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Gene Expression Profiling for Understanding Chemical Causation of Biological Effects for Complex Mixtures: A Case Study on Estrogens

AMY L. FILBY,* EDUARDA M. SANTOS, KAREN L. THORPE,† GERD MAACK, AND CHARLES R. TYLER

School of Biosciences, The Hatherly Laboratories, University of Exeter, Prince of Wales Road, Exeter, EX4 4PS, U.K.

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Gene expression profiling offers considerable potential for identifying chemical causation of effects induced in exposures to complex mixtures, and for understanding the mechanistic basis for their phenotypic effects. We characterized gene expression responses in livers and gonads of fathead minnow (*Pimephales promelas*) exposed (for 14–21 days) to estrogenic wastewater treatment works final effluents with varying potencies and assessed the extent to which these expression profiles mapped with those induced by individual steroid estrogens present in the effluents (17β-estradiol and 17α-ethinylestradiol) and, thus, were diagnostic of estrogen exposure. For these studies, we adopted a targeted approach (via real-time PCR) with a suite of 12 genes in liver and 21 genes in gonad known to play key roles in reproduction, growth and development (processes controlled by estrogens) and responses were compared with effects on phenotypic end points indicative of feminization. Gene responses to effluent were induced predominantly in a linear (monotonic) concentration-dependent manner but were complex with many genes responding differently between tissue types and sexes. The gene expression profiles for the estrogenic effluents and the individual steroid estrogens had many common features. There were marked differences in the profiles between the two effluents, however, that were not explained by differences in their estrogenic potencies, suggesting that these may have arisen as a consequence of differences in the contents of other chemicals, which may act directly or indirectly with the estrogen-response pathway to alter estrogen-induced gene expression. These data demonstrate that the patterns of gene expression induced by estrogenic effluents, although complex, can be diagnostic for some of the estrogens they contain and provide insights into the mechanistic basis for the phenotypic effects seen.

Introduction

There is heightened concern worldwide about the impacts of endocrine disrupting chemicals (EDCs) present in the aquatic environment that can alter physiological function in wildlife and humans (reviewed in (*1*)). Effluents from wastewater treatment works (WwTWs) are a major point source of EDCs into the aquatic environment, and exposure has been associated with a range of reproductive abnormalities, particularly in fish, including abnormal induction of the female yolk protein precursor vitellogenin (VTG) in males (*2*), a high incidence of intersex (*3*), lowered hormone levels (*4*), and reduced fertility (*5, 6*). WwTW effluents are highly complex mixtures of thousands of chemicals but their feminizing effects have largely been associated with chemicals that act through estrogen-signaling pathways, most notably natural and synthetic steroid estrogens (particularly estrone (E_1), 17β-estradiol (E_2) and 17α-ethinylestradiol (EE_2) (*7*). Recently, it has also been shown that these environmental estrogens are also, in part, responsible for further observed effects of effluents on wider aspects of health in exposed organisms, which include genotoxicity, immunotoxicity, and altered metabolism (*8*).

Gene expression profiling offers potential for identifying chemical causation of effects induced in exposures to real-world chemical mixtures, like effluents, and for determining the mechanistic basis for their phenotypic effects. For example, in relation to estrogens, studies in both mammals (*9, 10*) and fish (*11, 12*) have demonstrated that different estrogens share extensive commonalities in the gene expression fingerprints/profiles they induce, and that these are distinct from the fingerprints/profiles induced by chemicals with other modes of action (MOA) and, therefore, diagnostic of estrogen exposure. The extent to which the gene expression profiles induced by estrogenic effluents (which contain many pollutants with many MOAs) will reflect those induced by the estrogens they contain, however, has not been established.

Recent studies that have compared transcriptomic responses to various chemicals, including EE_2, dosed individually and in simple combinations (*13, 14*), have illustrated the complexities in the use of gene expression profiling for studying chemical mixtures, like effluents. For example, in the work of Finne et al. (*13*), exposure of trout hepatocytes to EE_2 in combination with the dioxin TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), the oxidative stressor paraquat and the mutagen 4-nitroquinoline-1-oxide lead to a 60% loss of the transcriptomic signature elicited by EE_2 alone and revealed combination effects that were not predicted by the results for the individual chemicals. Given the high number of chemicals effluents typically contain, there is a high likelihood for complex interactions and this complicates the application of gene expression profiling for understanding the mechanisms of mixture effects.

In this work, we carried out experiments to characterize gene expression responses to estrogenic WwTW effluents with different potencies in liver and gonad tissues and assessed the extent to which these expression profiles were similar to those induced by individual steroid estrogens present in the effluents and, thus, were diagnostic of estrogen exposure. For these studies, we adopted a targeted approach (via real-time PCR) with a suite of 12 genes in liver and 21 genes in gonad known to play key roles in reproduction, growth and development (processes that are regulated by estrogens). The studies were conducted using a toxicological model fish species, the fathead minnow (*Pimephales promelas*), and the responses in gene expression were compared with effects on phenotypic end points indicative of feminization (reported in *15*).

* Corresponding author phone: +44(0)1392 263752; fax: +44 (0)1392 263700; e-mail: a.l.filby@exeter.ac.uk.

† Current address: University of Basel, Programme MGU, Vesalgasse 1, CH-4051 Basel, Switzerland.

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Materials and Methods

Experimental Designs and Fish Sampling. The analyses conducted in this study used tissue generated in experiments described in full elsewhere (*15*). Briefly, in those experiments, sexually maturing (>150 days post hatch) male and female fathead minnow were exposed to two WwTW final effluents, one strongly estrogenic (in experiment 1, "effluent 1"); and the other moderately estrogenic (in experiment 2, "effluent 2") (for further details of the effluents used, see Supporting Information Table S1), or to individual steroid estrogens known to be present in WwTW effluents (in experiment 1, E_2 at 14 ng/L; in experiment 2, EE_2 at 18 ng/L) such that the gene expression profiles (relative to those of fish exposed to dilution–water only) could be compared. To enable concentration-related responses to effluent to be established for the study genes, in experiment 2 exposures were carried out to a series of graded effluent concentrations (25 and 50%, in addition to the 100% concentration). In each experiment, duplicate tanks of fish (eight males and eight females per tank) were exposed for each treatment and the exposures were run for 14 days (experiment 1) or 21 days (experiment 2) after which all fish were sacrificed by a lethal dose of anesthesia (500 mg/L MS-222 buffered to pH 7.4; Sigma, Poole, U.K.) for quantification of the phenotypic and gene expression end points.

