqAPASS analysis

Overall, the SeqAPASS Level 1 comparisons of zebrafish 17 β HSD 1, 3, and 12A primary amino acid sequences to sequences of fish from 26 Orders found these three isoforms to be well conserved (Table 1). Specifically, the 17 β HSD1 and 17 β HSD3 protein sequences were conserved in 21 fish Orders which had sequences that aligned with the target sequences of the two proteins. The 17 β HSD12A target sequence was conserved in fish from all 22 Orders surveyed.

Conservation of 17 β HSD7 proteins was not as universal as the other three isoforms, with no observed orthologs in four of the 26 fish Orders evaluated (Table 1). For example, no amino acid sequence comparable to zebrafish 17 β HSD7 was found in European seabass (Order Moronidae), rainbow smelt (Order Osmeriformes) or gilthead seabream (Order Spariformes). In seabass and smelt, 17 β HSD7 most closely aligned with retinol dehydrogenase 12. In seabream, 17 β HSD7 aligned most closely to carbonyl reductase-like 20 β -hydroxysteroid dehydrogenase. No sequence from fish of the Order Anguilliformes aligned with the zebrafish 17 β HSD7 query sequence.

Even when considering the slight 17 β HSD7 exception, all four 17 β HSD isoforms associated with E1 to E2 biotransformation in zebrafish appear to be comparatively well conserved across fish orders. Notably, rainbow trout consistently aligned predicted protein sequences in each of the SeqAPASS 17 β HSD evaluations.

Several individual amino acid residues critical to function of human 17 β HSD1 have been elucidated from structural and site-directed mutagenesis studies (R38, N115, S143, Y156, and K160 associated with catalytic activity; H222, and E283 associated with substrate recognition) (He et al. 2016). SeqAPASS Level 3 analysis comparing these amino acid residues to predicted and measured residues of 44 fish species showed that the amino acids were conserved in 43 species (Table 2). Only the predicted protein for spotted gar lacked amino acids similar to human H222 and E283, which both are involved in substrate recognition. The gar amino acid residues associated with catalytic activity of human 17 β HSD1 were conserved.

In the 43 species with conserved critical residues, six of the seven amino acids were exact matches to the human 17 β HSD1 (Table 2). The seventh human amino acid was glutamic acid (E) at position 283 on the protein, which had six different substitutions among the 43 fish species. Putative conservation of amino acid residues in SeqAPASS Level 3 is defined by both side chain classification and molecular weight as a surrogate for size. The assigned rules require both the classification and the molecular weight to be different from the template sequence to suggest that the amino acid substitution would lead to a change in some aspect of protein function. In the case of E283, the six substituted amino acids, leucine (L), isoleucine (I), phenylalanine (F), valine (V), proline (P), and threonine (T) have different side chain classifications than E, but the molecular weights are within the 30 g/mole size range of E. Therefore, the substitution of E by these other amino acids may not have a great effect on enzyme function. This suggests that from a functional perspective 17 β HSD1 is likely well conserved across the fish species included in the Level 3 analysis.

Table 3 summarizes basic attributes of the fish used for this study. Hepatosomatic index (HSI) was calculated by dividing liver weight by body weight then multiplying by 100. HSI for fish used in this study ranged from 0.84 to 1.35% (Table 3). Sex was determined by visual inspection of gonads

In vitro conversion of E1 to E2

In the metabolism studies, all the original E1 added to TEDG buffer without rainbow or lake trout tissue fractions was recovered as E1 after 20 h at 4°C. In contrast, concentrations of E1 measured in trout cytosol preparations dosed with 10 μ M of the steroid and incubated under the same conditions were not consistent, with losses of the parent compound of up to 74% (Table 3).

Measured 20 h E1 concentrations comprised 26 to 96% of the initial nominal dose in the rainbow trout cytosolic preparations, and 47 to 65% in the lake trout cytosol (Table 3). cytosol. Five of the rainbow 12 cytosols were tested twice to measure the response consistency. Inter-test E1 concentration variability was low among most cytosols tested on separate occasions, at 64% $\pm$ 19 (RT-2), 71% $\pm$ 3 (RT-3), 78% $\pm$ 8 (RT- 5), 44% $\pm$ 1 (RT-8)

and $40\% \pm 8$ (RT-10) (mean $\pm$ SD, $n=2$). Therefore, experimental variability does not explain the large range in recoveries measured among the different cytosol preparations.

In addition to E1, E2 was detected in all the cytosol preparations after 20 h. No E2 was detected in E1-dosed TEDG without cytosol indicating a biological component of the cytosol was responsible for the observed metabolism of E1 to E2. Concentrations of E2 ranged from 26 to 1464 pmoles/sample in the rainbow trout cytosolic samples and 226 to 406 pmoles/sample in the lake trout. As a side note, no 17 α -E2 was detected in any of the cytosol samples. The sum of recovered E1 + E2 accounted for 70 to 123% of the initial 2570 pmoles/sample in the 10 μ M E1 reaction mixture for 11 of 12 rainbow trout tested indicating that E2 was the main E1 metabolite (Table 3). The one outlier was RT-12 cytosol for which only 39% of the initial E1 was recovered as E1 + E2. Several unidentified chemical peaks unique to the RT-12 exposures were present on the HPLC chromatogram.

