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Estrogenic Wastewater Treatment Works Effluents Reduce Egg Production in Fish

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Estrogenic chemicals found within wastewater treatment work (WwTW) effluents have been shown individually to inhibit reproduction in fish, but the impact of the WwTW effluents themselves and the complex mixtures of environmental estrogens and other endocrine disrupting chemicals they contain has not been established. In this investigation, the effect of exposure to three WwTW effluents, with differing levels of estrogenic activity, was assessed on egg production in pair-breeding fathead minnow. Exposure to two of the three effluents tested resulted in a reduced egg production (by 28% for effluent I at a dilution of 50% and by 44% for effluent III at full strength), which was proportional to the estrogenic content of the effluents. The test effluents, however, had a greater effect on egg production than might be expected, on the basis of both the response they induced for induction of vitellogenin (an estrogen exposure biomarker) and when compared with an equivalent estrogen exposure to EE2. These data show that reliance on relatively simple biomarker responses for estrogenic activity alone, such as vitellogenin, can significantly underestimate the impacts of estrogenic WwTW effluents on fitness parameters such as reproductive health that are regulated by more complex estrogenic (and other endocrine) signaling mechanisms.

Introduction

Estrogenic chemicals prevalent in wastewater treatment work (WwTW) effluents are well-established as a principal cause of many reproductive abnormalities observed in wild fish, including induction of the female yolk protein precursor vitellogenin (VTG), intersexuality, and feminization of the male reproductive duct (*1, 2*). Laboratory experiments have further shown that exposure of fish to estrogenic chemicals can result in population-relevant effects including production of fewer viable offspring (*3*−*8*) and sex reversal in males exposed during early life (*9, 10*). The potential consequence of these effects for fish populations was recently demonstrated in an experimental lake in Canada, where continuous exposure to a potent estrogen (ethinylestradiol, EE2) for 4-years resulted in female-biased sex ratios, a reduced number of offspring, and ultimately in complete failure of a local fish population (*11*).

Concentrations of individual estrogens reported in WwTW effluents and their receiving waters are typically lower than those required to induce effects on population-relevant end points. It is well-documented, however, that estrogens occur in combination with WwTW effluents and that when present as mixtures they can be additive in inducing biological effects (*12*−*16*). Thus, concerns continue for the health of fish populations in rivers receiving chronic discharges of estrogenic effluents from WwTWs. Studies that investigate the impact of estrogenic effluents on the reproductive fitness of fish populations, however, have not been forthcoming. Even in the UK, where concentrations of some estrogenic chemicals measured in WwTW effluents are among the highest reported throughout the world, complete sex-reversal of males has not yet been demonstrated (*17*). Incidences of incomplete sex-reversal (intersexuality) are reported to be high in many UK Rivers, but the long-term consequences of intersex for reproductive fitness are not clear and male fertility has only been shown to be affected in the more severe cases, when gonadal ducts are blocked (*18*).

Experiments that more directly assess the effects of estrogenic WwTW effluents on the reproductive performance of fish have not been conducted. Internationally considerable efforts have been invested in the development of sensitive and robust tests for reproductive performance using freshwater fish species. As a result the adult reproduction test has been developed and this test has been demonstrated to be sensitive to the effects of estrogens and other endocrine active chemicals (*3, 5, 8, 19, 20*). This test in the fathead minnow, *Pimephales promelas*, has also been applied to assess the effects of Canadian bleached kraft mill effluents (*21*) and metal mine and municipal WwTWs (*22*) on egg production. In this study, we applied a laboratory-based flow-through exposure system (*23*) to evaluate the effects of exposure to three WwTW effluents, with different estrogenic potencies, on egg production in pair-breeding fathead minnow. The potential to use simple measures of estrogenic activity to signal for the observed effects on reproduction were also further evaluated.