Chemical Analyses. To confirm the estrogenic potencies and concentrations of individual steroidal estrogens (E_1 and E_2) in the effluents used, and the actual concentrations of the individual steroidal estrogen treatments (E_2 in experiment 1; EE_2 in experiment 2), chemical analyses were conducted which are described in full elsewhere (*15*). Briefly, water samples were collected regularly during the exposure periods from the effluent storage tank and from each exposure tank (pooled for the two replicate tanks for each treatment) (see ref *15* for details of sampling days) and extracted onto primed C_{18} solid-phase cartridges. Overall estrogenic potencies (E_2 equivalents) were determined using the recombinant yeast estrogen screen and E_1, E_2 and EE_2 concentrations were determined using GC-MS.

Phenotypic Effect Analyses. To assess for feminizing effects of the treatments, the following end points were measured: plasma VTG, development of the secondary sex characters (nuptial tubercles and dorsal fatpad) in males, and gonadosomatic index (GSI; a measure of gonadal weight as a direct proportion of the somatic weight), as described in full elsewhere (*15*).

Gene Expression Analyses. For the gene expression analyses, dissected gonads and livers from individual fish from a single replicate tank per treatment were snap frozen in liquid nitrogen and stored at −80 °C until use. Total RNA was extracted independently from the tissue samples (from eight and six fish of each sex for experiments 1 and 2, respectively), DNase-treated, and reverse transcribed to cDNA as previously described (*16*). Real-time quantitative PCR using SYBR Green chemistry was then performed in triplicate for each sample with the iCycler iQ Real-time Detection System (Bio-Rad Laboratories Inc., Hercules, CA) using previously established methods (*16*) for a suite of 12 genes in liver and 21 genes in gonad involved in reproduction, growth and development (liver: *estrogen receptor 1* (*esr1*), *estrogen receptor 2a* (*esr2a*), *estrogen receptor 2b* (*esr2b*), *androgen receptor* (*ar*), *growth hormone receptor* (*ghr*), *insulin-like growth factor 1* (*igf1*), *insulin-like growth factor 1 receptor* (*igf1r*), *thyroid hormone receptor α* (*thra*), *thyroid hormone receptor β* (*thrb*), *glucocorticoid receptor* (*gr*), *vitellogenin* (*vtg*), *cytochrome P450 1A* (*cyp1a*); gonad: *esr1*, *esr2a*, *esr2b*, *ar*, *cytochrome* P450 17 (*cyp17*), *cytochrome P450 19a* (*cyp19a*), *cytochrome P450 19b* (*cyp19b*), *steroidogenic acute regulatory protein* (*star*), *hydroxysteroid 11β-dehydrogenase* (*hsd11b*), *hydroxysteroid 17β-dehydrogenase* (*hsd17b*), *anti-Mullerian hormone* (*amh*), *vasa homologue* (*vasa*), *doublesex and mab-3 related transcription factor 1* (*dmrt1*), *nuclear receptor subfamily-5 group A member 2* (*nr5a2*; formerly known as *steroidogenic factor 1* (*sf1*)), *growth hormone* (*gh*), *ghr*, *igf1*, *igf1r*, *thra*, *thrb*, *gr*).

Relative expression levels (gene of interest: 'housekeeping' gene) were determined using a development of the arithmetic comparative $2^{-\Delta\Delta Ct}$ method (*17*) which includes efficiency correction (*18*). The "housekeeping" gene 18S rRNA was used for relative quantification because its expression did not change following any of the treatments in the tissues studied (see Supporting Information Figure S1). Real-time PCR primer sequences, product sizes, annealing temperatures, efficiencies, and accession numbers for each study gene can be found in Supporting Information Table S2.

Data Analyses. All data are shown as the mean ± SEM fold-increase in gene expression from the dilution–water control group. Statistical differences ($P < 0.05$) between treatments were assessed by one-way analysis of variance (ANOVA) followed by Dunn's post hoc test to identify differences compared to the water control, or a nonparametric alternative when the data failed to meet the criterias of normality and equal variance required for parametric analysis (SigmaStat 2.03; Jandel Scientific Software). Linear regression was performed to test for linear concentration–response relationships for the study genes to effluent 2 with a *P*-value cutoff of 0.05 (SigmaStat 2.03). Principal component analysis was performed to identify the main trends between individual gene expression profiles using GeneSpring 7.0 software (Silicon Genetics, U.S.).

Results

Chemical Analyses and Phenotypic Effects. Results for the chemical analyses and phenotypic effect measures for both the effluents (at 25, 50, and 100% concentrations), and individual estrogens are presented in detail elsewhere (*15*). Briefly, the estrogenic properties of effluents 1 and 2 were confirmed by yeast estrogen screen (38.2 ± 8.4 and 9.4 ± 5.1 ng E_2 equivalent/L, for effluents 1 and 2 respectively) and analytical chemistry of individual steroid estrogens (97.4 ± 22.2 and 6.3 ± 1.6 ng E_1/L, and 7.3 ± 1.7 and 6.3 ± 1.6 ng E_2/L, for effluents 1 and 2 respectively) (*15*). The concentrations of EE_2 were not measured in the effluents but have been previously measured as between <0.5–1.5 ng/L and <2.0 ng/L in effluents 1 and 2, respectively (see Supporting Information Table S1). The test concentrations of the individual steroid estrogen treatments (E_2, in experiment 1, and EE_2, in experiment 2) were confirmed by analytical chemistry as 13.8 ± 3.6 ng E_2/L and 18.0 ± 2.0 ng EE_2/L, respectively (*15*).

The phenotypic effects induced by the effluents were: induction of plasma VTG in males (effluent 1: from 25% concentration; effluent 2: from 50% concentration) and females (effluent 1: 100% concentration only; effluent 2: from 50% concentration), increased GSI in males (effluent 1: 50% concentration only; effluent 2: 100% concentration only), and altered GSI in females (decreased in a concentration-dependent manner by effluent 1, but increased by effluent 2, although only significantly so at 50% concentration) (*15*). Exposure to the individual steroid estrogens was associated with inductions of plasma VTG in both males and females (E_2 and EE_2), and reduced GSI in males (EE_2 only) and females (E_2 and EE_2) (*16*).