Expressed on a molar basis E2 accounted for 1.1 to 68.9% of the total measurable E1 + E2 in the rainbow trout liver cytosols dosed with 10 μ M E1 (Table 3). Variations in biotransformation of E1 to E2 were also observed in lake trout liver cytosols exposed under the same conditions (Table 3). However, the 20 h E2 concentration inter-test variability was low among five cytosols tested on separate occasions, $12.6\% \pm 4.5$ (RT-2), $23.2\% \pm 3.7$ (RT-3), $41.1\% \pm 2.6$ (RT-5), $53.3\% \pm 2.8$ (RT-8), and $57.6\% \pm 0.8$ (RT-10) (mean $\pm$ SD, $n=2$). So, as was true for measured E1 concentrations, cross-experiment variability does not account for the variability of E2 seen in the different cytosol preparations.

Kinetics of cytosolic E1 to E2 conversion

Cytosol from one of the experimental fish (RT-9) was used to more fully evaluate kinetics of the biotransformation of E1 to E2. Our ability to consistently detect E1 and E2 in the reaction mixture was a function of the initial E1 concentrations, related to instrumental detection limits (Table 4). However, the proportion of the amount of E2 detected as a function of the total E1 introduced to the system also was highly dependent upon the initial E1 concentration, suggesting that the generation of E2 likely was enzymatic in nature. For example, in the 0.5 μ M E1 treatment group, only about 11% of the total steroid nominally present in the system was detected after the 20 h incubation, but all that was detected was present as E2 (Table 4). At progressively higher nominal E1 treatments the relative proportion of total steroid occurring as E2 decreased, such that in the 100 μ M E1 incubation only 5.7% of the total measured steroid was present as E2. The data from Table 4 were used to evaluate the conversion of E1 to E2 in terms of enzyme saturation kinetics (Figure 1). The K_m (mean $\pm$ SD) for conversion of E1 to E2 in cytosol was 12.03 ± 1.63 μ M E1 and the V_{max} was 88.6 ± 3.7 pmole E2/mg protein $\cdot$ h or 1.48 ± 0.06 pmole E2/mg protein $\cdot$ min using the Michaelis-Menton model ($R^2 = 0.9813$).

Relative binding affinity assays

Apparent E1 RBA values among the nine rainbow trout cytosol preparations varied considerably, from 6.4 to 67.5% (Table 3). This did not appear to be due to experiment to experiment variability, as E1 RBA values for two cytosols tested on two different days were 14.2 and 15.6% for RT-2, and 57.6 and 61.5% for RT-10. Furthermore, RBA values for two

other chemicals tested in our lab at the same time as the E1 studies with two of the cytosol samples (RB-2, RB-10) were far less variable (estriol $3.2 \pm 0.3\%$, diethylstilbestrol $162.8 \pm 6.8\%$, mean [SD, n=2]).

As part of our routine testing, an E2 IC₅₀ was measured with each set of RBA determinations (Table 3). The E2 IC₅₀ values (6 to 19 nM) were much less variable than simultaneously-measured E1 IC₅₀ values (12 to 141 nM) for 10 of the rainbow trout cytosol preparations (Table 3).

In visually inspecting the data, variability of the apparent E1 IC₅₀ values appeared to be strongly influenced by the amount of E2 found in the cytosolic preparations after the 20 h incubation (Table 3). For example, when the cytosolic E2 concentration was 42.9% or less (RT-1, 2, 3, and 5) of the total E1 + E2 in the 10 μ M nominal E1 treatment (Table 3), the E1 and E2 IC₅₀ values did not overlap one another (based on the 95% confidence intervals; Table 3). Conversely, when the E2 proportion was 46.3% or higher (RT-6, 8, 9, 10, and 12) E1 and E2 IC₅₀ values overlapped one another, suggesting that the E2 contribution to the observed binding was overwhelming that of E1.

To further assess the influence of E2 on the apparent E1 RBA values we plotted the two variables as a function of one another, which yielded a significant positive correlation ($R^2=0.86$; Figure 2). Consistent with the IC₅₀ findings this indicates that E2 binding to the ER was overwhelming the effect of E1 binding to the ER.