Materials and Methods

Test Organisms. The fathead minnows used in each experiment were taken from stocks raised at the University of Exeter. Two weeks prior to the onset of each experiment, male and female fish with clearly defined secondary sex characters (nuptial tubercles and a dorsal fatpad on the males; an ovipositor on the females) were separated to prevent spawning activity and acclimated to the test conditions; dechlorinated water at 25 ± 1 °C; 16:8 h light:dark photoperiod. The fish were fed adult frozen brine shrimp (Tropical Marine Centre, Hertfordshire, U.K.) twice daily supplemented with a small quantity (<1% body weight/day) of Ecostart 17 1.0 mm fish food pellets (Biomar Ltd., Brande, Denmark) once daily.

Water Supply and Test Apparatus. The supply of water to the laboratory dosing system was prepared using a reverse-osmosis system with the addition of salts as described in OECD Guideline 203. The conductivity of the test water ranged between 235 and 255 μS/cm. Dissolved oxygen concentrations, temperature, and pH levels were determined in the individual tanks on days 0 and 1 and then as a minimum twice weekly throughout each experiment. In all experiments,

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the tanks were gently aerated, using a glass pipet, to ensure that the dissolved oxygen concentration remained >80% of the air saturation value throughout. Water temperatures ranged between 24 and 26 °C in all experiments, whereas pH levels remained between 7.5 and 7.7. Dilution water and test chemical flow rates were measured at least twice per week; flow-rates (15 mL/min) to the individual aquaria provided a 75% replacement time of 24 h. The test vessels had a working volume of 12 L and were constructed of glass, with a minimum of other materials (silicon rubber tubing and adhesive) in contact with the test solutions.

Waste Water Treatment Works Effluent. For experiments I, II and III, nine batches of effluent (2000 L per sampling occasion) were collected over a period of 3 weeks in November 2005, April 2006, and October 2006, respectively, in a stainless steel tanker. Each batch of effluent was collected from the final effluent stream at the respective WwTW between the hours of 8 and 10 a.m. and transported to the testing facility, where it was transferred into a fully enclosed stainless steel holding tank chilled to 8 °C. The effluent was pumped from the storage tank to the test aquaria via glass mixing tanks. Low dosing rates were used to allow the effluent to slowly acclimate to the desired test temperature of 25 °C before reaching the test aquaria. A full description of the effluent storage facility and dosing system is provided in Thorpe et al. (*23*).

Estrogen Control. To provide a reference estrogenic control, additional pairs of fish were exposed to EE2 in each experiment. EE2 (98% purity, Lot 024K1196) was purchased from Sigma, Poole, Dorset, U.K.. Solvent-free stock solutions were prepared every 3 days by adding 1 mL of a concentrated stock solution of EE2 (prepared in HPLC grade acetone; Fisher Scientific) to a 10 L glass vessel. After evaporation of the acetone, 10 L of dilution water was added and the solution was stirred for 2 h using a magnetic stirrer and follower. The solvent-free stock was then dosed to the glass mixing vessels, where it was mixed with the dilution water to provide a nominal test concentration of 15 ng/L.

Experimental Design; Pair-Breeding Test. To initiate each test, we placed male and female fish as pairs into eight replicate glass aquaria per concentration containing a spawning substrate (PVC half-guttering tile placed above a stainless steel mesh screened glass tray). The fish were acclimated to the test conditions for a minimum of 10 days, and the spawning substrates checked daily to confirm spawning activity. Egg number was then determined daily for each pair of fish, over a pre-exposure period of 3 weeks, to provide pair-specific data for egg production. Dosing of the DWC, EE2, or graded effluent concentrations (25, 50, and 100%) to the individual tanks was then initiated and the number of eggs spawned by each pair of fish determined daily for the 3-week exposure period. All experimental adult fish were sampled at the end of the exposure period.