Overall Gene Expression Responses To Effluent. Both effluents (at 100% concentration) induced changes in the expression of multiple genes in the livers and gonads of exposed male and/or female fish after 14 days (effluent 1) or 21 days (effluent 2) of exposure (Figure 1). In male liver, 10 genes were altered by exposure to effluent 1 and 6 genes by effluent 2. In female liver, effluent 1 did not induce any changes in gene expression but seven genes were altered by

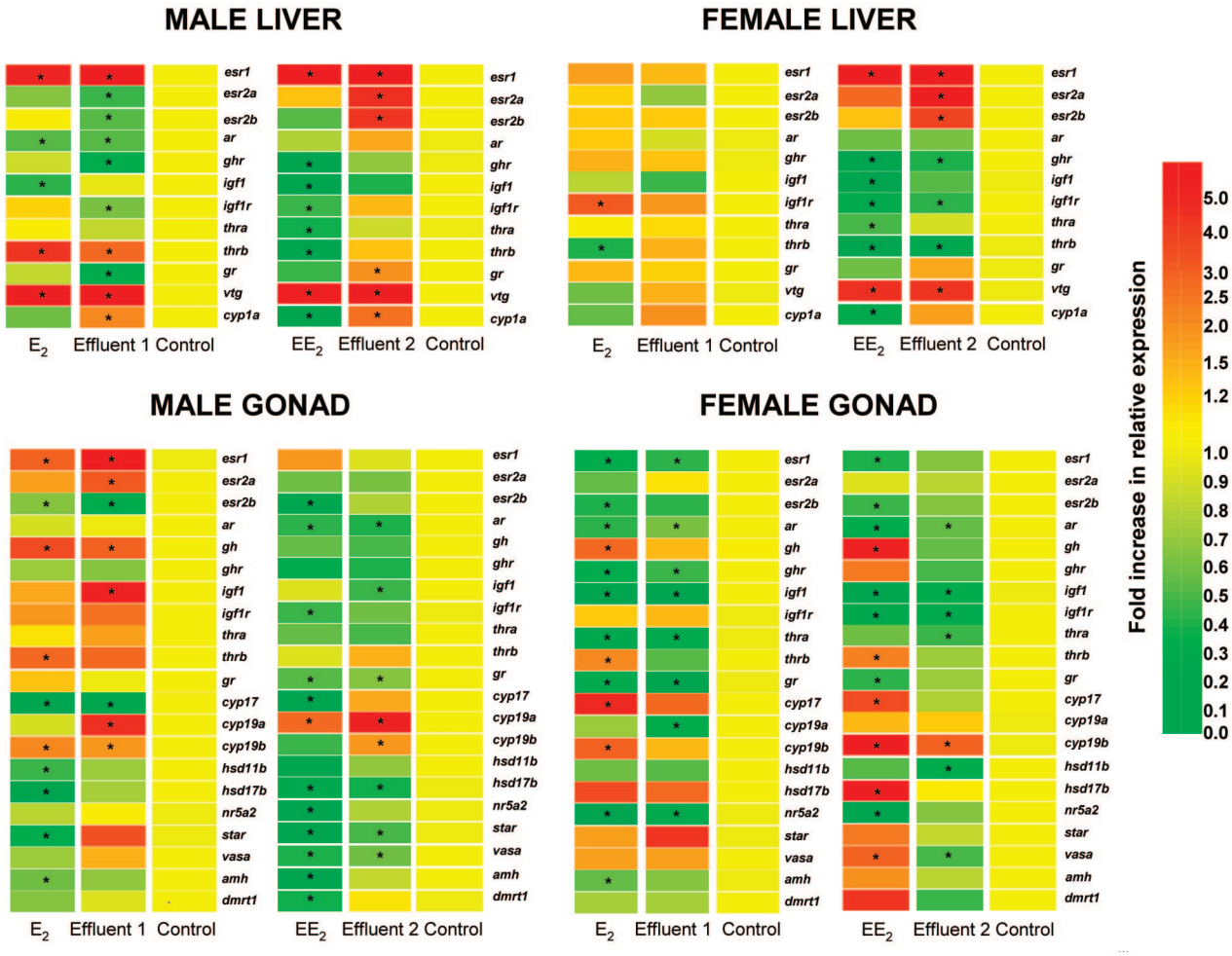

FIGURE 1. Heat maps showing gene expression responses to effluents (effluents 1 and 2, in experiments 1 and 2, respectively) and estrogens (E_2 at 14 ng/L and EE_2 at 18 ng/L, in experiments 1 and 2, respectively) in liver and gonad tissues of male and female fathead minnow. Results are represented as the mean fold increase in relative expression (gene of interest: 18S rRNA) from the water controls. Eight or six fish of each sex were analyzed per treatment after 14 or 21 days of exposure, for experiments 1 and 2 respectively. For each gene, statistically significant differences in expression from the water controls are denoted by an asterix ($P < 0.05$).

effluent 2. In male gonad, both effluents induced changes in the expression of eight genes in exposed fish while, in female gonad, effluents 1 and 2 altered the expression of eight and seven genes, respectively. Overall, more genes were down-regulated than up-regulated by the effluent treatments (16 and 15 genes down-regulated, compared to 10 and 13 genes up-regulated by effluents 1 and 2, respectively).

For each effluent, many of the genes responded differently between tissue types and/or sexes (Figure 1). For example, in males *esr2a* was down-regulated by effluent 1 in liver, but up-regulated in gonad, while the opposite was true for *gr* following exposure to effluent 2, and hepatic *cyp1a* was up-regulated by both effluents in males but unchanged in females.

Concentration–Response Relationships for Gene Expression Responses To Estrogenic Effluent. Overall, the number of genes whose expression was altered across the two tissues increased with increasing concentrations of effluent 2 (6 and 10 genes were induced by the 25 and 50% concentrations, respectively, compared with 28 at the 100% concentration). 79% (22/28) of the individual genes altered by the 100% concentration responded in a linear concentration-dependent manner (for examples, see Figure 2a), and the remainder in a nonlinear manner (Figure 2b). Additionally, there were five genes that were unchanged by exposure to 100% effluent, but were up- or down-regulated at lower effluent concentrations (Figure 2c).

Comparison of Gene Expression Profiles for Effluents with Different Estrogenic Potencies. Of the genes whose expression was altered across liver and gonad by the exposure to full-strength effluent 1 in experiment 1 (26 genes) and to effluent 2 in experiment 2 (28 genes), only eight were common to both effluents (five up-regulated and three down-regulated genes; Figure 3a). Of these genes, there was a notable difference in the magnitude of change between the two effluents only for *vtg*, *igf1* and *thra* (*vtg*: 3707-fold compared to 22-fold up-regulated in male liver; *igf1*: down-regulated to 18 and 33% of the control levels in female gonad; *thra*: down-regulated to 29 and 45% of the control levels in female gonad, for effluents 1 and 2, respectively). For the remaining genes, the magnitudes of induction/inhibition were similar for the two effluents. In contrast, 14 genes that were altered by the exposure to effluent 1 were not affected by that to effluent 2 (4 up-regulated and 10 down-regulated) and 16 genes that were altered by the exposure to effluent 2 were not affected by the exposure to effluent 1 (5 up-regulated and 11 down-regulated). Four additional genes were altered by both effluent exposures but in an opposite manner: *esr2a*, *esr2b* and *gr* were down-regulated by effluent 1 but up-regulated by effluent 2 in male liver, and *igf1* was up-regulated by effluent 1 but down-regulated by effluent 2 in testis. For lists of the genes regulated similarly and differently by effluent 1 and effluent 2 with the associated fold-changes and *P*-values, see Supporting Information Table S3.