To further explore the influence of the E1 to E2 conversion on measured RBAs in the fish, the binding assay was modified to use the nuclear cell fraction in place of cytosol. The nuclear fraction contains ER α and ER β (Ascenzi et al. 2006) but does not contain the cytosolic 17 β HSDs. Consistent with this, in the E1 metabolism studies E2 was not detected in either of the two nuclear extracts tested (Table 3). Nuclear E2 IC₅₀s were very consistent not only between the two nuclear preparations tested (RT-1, RT-12) but with the cytosolic preparations from RT-1 and RT-12, ranging from 8.2 to 15 nM. The E1 interactions in nuclear extract were far more consistent than in cytosol. Specifically, the nuclear E1 IC₅₀s were virtually the same for the two fish tested, 250 nM in RT-1 and 260 nM in RT-12, whereas cytosolic E1 IC₅₀s for the same two fish which were 141 and 12, respectively. Additionally, the nuclear E1 IC₅₀s were similar to the RT-1 cytosolic IC₅₀, which had only 1.1% conversion of E1 to E2 (Table 3). Consistent with the IC₅₀ values, nuclear E1 RBAs were very consistent, 3.4 and 5.8% in RT-1 and RT-12, respectively, whereas the cytosolic E1 RBAs varied greatly, 6.4 and 67.5%, in these same fish.

DISCUSSION

Environmental risk context

One of the most notable impacts of EACs in the environment involves feminization of male fish. A wide range of chemicals have been proposed as potentially responsible for the phenomenon, but among those most frequently implicated have been the steroids E2 and EE2. This is not unreasonable given their common occurrence in the environment, particularly around WWTPs (Desbrow et al 1998; Routledge et al. 1998), and their

comparatively strong affinity for ER α isoforms in basically all vertebrates (Ankley et al. 2016). Estrone is a lower-potency steroid also commonly found in aquatic environments associated with WWTPs and animal feedlots, often at concentrations far exceeding those reported for E2 and EE2 (Matthiessen et al. 2006; Sarmah et al. 2006; Chen et al. 2010; Alvarez et al. 2013; Blazer et al. 2014; Cavallin 2016 et al.; Ma et al. 2016). Ankley et al. (2017) noted the occurrence of elevated E2 in the plasma of male fathead minnows caged at sites with elevated E1 concentrations and hypothesized that the E2 was a product of the conversion of E1 by 17 β HSDs. Through controlled lab studies, including one in which the males were exposed to ¹³C-E1, Ankley et al. (2017) demonstrated conclusively that the males were converting water-borne E1 to plasma E2 in quantities sufficient to induce responses associated with feminization, such as induction of VTG. A potential implication of this is that E1 may be a much more important environmental estrogen than previously supposed in terms of endocrine effects in fish. Of course, a critical consideration relative to this possibility is the degree to which a broad variety of fish species are able to convert E1 to E2. The studies described in the present paper aim to assess this consideration.

Based on steroid synthesis studies conducted primarily in rodents and humans, it is probable that the E1 to E2 conversion is catalyzed by one or more cytosolic 17 β HSDs (Ryan and Engel 1953a, 1953b; Peltoketo et al. 1999; Lukacik et al. 2006; Moeller and Adamski 2006). While knowledge concerning 17 β HSDs in fish is sparse, studies in one species, zebrafish, indicate structurally and functionally similar enzymes to rodent 17 β HSDs isoforms 1, 3, 7, 12A, all of which ostensibly can convert E1 to E2 (Mindnich et al. 2004, 2005, 2009). Consequently, one facet of our assessment involved the use of SeqAPASS to assess occurrence and structural conservation of 17 β HSDs across a range of fish species that reside in freshwater and marine environments that could be polluted by E1.

The second aspect of our assessment involved direct evaluation of the conversion of E1 to E2 by hepatic tissue preparation from two ecologically and commercially important salmonid species, rainbow and lake trout. In these studies, we were able not only to document and characterize the E1 to E2 conversion, but showed the practical consequences of the reaction in terms of estimating potency of E1 in a RBA assay, arguably one of the most commonly employed measures of the estrogenic potential of test chemicals.

Evaluation of cross-species structural conservation of 17 β HSDs

The SeqAPASS tool was developed to enhance cross-species extrapolation of the potential toxicity of chemicals that exert toxicity through protein interactions, with the assumption that the greater the degree of structural conservation of a protein target the greater the likelihood of similar among-species susceptibility to a given chemical(s) (LaLone et al. 2013). An especially powerful attribute of SeqAPASS is the ability to effectively “mine” the extensive NCBI protein sequence database in an efficient manner, thereby providing comparative data for millions of protein sequences and thousands of species (LaLone et al. 2016). Previously LaLone et al., (2018) used SeqAPASS to evaluate structural conservation of 17 β HSD1 and 17 β HSD3 across all vertebrate classes. For those analyses human 17 β HSD isoforms were used as the query sequences for the similarity assessments. Overall, the proteins were well conserved across most vertebrate phyla: Actinopterygii (bony fishes),

Amphibia, Coelacanthiformes, Testudines, Crocodylia, Aves, and Mammalia. Exceptions to this were observed for Lepidosauria (a reptile subclass that includes snakes and lizards), and Chondrichthyes (cartilaginous fish such as sharks) where retinol dehydrogenase 8 aligned best with the human 17 β HSD1.