Characterization of the Estrogenic Content of the WwTW Effluents. During the exposure phase of each experiment, water samples were collected from the fish exposure tanks into solvent-cleaned flasks for measurement of estrogenic activity in the recombinant yeast screen (rYES) and for the analytical chemistry determinants. The samples for the rYES were collected on days 2, 4, 7, 10, 14, 17, and 21; on each occasion, a total volume of 700 mL was collected from two tanks per treatment (i.e., 350 mL from each tank). The samples for the analytical chemistry were collected on days 4, 7, 14, and 21 and on each occasion a total volume of 2.5 L was collected from two tanks per treatment (i.e., 1.25 L from each tank). Samples were also collected daily from the effluent storage tank for analysis in the rYES (700 mL) and on the morning following delivery of each new batch of effluent for the analytical determinants (2.5 L). Immediately after collection, the samples were spiked with 0.05% methanol and extracted onto preconditioned Sep-Pak Classic C18 Cartridges (Waters Ltd., Hertfordshire, UK). For measurement of estrogenic activity in the rYES and for measurement of E1 and E2 concentrations via GCMS, the extracts were treated as described in Thorpe et al. (*15*).

To assess the efficiency of the extraction for each procedure, we extracted spiked (0.05% of an estrogenic mixture containing 4 μg estradiol-17β(E2)/L, 1.6 μg EE2/L, 8 μg estrone(E1)/L, and 800 μg nonylphenol/L prepared in methanol) dilution water and effluent samples under the same conditions. Dilution water spiked with 0.05% methanol was also extracted under the same conditions. Recoveries of total estrogenic activity in the rYES and measured concentrations of E1 and E2 in the spiked effluent samples were 84, 93, and 189%, respectively, for effluent I; 97, 249, and 496%, respectively, for effluent II; 95, 112, and 107%, respectively, for effluent III.

Fish Sampling. Fish were sacrificed in a lethal dose of MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt; Sigma), buffered to pH 7.4. Total length and wet body weight of the fish were recorded to the nearest 1 mm and 0.01 g, respectively, and the condition factor derived by expressing the cube of the total fish length as a percentage of the body weight. Blood was collected by cardiac puncture using a heparinized syringe (1000 units of heparin/mL). It was then centrifuged (7000 g; 5 min, 15 °C) and the plasma was removed and stored at −20 °C for later analysis of VTG using a carp ELISA (*24*). The gonads were removed and wet weighed to the nearest 0.01 mg; the gonadosomatic index was derived by expressing the gonad weight as a percentage of the total body weight. The numbers of tubercles on the snout of each fish were recorded, and the dorsal fat pad was removed and wet weighed to the nearest 0.01 mg.

Statistical Analyses. All biological results are expressed as mean ± standard error of the mean (SEM). To investigate effects of exposure on body weight, length, gonadosomatic index, secondary sex characters (tubercle number and dorsal fatpad weight) and log10 transformed plasma VTG concentrations, data were compared to the DWC using SPSS version 13.0. All data met the assumptions of normality and homogeneity of variance and were analyzed using one-way analysis of variance (ANOVA) followed by a Dunnett test. To investigate effects on reproductive activity, we compared mean cumulative egg production over the 21 day pre-exposure and exposure periods for each treatment group using the Kolmogorov−Smirnov test (KS-test).

For the rYES, estradiol equivalent concentrations (E2EQ/L) were calculated by determining the dilution of effluent concentrate required to produce the median effect concentration of E2. For less-potent effluents, the concentration of E2 required to produce the level of effect observed for the highest concentration of effluent (100% of a 700-fold concentrate) tested was determined.

Results

Characterization of Estrogenic Content and Physical Parameters for Each Effluent. A combination of in vitro analysis (using the rYES) for measurement of total estrogenic activity and analytical measurements for E1 and E2 (the two principal estrogens prevalent in WwTW effluents) was used to characterize estrogenic content in both the batches of effluent delivered and in the water within the exposure system. The mean estimates derived from these measurements are summarized in Table 1. The variation in estrogenic content between the individual batches of effluent delivered to the laboratory and the persistence of the measured estrogenic activity for each batch of effluent during the 2−3 day holding period are detailed in Figure 1. Mean measured concentrations of EE2 were 8.2 ± 2.3, 14.0 ± 1.0, and 15.7 ± 1.3 ng/L in experiments I, II, and III, respectively.