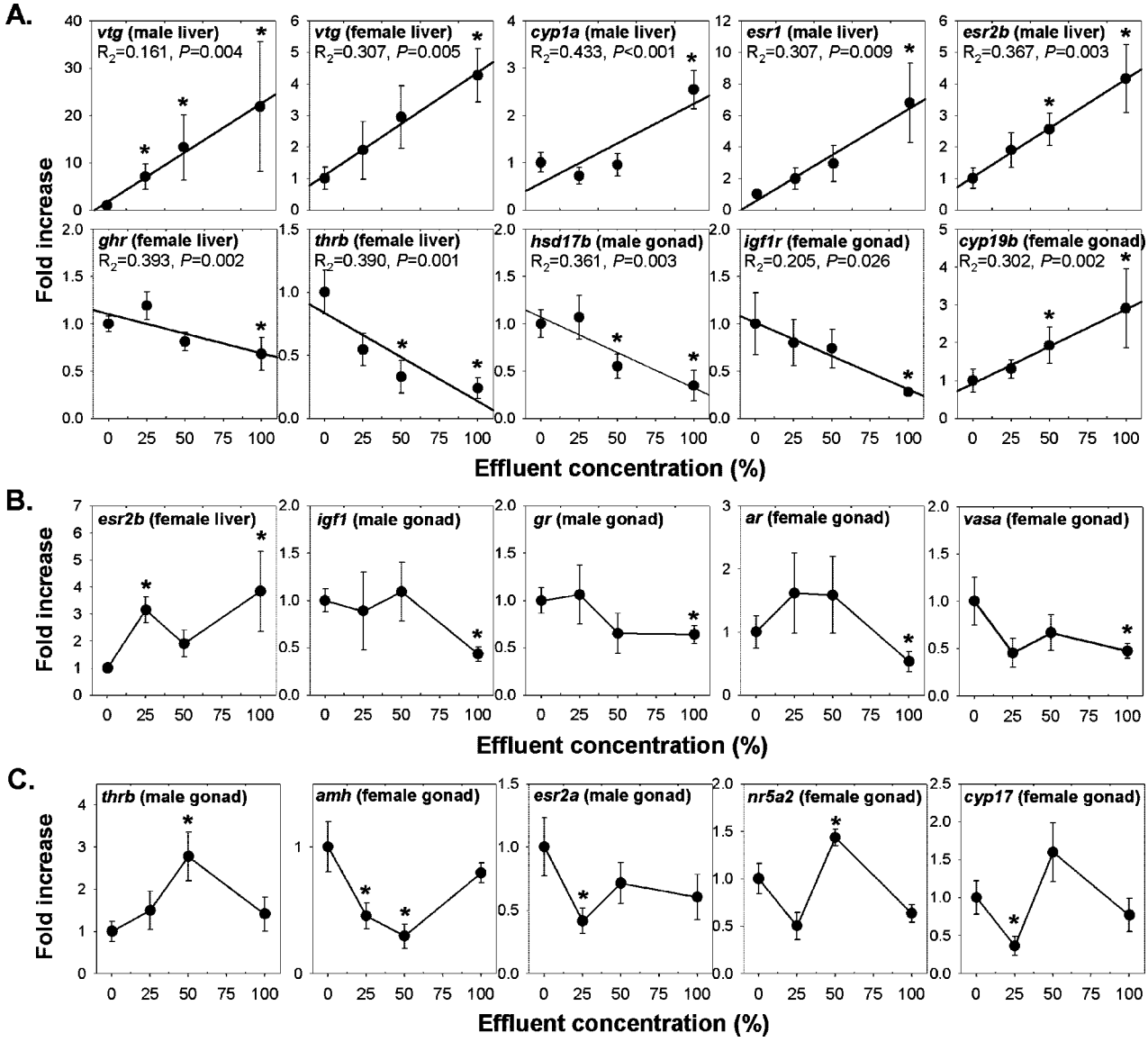

FIGURE 2. Differences in concentration–response relationships for gene expression responses to effluent (effluent 2; 25%, 50%, 100% concentrations) in liver and gonad of male and female fathead minnow. (A) Examples of genes with linear concentration–response relationships, (B) Genes that were significantly changed in their expression by 100% effluent but did not have linear concentration–response relationships, (C) Genes that were not significantly changed in their expression by effluent at 100% concentration but that responded at lower concentrations. Six fish of each sex were analyzed per treatment after 21 days of exposure. Results are represented as mean ± SEM fold increase in relative expression (gene of interest: 18S rRNA) from the water controls. Linear responses were assessed for using linear regression ($P < 0.05$). Statistically significant differences in expression from the water controls are denoted by an asterix ($P < 0.05$).

Comparison of Gene Expression Profiles Induced by the Steroid Estrogens. The gene expression profiles induced by the two steroid estrogens, E_2 (used in experiment 1) and EE_2 (used in experiment 2), have been presented independently previously (*19, 20*). In general, the gene expression profiles for the two estrogens were relatively similar. Of the genes whose expression was altered across liver and gonad by exposure to E_2 and EE_2, 18 were common to both estrogens (6 up-regulated and 12 down-regulated genes; Figure 3b). However, in total EE_2 altered the expression of a greater number of genes than E_2 (41 genes compared to 30 genes were altered by EE_2 and E_2, respectively) and typically with a greater magnitude of effect. There were only two genes for which E_2 and EE_2 had opposite effects (*thrb* in male liver and *igf1r* in female liver were up-regulated by E_2 but down-regulated by EE_2). For lists of the genes regulated similarly and differently by E_2 and EE_2 with the associated fold-changes and *P*-values, see Supporting Information Table S4).

Comparison of Gene Expression Profiles Induced by the Effluents and the Steroid Estrogens. In experiment 1 (which used E_2 as the test estrogen and effluent 1 as the test effluent), 50% (6/12) of the genes up-regulated by the estrogen were also up-regulated by the effluent and 56% (10/18) of the genes down-regulated by the estrogen were also down-regulated by the effluent (Figure 4a). In experiment 2 (which used EE_2 as the test estrogen and effluent 2 as the test effluent), 55% (6/11) of the genes up-regulated by the estrogen were also up-regulated by the effluent and 37% (11/30) of the genes down-regulated by the estrogen were also down-regulated by the effluent (Figure 4a), There were only two genes in one of the experiments for which the estrogen and the effluent had opposite effects (in experiment 2, *cyp1a* in male liver was up-regulated by effluent 2, but down-regulated by EE_2, whereas the opposite was true for *vasa* in female gonad).