In this study the subclass Actinopterygii was more extensively examined for not only 17 β HSD1 and 17 β HSD3, but also 17 β HSD7, and 17 β HSD12A, the various isoforms associated with conversion of E1 to E2 in zebrafish (Mindnich et al. 2004, 2005, 2009). Because our emphasis in this study was on conservation of this specific functionality (i.e., the E1 conversion) in the context of observations made in zebrafish, in the current study zebrafish rather than human 17 β HSD sequence data were used as the query sequences in the Level 1 analysis. Overall, the newer analysis highlighted strong structural conservation of the four isoforms across bony fishes, with some minor exceptions for 17 β HSD7 for representatives of the Moronidae, Osmeriforme and Spariforme Orders.

The SeqAPASS tool also enables higher-level evaluations of sequence conservation relative to the occurrence/position of specific amino acids known to be involved in function of a given protein. While identification of critical amino acid for 17 β HSDs has not been done in any fish species, data are available for isoforms of the enzyme in humans. Specifically, a total of seven individual amino acid residues important to protein function have been identified for human 17 β HSD1; five are involved in aspects of catalytic activity and two contribute to aspects of substrate recognition (He et al. 2016). A Level 3 SeqAPASS analysis of the Actinopterygi subclass using human 17 β HSD1 as the template sequence revealed that all five key catalytic amino acids are well-conserved. Substrate recognition residue H222 also was well conserved but residue E283 (E) had many other amino acids substituted among the fish tested. In many instances the alternative amino acids observed at this position in fish were structurally similar enough to E that substrate binding may not be affected. But there were a few instances where this would not be the case; for example, spotted gar did not have amino acid residues comparable to either H222 or E283 in human 17 β HSD1.

In summary, we can conclude from the SeqAPASS analysis that 17 β HSDs 1, 3, 7 and 12A are well conserved among (bony) fish species, suggesting that the ability to convert E1 to E2 should be wide-spread in this subclass. However, it is possible, due to the variations seen in the substrate recognition amino acids residues of 17 β HSD1—at least in some fish species—that reactions catalyzed by the enzyme may occur with other substrates other than E1.

Evaluation of E1 to E2 biotransformation

The lab results from this study clearly demonstrate biotransformation of E1 to E2 in by hepatic cytosol from both rainbow and lake trout. We also found that the conversion followed saturation kinetics whereby increased substrate did not result in increased product; the substrate-product relationship yielded an enzyme saturation curve consistent with Michaelis-Menton kinetics. Further, we showed that the E1 to E2 conversion was associated with cytosolic but not nuclear preparations, clearly indicating the cellular fraction containing the enzyme(s) catalyzing the reaction. So, while we did not directly demonstrate that the E1 to E2 conversion was catalyzed by 17 β HSDs in the trout, based on our observations and existing mammalian and zebrafish literature (Ryan and Engel 1953a, 1953b; Peltoketo et al.

1999; Lukacik et al. 2006; Moeller and Adamski 2006; Mindnich et al. 2004,2005, 2009), the conclusion that these enzymes were responsible for the reaction seems all but certain.

Although direct comparison of our results with reported biotransformation kinetic values from other studies is difficult because of the wide range of reaction conditions employed, there are several relevant observations that can be made. For example, the rate of E2 formation of 88.6 pmole/mg cytosolic protein • h in this study was within the range of values reported for 17 β HSD1-catalyzed E2 formation in three of human placental choriocarcinoma cell lines: 67 pmole/mg protein • h in JAR cells, 935 pmole/mg protein • h in JEG-3 cells, and 2382 pmole/mg protein • h in BeWo cells (Lewintre et al. 1994). Since the trout assays were performed at 4°C versus 37°C in the human cell lines, it is not surprising the trout rate was at the low end of the range. Similarly, the total extent of E2 formation of a 0.5 μ M E1 over the course of 20 h noted in this study was very comparable to that seen in numerous mammalian cell lines exposed to the same initial E1 concentration (Miettinen et al. 1996; Piao et al. 1997).

A potentially important observation from our study was the large among-fish variation in the degree of conversion of E1 to E2. In the rainbow trout the amount of E1 converted into E2 over the course of a 20-h incubation ranged from about 4 to 74%. The among-fish differences were not due to experimental variability, so likely were related to physiological factors that may influence the types and/or levels of 17 β HSD isoforms present in the tested cytosols. In any case, from a pragmatic perspective, the large variation seen among the fish could have repercussions in terms of inherent individual susceptibility to possible endocrine perturbations associated with environmental exposure to E1, so understanding the basis of this variation is important.