TABLE 1. Mean Measured Concentrations of Estrone and Estradiol and Total Estrogenic Activity (rYES analysis) for the Nine Batches of the Three Test Effluents, And from the Fish Aquaria Receiving Either the 100% Effluent or Dilution Water Only (n = 4 measurements for each tank)

	estrone (ng/L)	estradiol (ng/L)	E2EQ (ng/L)
Effluent I			
delivered effluent	62.5 ± 15.5	4.4 ± 0.5	11.7 ± 1.9
100% effluent exposure tank	92.7 ± 64.2	6.3 ± 1.9	21.2 ± 2.9
dilution water control tank	2.7 ± 0.9	0.5 ± 0.2	2.5 ± 1.0
Effluent II			
delivered effluent	1.6[a]	0.9 ± 0.2	0.7 ± 0.1
100% effluent exposure tank	2.6 ± 0.3	1.1 ± 0.2	2.0 ± 0.4
dilution water control tank	3.8 ± 1.5	1.4 ± 0.2	3.7 ± 0.5
Effluent III			
delivered effluent	3.8 ± 0.7	0.8 ± 0.2	6.7 ± 2.4
100% effluent exposure tank	4.1 ± 0.7	2.5 ± 0.8	18.0 ± 4.0
dilution water control tank	2.1 ± 0.3	0.8 ± 0.3	8.3 ± 1.5

[a] Because of analytical technical difficulties, E1 was measured in only one of the delivered batches of effluent II.

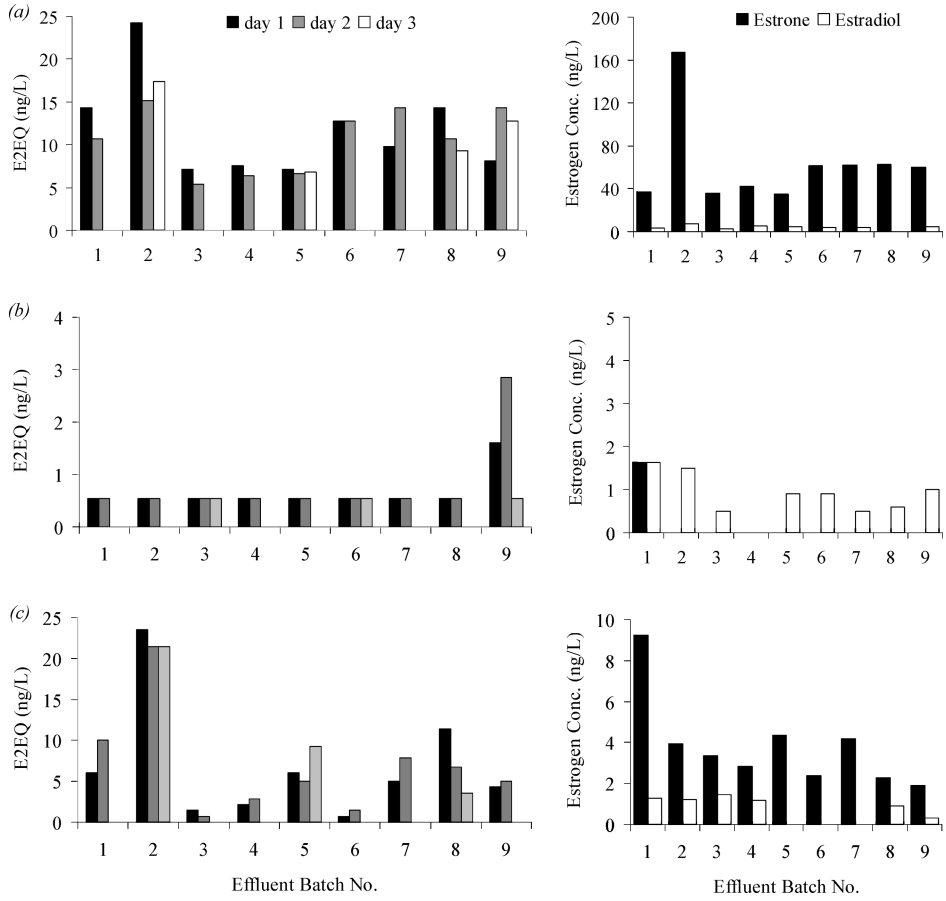

FIGURE 1. Daily assessments of estrogenic activity (E2EQs, as determined in the rYES) for each batch of effluent collected from WwTWs (a) I, (b) II, and (c) III during the 2−3 day storage period (left). The concentrations of estrone and estradiol measured in each batch of effluent are also shown (right; missing data points indicate that the estrogen was not detected in the effluent sample).