Further comparisons (within each experiment) of the gene expression profiles for individual fish exposed to the water

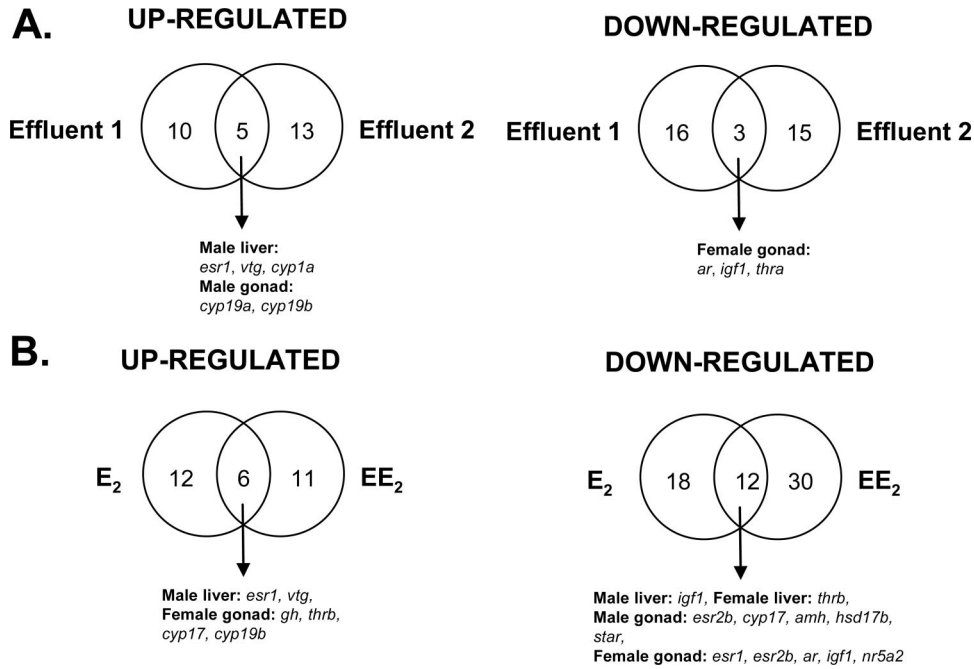

FIGURE 3. Venn diagrams obtained from comparisons of the genes significantly ($P < 0.05$) up- and down-regulated by (A) effluent 1 (in experiment 1) and effluent 2 (in experiment 2) and (B) E_2 at 14 ng/L (in experiment 1) and EE_2 at 18 ng/L (in experiment 2) across liver and gonad tissues of exposed male and female fathead minnow. Eight or six fish of each sex were analyzed per treatment after 14 or 21 days of exposure, for experiments 1 and 2 respectively.

control, the effluent and the estrogen using principal component analysis (Figure 4b) generally clustered all individuals according to treatment and identified differences between treatments within each experiment. In both experiments, the gene expression profiles of the effluent-exposed fish clustered more closely with those of the fish exposed to the steroid estrogens than with the water controls and this was especially true for the analyses on gonad. A further finding was that the gene expression profiles of the fish exposed to EE_2 were more closely clustered with each other than for the fish exposed to the other treatments.

Discussion

This study represents the first application of gene expression profiling in fish to characterize responses to estrogenic WwTW effluents. We have shown that the gene expression profiles induced by estrogenic effluents, although complex, can be diagnostic for the estrogens they contain and can be interrogated to provide insights into the mechanistic basis for phenotypic effects observed.

General Characteristics of Gene Expression Responses To the Effluents. The data presented demonstrate that expression of key genes involved in reproduction, growth, and development is altered in multiple tissues of fathead minnow exposed to estrogenic WwTW effluents. The complexity observed in these responses (with most genes showing differential responses between tissues and/or sexes) is in agreement with transcriptomic responses of fish to a range of pollutants (e.g., refs *21* and *22*) and highlights potential differences in the responses of males and females to effluents, and in the chemical sensitivities of different body tissues.

The responses in gene expression on exposure to effluent were, however, predominantly elicited in a linear (monotonic) concentration-dependent manner, which suggests that a simple "single dose" approach may be appropriate for use in identifying gene expression responses to effluents and could be used to predict responses at lower or higher effluent concentrations. Furthermore, although most of the study genes were only affected by effluent at full-strength, the concentration–response analyses highlight the greater sensitivity of gene expression end points compared to most phenotypic end points for assessing responses to effluents. The nonmonotonic responses for a few of the genes studied (e.g., *esr2a* and *thrb* in testis, and *amh*, *nr5a2*, and *cyp17* in ovary, which responded only at the lower effluent concentrations) likely reflect differences in their normal physiological roles, and positions within regulatory networks, compared with the other study genes. For example, the regulation of *nr5a2* and *cyp17* at low concentrations may reflect a compensatory mechanism of the steroidogenic pathway which at high concentrations may have little effect.

Gene Expression Profiling for Diagnosing Estrogen Responses in Complex Mixtures. To determine whether the gene expression profiles induced by the effluents were diagnostic for the estrogens they contained, we compared their effects with those of individual steroid estrogens known to be present in the effluents. These analyses were conducted within each experiment due to the slightly different exposure durations used for the two experiments. Despite the different exposure durations, the gene expression profiles for the two estrogens used (E_2 in experiment 1 and EE_2 in experiment 2) shared a reasonably high degree of similarity in the tissues examined, as observed previously for steroid estrogens (*9*–*12*). The differences observed (most notably the greater overall effect of EE_2) likely relate to the greater (11–30-fold) in vivo potency of EE_2 compared with E_2 (e.g., ref *24*), and the differences in the test concentrations (14 ng/L for E_2 and 18 ng/L for EE_2, the latter of which induced a 7.5-fold greater vitellogenic response (*15*)), but possible differences in their MOAs (due to the subtle differences in their chemical structures), as well as the different exposure periods employed, may also have contributed to the differences seen.