In considering the key role of 17 β HSDs in reproduction, it seems plausible that among-fish differences in E1 biotransformation could be related to sex and/or reproductive status of the animals. The best-studied of the HSD isoforms in the context of reproduction in mammals is 17 β HSD1, where it is detected at low quantities in the liver of females (Nokelainen et al. 1996; Gheresevich et al. 1994; Mäentausta et al. 1990) but is abundantly expressed in ovary granulosa cells and placenta syncytiotrophoblasts (Vihko et al. 2001). Levels of 17 β HSD1 in the ovary and placenta of mammals fluctuate greatly in conjunction with changes in reproductive status. For example, 17 β HSD1 expression is markedly increased by follicle-stimulating hormone and decreased by luteinizing hormone, presumably to control relative levels of E1 versus the more potent E2 (Gheresevich et al. 1994). Of course, most fish lack placentas, but their liver is a very active organ from a reproductive perspective, particularly relative to production of VTG. Hence, it is not surprising that expression of the 17 β HSD1 isoform appears to be greater in the liver of fish than mammals (Mindnich et al. 2009; Ankley et al. 2017).

Although the sample size is admittedly small, data from the present study do suggest that observed variations in E1 metabolism were indeed related to sex and reproductive status of the rainbow trout. Both males and females were able to convert E1 into E2, but females were tended to be far more efficient relative to E1 metabolism, as well as more variable. There were three male rainbow trout included in the *in vitro* metabolism study, and all of them

converted around 30% of the 10 μ M E1 over the 20 h incubation (Table 3). In contrast, the six largest rates of conversion of E1 to E2 were observed in six of the nine females examined, with five of these exceeding 60% conversion of the E1 (Table 3). Significantly, females exhibited both the smallest and largest amount of E1 metabolism at around 4% (RT-1) and 74% (RT-12). Interestingly, these two fish also had about a 30% difference in their relative liver weight (HSI) with RT-1 having the largest HSI measured (1.35%) and RT-12 one of the smaller values (0.99%). The relative weight of the liver in female fish can be indicative of vitellogenesis, so it is possible that the differences between the fish relative to E1 metabolism could be related to hepatic production of VTG. Given our small sample size, this hypothesis is clearly speculative but the influence of reproductive status on E1 metabolism (and 17 β HSD profiles) in fish is worthy of further investigation.

Consequences of E1 metabolism on estimates of estrogenic potency

In the present study, estimates of the apparent RBA values for E1 (relative to E2) varied by about an order of magnitude across the individual rainbow trout. The extant literature is replete with inconsistent estimates of E1 estrogenic potency. For example, reported mammalian uterine cytosol and recombinant ER E1 RBA values have ranged from 0.7 to 66% (Korenman 1969; Kuiper et al. 1997; Blair et al. 2000; Gutendorf and Westendorf 2001). Our data strongly suggest the observed variation in RBA values in the present study was due to the differential metabolism of E1 to E2 in individual animals. This is supported by multiple lines of evidence, including: (a) the observed among-fish variation in E1 but not E2 IC₅₀ values (Table 3); (b) the positive association between E1 RBA values and measured E2 concentrations in the cytosol (Figure 2); and (c) the fact that E1 RBA values measured in nuclear fractions from the liver (which by definition lack 17 β HSDs) were less variable, and comparatively low (Table 3). It seems probable, therefore, that an important source of variability in E1 potency estimates in the literature arises from differential conversion of the steroid to E2 in metabolically-competent, but poorly characterized test systems.

A challenge therefore is defining what the “true” values are for an E1 RBA in fish, as this clearly will be a function of the test system employed. For example, in a relatively comprehensive transcriptional-activation study using transfected mammalian cells, Lange et al. (2012) found that E2 was consistently more potent than E1 by factors ranging from 19 to 65-fold, using cloned receptors from zebrafish, medaka, roach, fathead minnow, three-spined stickleback and common carp. This is at odds with comparative *in vivo* studies where E1 has the potential to be converted to E2. For example, Thorpe et al. (2003) reported that E2 was only about 2-fold more effective than E1 in inducing VTG in juvenile rainbow trout, and Van den Belt et al. (2004) reported similar effects concentrations for induction of VTG in zebrafish by E1 and E2. Similarly, both Panter et al. (1998) and Dammann et al. (2011) conducted 21-d tests with the fathead minnow that examined a variety of endpoints (including induction of VTG) and did not find E1 to be any less potent than E2. Overall these observations highlight, in general, the challenge of extrapolating data from *in vitro* systems with variable metabolic capabilities to *in vivo* responses.

From the standpoint specifically of E1, our study clearly shows the potential for underestimating the risk of this common environmental estrogen to aquatic vertebrates. In

the absence of biotransformation, the estrogenic potential of E1 is low, at around 3 to 6% of the potency of E2. However, the apparent estrogenic potency of E1 can be increased by an order-of-magnitude through the formation of E2. Furthermore, the 17 β HSDs likely responsible for biotransformation of E1 to E2 in the two salmonid species evaluated in this study are probably well-conserved across most bony fishes, suggesting that this biotransformation would be a wide-spread phenomenon. Further evaluation of this possibility is warranted.