Conductivity, pH, and dissolved oxygen concentration were measured for each batch of effluent on arrival at the testing laboratory. Conductivity ranged between 1153 and 1450 μS/cm for effluent I, between 608 and 858 μS/cm for effluent II, and between 1040 and 1261 μS/cm for effluent III. The pH values were comparable for all effluents tested and ranged between 7.6 and 8.4, and dissolved oxygen concentrations were above 80% for all effluents on arrival at the testing laboratory.

Biological Effects of Effluent Exposure. There was no evidence that exposure for 21 days to the WwTW effluents or EE2 affected survival, growth, condition, or relative gonadal weight of the male or female fish. The appearance of the male secondary sex characters was also unaffected by exposure to the effluents, but a significant reduction in tubercle number was observed in males exposed to EE2 in each experiment.

Assessments of effluent exposure on egg production demonstrated that effluent I inhibited reproduction at the two highest test concentrations; 28 and 32% reductions in mean cumulative egg production over the 21 day exposure were observed for breeding pairs exposed to 50 and 100% concentrations, respectively, of effluent I ($p < 0.05$; Figure 2a). For effluent III, there was a 44% reduction in mean

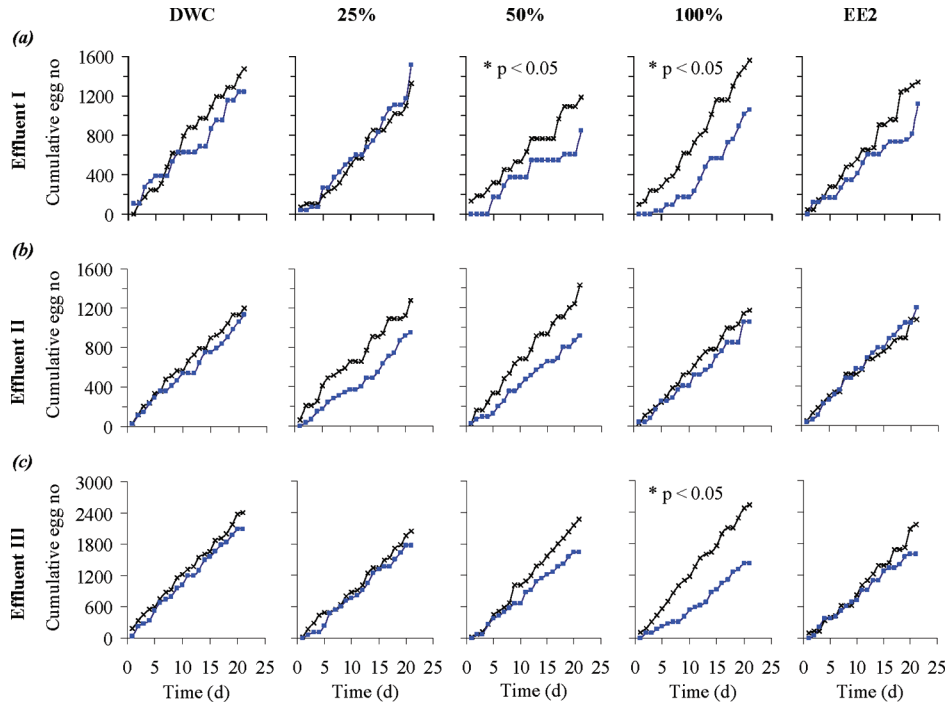

FIGURE 2. Mean cumulative egg number in pairs of fathead minnow exposed for 21 days to either a dilution water control (DWC), ethinylestradiol (EE2), or graded concentrations (25, 50, and 100%) of effluent collected from WwTWs (a) I, (b) II, and (c) III. *$p < 0.05$ indicates differences in mean cumulative egg production between the pre-exposure (black lines) and exposure (blue lines) periods for the eight breeding-pairs assigned to each treatment group.

cumulative egg production, relative to the pre-exposure period, for pairs of fish exposed to the undiluted effluent (100%; $p < 0.05$; Figure 2c). There was, however, no evidence for an effect of effluent II on mean cumulative egg production ($p > 0.05$; Figure 2b). In each of the three experiments, there was no evidence for an effect of EE2 ($p > 0.05$) on reproduction.