Strong similarities in the gene expression profiles for the effluents and steroid estrogens suggest gene expression profiling can be used to diagnose exposure to estrogens, even when they are present within highly complex mixtures, like effluents. This is in agreement with the previous finding that the gene expression profiles for

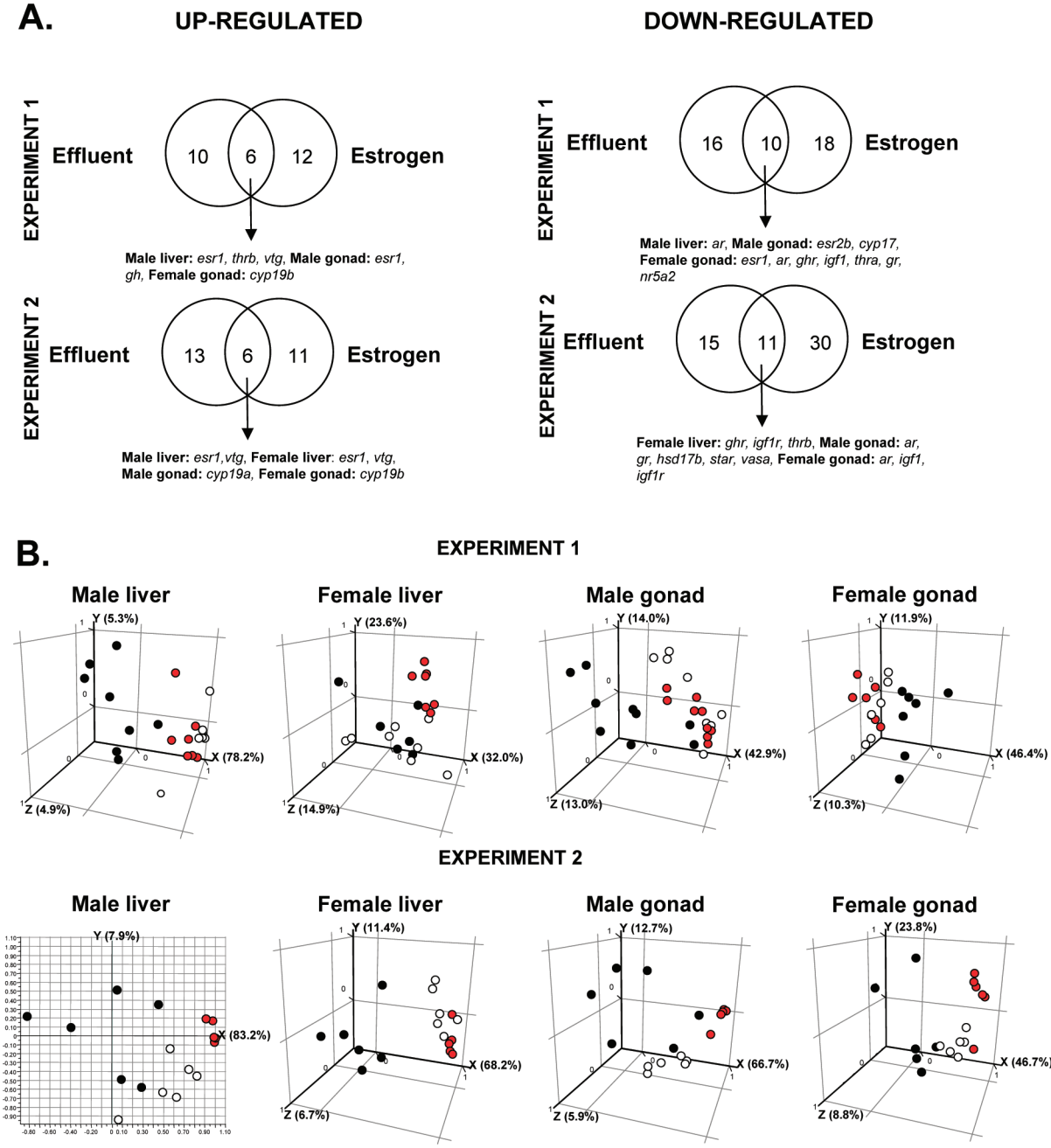

FIGURE 4. Comparison of gene expression responses to effluents with those to steroid estrogens in exposed fathead minnow. (A) Venn diagrams obtained from the comparisons of the genes significantly ($P < 0.05$) up- and down-regulated by effluent (experiment 1: effluent 1; experiment 2: effluent 2) and estrogen (experiment 1: E_2 at 14 ng/L; experiment 2: EE_2 at 18 ng/L) across liver and gonad tissues of males and females. (B) Principal component analysis of all genes quantified in liver and gonad tissues of males and females comparing the responses of the control fish (black) with fish exposed to effluent (white; experiment 1: effluent 1; experiment 2: effluent 2) or estrogen (red; experiment 1: 14 ng E_2/L; experiment 2: 18 ng EE_2/L). The percentage of variance associated with each principal component is stated on each axis (X-, Y-, and Z-axis for principal components 1, 2, and 3, respectively). Eight or six fish of each sex were analyzed per treatment after 14 or 21 days of exposure, for experiments 1 and 2 respectively.

estrogenic pulp mill effluents and individual estrogens (particularly E_2 and the phytoestrogen β-sitosterol) in human ER-positive MCF-7 breast cancer cells were closely related (*25*). The genes altered similarly by the effluents and steroid estrogens included many genes that have been previously characterized as estrogen-responsive in other studies, such as the ER subtype *esr1* (up-regulated in liver (*26*)), hepatic *vtg* (up-regulated (*26*)), the *cyp19* genes which code for the estrogen-synthesizing enzyme P450 aromatase (up-regulated (*27*)), *ar* (down-regulated (*23*)), and the steroidogenic enzyme genes *cyp17*, *hsd17b* and *star* (down-regulated in males, in which they play key roles in androgen production (*28*)). Other changes in gene expression induced by the effluents and the estrogens, including the up-regulations of *gh* in testis, which is believed to exert a local autocrine/paracrine role in the onset of spermatogenesis (*29*), and the thyroid hormone receptor *thrb* in liver of males, which plays important roles in the regulation of growth and metabolism (*30*), have, however, not been previously characterized as responses to estrogens.

Some genes whose expression was altered by the steroid estrogens were unchanged in fish exposed to the effluents. These genes included the somatotropic hormone *igf1* (which was down-regulated by E_2 and EE_2 in liver), the steroidogenic

enzymes *cyp17* and *hsd17b* (which were up-regulated by E_2 and/or EE_2 in ovary) and *hsd11b* (which was down-regulated by E_2 in testis), and the sex differentiation genes *amh* and *dmrt1* (which were down-regulated by E_2 and/or EE_2 in testis). The lack of effect of the estrogens in the effluents on these genes may result from antagonistic effects of other chemicals present in the effluents and/or the fact that the test concentration adopted for EE_2 was at the very top end of the concentration range in which it is found in European effluents (*7, 31, 32*). Indeed, the EE_2 treatment had a greater effect on gene expression than the effluents (both overall, as shown by the principal component analyses where variation between individuals was greatly reduced in EE_2-treated fish, and on individual genes (e.g., *esr1*, *vtg*), in terms of magnitude of effect), while this was not observed for the E_2 treatment, the test concentration of which was analogous in terms of overall estrogenic potency to that measured in effluent 1.