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Figure 1.

Enzyme kinetic graph. Graph represents the formation of estradiol during the 20 h incubation relative to the initial exposure of estrone. Mean $\pm$ SD, n=2

Figure 2. Correlation between preliminary estrone relative binding affinity for cytosolic hepatic rainbow trout ER and percent E2 as related to the total measured E1 + E2 concentrations after 20 h incubation. Each square represents a single rainbow trout. Data from RT-12 was not included in this calculation due to the low recovery of chemical with this cytosol.

Table 1.Conservation of 17 β HSDs across Orders of fish (class Actinopteri) based on SeqAPASS Level 1 evaluation

		Zebrafish Query Protein			
		(Total # Species with conserved sequences; Total # of ortholog candidates)			
		17 β HSD1 (46; 29)	17 β HSD3 (48; 43)	17 β HSD7 (49; 45)	17 β HSD12A (49; 41)
Orders	Anguilliformes	Yes	-	-	-
	Beloniformes	Yes	Yes	Yes	Yes
	Carangiformes	Yes	Yes	Yes	Yes
	Centropomidae	Yes	Yes	Yes	Yes
	Characiformes	Yes	Yes	Yes	Yes
	Cichliformes	Yes	Yes	Yes	Yes
	Clupeiformes	Yes	Yes	Yes	Yes
	^a Cypriniformes	Yes	Yes	Yes	Yes
	Cyprinodontiformes	Yes	Yes	Yes	Yes
	Esociformes	Yes	Yes	Yes	Yes
	Gobiiformes	-	Yes	Yes	Yes
	Labriformes	Yes	Yes	Yes	Yes
	Moronidae	-	-	No	-
	Osmeriformes	-	-	No	-
	Osteoglossiformes	-	Yes	Yes	Yes
	Perciformes	Yes	Yes	Yes	Yes
	Pleuronectiformes	Yes	Yes	Yes	Yes
	Pomacentridae	Yes	Yes	Yes	Yes
	^b Salmoniformes	Yes	Yes	Yes	Yes
	Sciaenidae	Yes	Yes	Yes	Yes
	Semionotiformes	Yes	Yes	Yes	Yes
	Siluriformes	Yes	Yes	Yes	Yes
	Spariformes	-	-	No	-
	Synbranchiformes	Yes	-	Yes	Yes
	Syngnathiformes	Yes	Yes	Yes	Yes
	Tetraodontiformes	Yes	Yes	Yes	Yes

^aThe query species used in each SeqAPASS evaluation, zebrafish (*Danio rerio*), belongs to the Order Cypriniformes.

^bRainbow trout (*Oncorhynchus mykiss*) and lake trout (*Salvelinus namaycush*) belong to the Order Salmoniformes.

Grey box with “Yes” indicates sequence conservation compared to the zebrafish 17 β HSD query sequence; Black box with “No” indicates that the sequence was not conserved; Dashed line indicates that no sequence from that Order aligned with the zebrafish 17 β HSD query sequence.

Table 2.

SeqAPASS Level 3 comparison of critical amino acids in human 17 β HSD1 sequence information for the protein in bony fishes

NCBI Accession	Scientific Name	Common Name	17 β HSD1 Sequence	^a R38	N115	S143	Y156	K160	H222	E283
P14061.3	<i>Homo sapiens</i>	Human	Measured	R	N	S	Y	K	H	E
NP_991147.1	<i>Danio rerio</i>	Zebrafish	Measured	R	N	S	Y	K	H	L
XP_016427938.1	<i>Sinocyclocheilus rhinoceros</i>	none	Predicted	R	N	S	Y	K	H	L
XP_016344065.1	<i>Sinocyclocheilus anshuiensis</i>	none	Predicted	R	N	S	Y	K	H	L
XP_016108943.1	<i>Sinocyclocheilus grahami</i>	none	Predicted	R	N	S	Y	K	H	L
XP_018954756.1	<i>Cyprinus carpio</i>	Common carp	Predicted	R	N	S	Y	K	H	L
AKP99413.1	<i>Carassius gibelio</i>	Silver crucian carp	Measured	R	N	S	Y	K	H	L
XP_007234532.1	<i>Astyanax mexicanus</i>	Mexican tetra	Measured	R	N	S	Y	K	H	F
XP_017576724.1	<i>Pygocentrus nattereri</i>	Red-bellied piranha	Predicted	R	N	S	Y	K	H	L
XP_017341929.1	<i>Ictalurus punctatus</i>	Channel catfish	Predicted	R	N	S	Y	K	H	L
AIZ03371.1	<i>Clarias batrachus</i>	Walking catfish	Measured	R	N	S	Y	K	H	F
XP_021480528.1	<i>Oncorhynchus mykiss</i>	Rainbow trout	Measured	R	N	S	Y	K	H	I
XP_014059789.1	<i>Salmo salar</i>	Atlantic salmon	Predicted	R	N	S	Y	K	H	I
XP_020340270.1	<i>Oncorhynchus kisutch</i>	Coho salmon	Measured	R	N	S	Y	K	H	I
AAR88433.1	<i>Anguilla japonica</i>	Japanese eel	Measured	R	N	S	Y	K	H	L
XP_020463062.1	<i>Monopterus albus</i>	Swamp eel	Measured	R	N	S	Y	K	H	L
XP_010737963.1	<i>Larimichthys crocea</i>	Large yellow croaker	Predicted	R	N	S	Y	K	H	I
XP_010873150.2	<i>Esox lucius</i>	Northern pike	Predicted	R	N	S	Y	K	H	I
XP_008294638.1	<i>Stegastes partitus</i>	Bicolor damselfish	Predicted	R	N	S	Y	K	H	L
XP_019950004.1	<i>Paralichthys olivaceus</i>	Japanese flounder	Predicted	R	N	S	Y	K	H	I
XP_018558025.1	<i>Lates calcarifer</i>	Barramundi perch	Predicted	R	N	S	Y	K	H	I
XP_004552340.1	<i>Maylandia zebra</i>	Zebra mbuna	Predicted	R	N	S	Y	K	H	I
XP_019722057.1	<i>Hippocampus comes</i>	Tiger tail seahorse	Predicted	R	N	S	Y	K	H	L
XP_014188513.1	<i>Haplochromis burtoni</i>	Burton's mouthbrooder	Predicted	R	N	S	Y	K	H	I
XP_020488333.1	<i>Labrus bergylta</i>	Ballan wrasse	Measured	R	N	S	Y	K	H	I
XP_022061236.1	<i>Acanthochromis polyacanthus</i>	Spiny chromis	Measured	R	N	S	Y	K	H	I
XP_022595311.1	<i>Seriola dumerili</i>	Greater amberjack	Measured	R	N	S	Y	K	H	I
NP_001266724.1	<i>Oreochromis niloticus</i>	Nile tilapia	Measured	R	N	S	Y	K	H	I
XP_005726130.1	<i>Pundamilia nyererei</i>	Cichlids	Predicted	R	N	S	Y	K	H	I
XP_008328249.1	<i>Cynoglossus semilaevis</i>	Tongue sole	Predicted	R	N	S	Y	K	H	I
XP_017284948.1	<i>Kryptolebias marmoratus</i>	Mangrove rivulus	Predicted	R	N	S	Y	K	H	V
XP_003964840.1	<i>Takifugu rubripes</i>	Torafugu	Predicted	R	N	S	Y	K	H	I

NCBI Accession	Scientific Name	Common Name	17 β HSD1 Sequence	^a R38	N115	S143	Y156	K160	H222	E283
XP_012727483.1	<i>Fundulus heteroclitus</i>	Mummichog	Predicted	R	N	S	Y	K	H	I
XP_010770523.1	<i>Notothenia coriiceps</i>	Black rockcod	Predicted	R	N	S	Y	K	H	T
XP_015804147.1	<i>Nothobranchius furzeri</i>	Turquoise killifish	Predicted	R	N	S	Y	K	H	I
XP_005796759.2	<i>Xiphophorus maculatus</i>	Southern platyfish	Predicted	R	N	S	Y	K	H	I
XP_015241432.1	<i>Cyprinodon variegatus</i>	Sheepshead minnow	Predicted	R	N	S	Y	K	H	I
XP_014824958.1	<i>Poecilia mexicana</i>	Shortfin molly	Predicted	R	N	S	Y	K	H	I
XP_014896330.1	<i>Poecilia latipinna</i>	Sailfin molly	Predicted	R	N	S	Y	K	H	L
XP_007558204.1	<i>Poecilia formosa</i>	Amazon molly	Predicted	R	N	S	Y	K	H	I
XP_013877431.1	<i>Austrofundulus limnaeus</i>	New World rivulines	Predicted	R	N	S	Y	K	H	I
XP_004071345.1	<i>Oryzias latipes</i>	Japanese medaka	Measured	R	N	S	Y	K	H	I
XP_008415271.1	<i>Poecilia reticulata</i>	Guppy	Predicted	R	N	S	Y	K	H	I
XP_015217570.1	<i>Lepisosteus oculatus</i>	Spotted gar	Predicted	R	N	S	Y	K	^b	-

Abbreviations: NCBI = National Center for Biotechnology Information; R=arginine, N=asparagine, S=serine, Y=tyrosine, K=lysine, H=histidine, E=glutamic acid, L=leucine, F=phenylalanine, I=Isoleucine, V=valine, T=threonine.