The greatest differences of the effluents compared to the individual estrogens on gene expression were their opposite effects on the hepatic phase I biotransformation enzyme *cyp1a* (up-regulated by both effluents in males but down-regulated by EE_2 in both males and females) and the germ cell-specific RNA helicase *vasa* in ovary (down- and up-regulated by effluent 2 and EE_2, respectively). For *cyp1a*, the lack of an inhibitory effect of estrogen when present in effluent (an effect well characterized for estrogens when dosed individually (e.g., ref *33*)) may be explained by a strong induction of this gene by AhR agonists, such as PCBs, PAHs, and dioxins, also likely to be present in the effluents, since *cyp1a* is a gene under positive control of the AhR (reviewed in ref *34*). Indeed, estrogens do not appear to completely antagonize the *cyp1a*-inducing properties of AhR agonists in effluents unless they are present at high concentrations (*8*).

Gene Expression Profiling for Assigning the Estrogenic Potency of Effluents. Although the data demonstrate the ability of gene expression profiling to diagnose estrogenic effects of effluents, the relative estrogenic potencies of the effluents were not generally signaled by differences in the numbers of genes they induced, or in the magnitudes of their responses (exceptions were for *vtg*, *igf1*, and *thra*, the expression of which was altered to a greater magnitude by the more estrogenic effluent). We cannot rule out the possibility that the differences in the exposure durations for effluents 1 and 2 may provide an explanation for some of the differences observed in their gene expression profiles. However, it is likely that the many of differences observed resulted from differences between the two effluents in their contents (and/or concentrations) of other chemicals (aside from estrogens), which may act, directly or indirectly, on estrogen-response pathways to alter estrogen-induced gene expression. In addition, since different estrogens are known to have differential binding affinities and transactivational potencies for different ER subtypes (e.g., ref *35*), the differential ER induction by the effluents (in liver all three ER genes were induced by effluent 2, but only *esr1* was induced by effluent 1) may reflect a broader range of estrogens in effluent 2 which may have also contributed to the differences in transcriptomic effect observed for the two effluents.

Gene Expression for Understanding the Mechanistic Basis for the Phenotypic Effects of the Effluents. Comparisons of the gene expression profiles for the two different effluents, and for the effluents and steroid estrogens, could potentially help identify the mechanistic basis for the differences observed in the phenotypic effects of the different treatments. For example, both the effluents had stimulatory effects, while EE_2 had an inhibitory effect, on gonad growth in males. This inhibitory effect exclusive to EE_2 may relate to its inhibitory effect on some of the genes involved in testicular development and function in males, notably *nr5a2* (*36*), *amh* (*37*), and *dmrt1* (*38*), since these genes were unchanged in the testis of males exposed to the effluents. As a further example, ovarian growth was inhibited in a concentration-related manner by effluent 1, but stimulated by effluent 2 (at 50% concentration), and the molecular responses of the ovary to effluent 1 included inhibitions of *esr1*, *ghr*, *gr*, *cyp19a*, and *nr5a2*. In contrast, *esr1*, *ghr*, *gr*, and *cyp19a* were unchanged in the ovaries of fish exposed to 50% effluent 2, but *nr5a2* was up-regulated and *amh* was down-regulated exclusively at the lower concentrations of this effluent. Indeed, *esr1*, *ghr*, *gr*, *cyp19a*, and *nr5a2* are all known to have stimulatory roles in oocyte development and ovarian growth, so their inhibition by effluent 1 may result in reduced ovarian growth. In contrast, *amh* is a negative regulator of ovarian development in adult females (*37*), so down-regulation of this gene by effluent 2 may promote ovarian growth.

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Supporting Information Available

Validation of 18S rRNA as a suitable "housekeeping" gene for use in normalization of the real-time PCR data (Figure S1), details of the effluents used in the studies (Table S1), real-time PCR assays (Table S2) and lists comparing genes up- and down-regulated by effluent 1 and effluent 2 (Table S3) and the individual estrogens E_2 and EE_2 (Table S4).

Literature Cited

(1) Tyler, C. R.; Jobling, S.; Sumpter, J. P. Endocrine disruption in wildlife: a critical review of the evidence. *Crit. Rev. Toxicol.* **1998**, *28*, 319–361.

(2) Purdom, C. E.; Hardiman, P. A.; Bye, V. J.; Eno, N. C.; Tyler, C. R.; Sumpter, J. P. Oestrogenic effects of effluents from sewage treatment works. *Chem. Ecol.* **1994**, *8*, 275–285.

(3) Jobling, S.; Nolan, M.; Tyler, C. R.; Brighty, G.; Sumpter, J. P. Widespread sexual disruption in wild fish. *Environ. Sci. Technol.* **1998**, *32*, 2498–2506.

(4) Folmar, L. C.; Denslow, N. D.; Rao, V.; Chow, M.; Crain, D. A.; Enblom., J.; Marcino, J.; Guillette, L. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environ. Health Perspect.* **1996**, *104*, 1096–1101.

(5) Jobling, S.; Beresford, N.; Nolan, M.; Rodgers-Gray, T.; Brighty, G. C.; Sumpter, J. P.; Tyler, C. R. Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biol. Reprod.* **2002**, *66*, 272–281.

(6) Jobling, S.; Coey, S.; Whitmore, J. G.; Kime, D. E.; Van Look, K.J.W.; McAllister, B. G.; Beresford, N.; Henshaw, A. C.; Brighty, G.; Tyler, C. R.; Sumpter, J. P. Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biol. Reprod.* **2002**, *67*, 515–524.

(7) Desbrow, C.; Routledge, E. J.; Brighty, G. C.; Sumpter, J. P.; Waldock, M. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environ. Sci. Technol.* **1998**, *32*, 1549–1558.

(8) Filby A. L., Neuparth T., Thorpe K. L., Owen R., Galloway T. S., Tyler C. R. Health effects of estrogens in the environment, considering complex mixture effects. *Environ. Health Perspect.* **2007**,in press.

(9) Watanabe, H.; Suzuki, A.; Kobayashi, M.; Lubahn, D. B.; Handa, H.; Iguchi, T. Similarities and differences in uterine gene expression patterns caused by treatment with physiological and non-physiological estrogens. *J. Mol. Endocrinol.* **2003**, *31*, 487–497.