^a Amino acid residues and positions from human 17 β HSD1 (P14061) identified as critical amino acids (He 2016)

^b No corresponding amino acid residue found

Table 3.

In vitro evaluation of E1 metabolism and binding affinity

Fish ^a	Sex	Body Weight (g)	HSI ^b (%)	Cellular Fraction	METABOLISM				BINDING			
					E1 pmoles/sample Mean ^g	E2 pmoles/sample Mean ^g	E1 % ^c of Nominal Dose	E1 + E2 % ^d of Nominal Dose	$\frac{[E2]}{[E1] + [E2]}$ (%) ^e Mean ^g	E1 IC50 (nM) Mean ^h	E2 IC50 (nM) Mean ^h	E1Mean ^f RBA (%)
RT-1	F	400	1.35	Cytosol	2473 (443)	26 (4)	96	97	1.1 (0.4)	141 (110–900)	9 (6–13)	6.4
				Nuclear	2989 (117)	0 (0)	116	116	0.0 (0.0)	250 (94–689)	15 (9–24)	5.8
RT-2	F	608	0.89	Cytosol	1979 (17)	226 (2)	77	86	15.7 (0.8)	58 (46–73)	8 (6–11)	14.2
RT-3	M	857	0.88	Cytosol	1891 (587)	467 (12)	74	96	31.9 (2.4)	25 (18–35)	6 (4–11)	25.0
RT-4	F	322	1.24	Cytosol	1827 (326)	416 (110)	77	87	35.0 (3.9)	nt nt	nt nt	nt
RT-5	M	404	0.91	Cytosol	1857 (14)	510 (100)	72	92	42.9 1.8	26 (15–45)	7 (4–13)	27.7
RT-6	M	350	1.22	Cytosol	1702 (103)	1464 (6)	66	123	46.3 (1.4)	17 (11–25)	8 (6–11)	45.6
RT-7	F	317	1.07	Cytosol	1740 (82)	710 (71)	68	95	52.5 (3.5)	nt nt	nt nt	nt
RT-8	F	567	0.90	Cytosol	1142 (254)	662 (122)	43	70	55.2 (1.5)	32 (15–71)	19 (12–29)	57.9
RT-9	F	368	1.20	Cytosol	1004 (192)	929 (80)	39	75	48.2 (2.6)	14 (4–45)	8 (5–13)	58.7
RT-10	F	608	0.97	Cytosol	888 (84)	1115 (90)	35	78	58.2 (1.5)	21 (14–31)	12 (7–20)	57.6
RT-11	F	289	1.14	Cytosol	1007 (88)	1323 (273)	39	91	68.9 (4.1)	nt nt	nt nt	nt
RT-12	F	483	0.99	Cytosol	658 (144)	348 (48)	26	39	34.8 (1.8)	12 (6–21)	8 (6–12)	67.5
				Nuclear	2587 (361)	0 (0)	101	101	0.0 (0.0)	260 (41–1615)	9 (6–13)	3.4
LT-1	F	1780	1.18	Cytosol	1662 (8)	406 (78)	65	80	47.7 (3.0)	nt nt	nt nt	nt
LT-2	M	4080	0.84	Cytosol	1208 (331)	226 (123)	47	56	72.5 (3.4)	nt nt	nt nt	nt

^aRT=rainbow trout, LT=lake trout

^bHepatosomatic index (HSI)

^cDose recovery-20 hour E1 pmoles/sample divided by the nominal dose of 2570 pmoles E1/sample*100

^dDose recovery-20 hour E1 pmoles/sample + E2 pmoles/sample divided by the nominal dose of 2570 pmoles E1/sample*100

^eContribution of the molar E2 concentration relative to the sum of molar E1 and E2 concentrations measured after 20 h incubation at 4 °C, 10 µM initial dose. E2 µM concentrations divided by the sum of E1 µM + E2 µM *100

^fNo binding of solvent carrier (ethanol) to the rER was detected in any of the experiments.

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^gValues within parenthesis are standard deviation, N=2

^hValues within parenthesis are 95% confidence intervals, N=2 nt=not tested

Table 4.

Chemical analysis cytosol dosed with E1

Fish	Nominal [E1] (μM)	$\frac{[E2]}{[E1 + E2]}$ ^a (%)	E1 + E2 ^b % of nominal dose	Measured [E2] (μM)
RT-9	0.1	ND	ND	-
	0.5	100.0	11.4	0.057
	1.0	81.9	21.8	0.16
	5.0	65.0	57.0	1.8
	7.5	55.2	70.9	2.9
	10.0	48.2	75.3	3.6
	50.0	11.4	111.5	6.3
	100.0	5.7	109.2	6.2

^a E2 20 h concentration as a percentage of the 20 h E1 + E2 concentrations sum^b Dose recovery-20 hour E1 + E2 concentrations divided by the nominal 10