(10) Moggs, J. G.; Ashby, J.; Tinwell, H.; Lim, F. L.; Moore, D. J.; Kimber, I.; Orphanides, G. The need to decide if all estrogens are intrinsically similar. *Environ. Health Perspect.* **2004**, *112*, 1137–1142.
(11) Larkin, P.; Sabo-Attwood, T.; Denslow, N. D. Gene expression analysis of largemouth bass exposed to estradiol, nonylphenol, and *p,p'*DDE. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2002**, *133*, 543–557.
(12) Moens, L. N.; van der Ven, K.; Van Remortel, P.; Del-Favero, J.; De Coen, W. M. Expression profiling of endocrine disrupting compounds using a customized *Cyprinus carpio* cDNA microarray. *Toxicol. Sci.* **2006**, *93*, 298–310.
(13) Finne, E. F.; Cooper, G. A.; Koop, B. F.; Hylland, K.; Tollefsen, K. E. Toxicogenomic responses in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to model chemicals and a synthetic mixture. *Aquat. Toxicol.* **2007**, *81*, 293–303.
(14) Krasnov, A.; Afanasyev, S.; Oikari, A. Hepatic responses of gene expression in juvenile brown trout (*Salmo trutta lacustris*) exposed to three model contaminants applied singly or in combination. *Environ. Toxicol. Chem.* **2007**, *26*, 100–109.
(15) Thorpe K. L.; Benstead R.; Eccles P.; Maack G.; Williams T.; Tyler C. R. Effective assessment of the estrogenic activity of effluents using a laboratory flow-through exposure system. *Environ. Sci. Technol.* **2007**,submitted.
(16) Filby, A. L.; Tyler, C. R. Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). *Biol. Reprod.* **2005**, *73*, 648–662.
(17) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408.
(18) Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, e45.
(19) Filby, A. L.; Thorpe, K. L.; Tyler, C. R. Multiple molecular effect pathways for an environmental oestrogen in fish. *J. Mol. Endocrinol.* **2006**, *37*, 121–134.
(20) Filby, A. L.; Thorpe, K. L.; Maack, G.; Tyler, C. R. Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. *Aquat. Toxicol.* **2007**, *81*, 219–231.
(21) Klaper, R.; Rees, C. B.; Drevnick, P.; Weber, D.; Sandheinrich, M.; Carvan, M. J. Gene expression changes related to endocrine function and decline in reproduction in fathead minnow (*Pimephales promelas*) after dietary methylmercury exposure. *Environ. Health Perspect.* **2006**, *114*, 1337–1343.
(22) Pinto, P. I.; Singh, P. B.; Condeca, J. B.; Teodosio, H. R.; Power, D. M.; Canario, A. V. ICI 182,780 has agonistic effects and synergizes with estradiol-17 beta in fish liver, but not in testis. *Reprod. Biol. Endocrinol.* **2006**, *4*, 67.
(23) Seo, J. S.; Lee, Y. M.; Jung, S. O.; Kim, I. C.; Yoon, Y. D.; Lee, J. S. Nonylphenol modulates expression of androgen receptor and estrogen receptor genes differently in gender types of the hermaphroditic fish *Rivulus marmoratus*. *Biochem. Biophys. Res. Commun.* **2006**, *346*, 213–223.
(24) Van den Belt, K.; Berckmans, P.; Vangenechten, C.; Verheyen, R.; Witters, H. Comparative study on the in vitro/in vivo estrogenic potencies of 17β-estradiol, estrone, 17α-ethynylestradiol and nonylphenol. *Aquat. Toxicol.* **2004**, *66*, 183–195.
(25) Wang, D. Y.; McKague, B.; Liss, S. N.; Edwards, E. A. Gene expression profiles for detecting and distinguishing potential endocrine-disrupting compounds in environmental samples. *Environ. Sci. Technol.* **2004**, *38*, 6396–6406.
(26) MacKay, M. E.; Raelson, J.; Lazier, C. B. Up-regulation of estrogen receptor mRNA and estrogen receptor activity by estradiol in liver of rainbow trout and other teleostean fish. *Comp. Biochem. Physiol. C.* **1996**, *115*, 201–209.
(27) Callard, G. V.; Tchoudakova, A. V.; Kishida, M.; Wood, E. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. *J. Steroid Biochem. Mol. Biol.* **2001**, *79*, 305–314.
(28) Govoroun, M.; McMeel, O. M.; Mecherouki, H.; Smith, T. J.; Guiguen, Y. 17β-estradiol treatment decreases steroidogenic enzyme messenger ribonucleic acid levels in the rainbow trout testis. *Endocrinology* **2001**, *142*, 1841–1848.
(29) Harvey, S.; Baudet, M. L.; Murphy, A.; Luna, M.; Hull, K. L. Aramburo C. Testicular growth hormone (GH): GH expression in spermatogonia and primary spermatocytes. *Gen. Comp. Endocrinol.* **2004**, *139*, 158–167.
(30) Blanton, M. L.; Specker, J. L. The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction. *Crit. Rev. Toxicol.* **2007**, *37*, 97–115.
(31) Aherne, G. W.; Briggs, R. The relevance of the presence of certain synthetic steroids in the aquatic environment. *J. Pharm. Pharmacol.* **1989**, *41*, 735–736.
(32) Ternes, T. A.; Stumpf, M.; Mueller, J.; Habener, K.; Wilken, R. D.; Servos, M. Behaviour and occurrence of estrogens in municipal sewage treatment plants. I. Investigations in Germany, Canada and Brazil. *Sci. Total Environ.* **1999**, *225*, 81–90.
(33) Navas, J.; Segner, H. Estrogen-mediated suppression of cytochrome P4501A (CYP1A) expression in rainbow trout hepatocytes: role of estrogen receptor. *Chem.-Biol. Interact.* **2001**, *138*, 285–298.
(34) Bucheli, T. D.; Fent, K. Induction of cytochrome P450 in fish as a biomarker for environmental contamination in aquatic ecosystems. *Crit. Rev. Environ. Sci. Technol.* **1995**, *25*, 200–268.
(35) Kuiper, G. G. J. M.; Carlsson, B.; Grandien, K.; Enmark, E.; Haggblad, J.; Nilsson, S.; Gustafsson, J. A. Comparison of ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* **1997**, *138*, 863–870.
(36) von Hofsten, J.; Olsson, P. E. Zebrafish sex determination and differentiation: involvement of FTZ-F1 genes. *Reprod. Biol. Endocrinol.* **2005**, *3*, 63.
(37) Josso, N.; Racine, C.; di Clemente, N.; Rey, R.; Xavier, F. The role of anti Mullerian hormone in gonadal development. *Mol. Cell. Endocrinol.* **1998**, *145*, 3–7.
(38) Kobayashi, T.; Matsuda, M.; Kajiura-Kobayashi, H.; Suzuki, A.; Saito, N.; Nakamoto, M.; Shibata, N.; Nagahama, Y. Two DM domain genes, DMY and DMRT1, involved in testicular differentiation and development in the medaka, *Oryzias latipes*. *Dev. Dyn.* **2004**, *231*, 518–526.

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