Analysis of plasma VTG in males confirmed the estrogenic activity of each of the test effluents and for each effluent a concentration-related relationship with the dilution of effluent was observed (Figure 3). Effluent I induced the highest estrogenic response and a significant increase in VTG concentrations in males, relative to the controls, was observed for all the dilutions tested (25, 50, and 100% effluent; $p < 0.01$). Exposure to the undiluted effluent resulted in 1765-fold increase in VTG concentrations to a level of 225 000 ng/mL in males. For effluent II, increases in plasma VTG concentrations were only significant in males exposed to the full strength effluent (100%; $p < 0.05$) and the magnitude of response was relatively small (9-fold increase to 365ng/ml). Exposure to effluent III increased VTG in males exposed to both the 50 and 100% concentrations, with the undiluted effluent inducing a 46-fold increase in plasma VTG concentrations to a level of 7110 ng/mL ($p < 0.05$). There was no evidence for an increase in plasma VTG concentrations in the effluent-exposed females, but in contrast to expectation, exposure to effluent III was observed to reduce VTG concentrations in females by 1.8-fold to 270 μg/mL ($p < 0.05$).

Exposure to EE2 consistently induced a higher vitellogenic response in males than for exposure to the effluents, with a 9785-fold increase (1240 μg/mL) in VTG concentrations in experiment I, a 15031-fold increase (620 μg/mL) in experiment II, and a 3517-fold increase (545 μg/mL) in experiment III (Figure 3). EE2 was also consistently more potent in inducing VTG in the females compared with the effluents, with a 4-fold increase (2340 μg/mL) in experiment I, a 1.6 fold increase (430 μg/mL) in experiment II, and a 1.2 fold increase (570 μg/mL) in experiment III (Figure 3).

Discussion

The FHM pair-breeding assay has previously been used to successfully assess the effects of exposure to individual or controlled mixtures of estrogens and other endocrine-active chemicals to show that these chemicals can cause concentration-dependent decreases in egg production (*3, 8, 16, 19, 20, 25*). The results of this investigation demonstrate that the test can be equally applied in a laboratory setting to quantify the effects of estrogenic effluents on egg production. The chilled effluent storage facility employed, in combination with a continuous flow-through exposure regime, enabled the estrogenic content of the test effluents to be stabilized and the effects of effluents with differing estrogenic potencies to be studied. Chemical analysis, in combination with in vitro assessments of estrogenic activity, revealed variations in estrogenic content between the nine individual batches of effluent collected for each WwTW, consistent with the short-term temporal variations in the estrogenic content of WwTW effluents reported by Martinovic et al. (*26*). This emphasizes the importance of considering fluctuations in the estrogenic content of WwTW effluents (through using multiple batches of effluents) for laboratory-based exposure studies to ensure they are representative estimates of the effluent under test. Despite the observed variations in estrogenic content between the batches of effluent, the mean measured concentrations of E1 and E2 in each WwTW effluent were consistent with those measured in an earlier series of experiments with effluents from these same WwTWs; <3.5-fold difference for E1 and <1.7-fold difference for E2 (*23*). Similarly, the mean estrogenic activity as determined in vitro (rYES) and in vivo (VTG induction) was comparable between the two sets of experiments for effluents I and III; <3.4-fold difference for both effluents. Effluent II, however, showed a markedly lower estrogenic activity (11-fold lower activity in the rYES and a 67-fold lower induction of VTG) in this experiment when compared with its activity as assessed in a previous

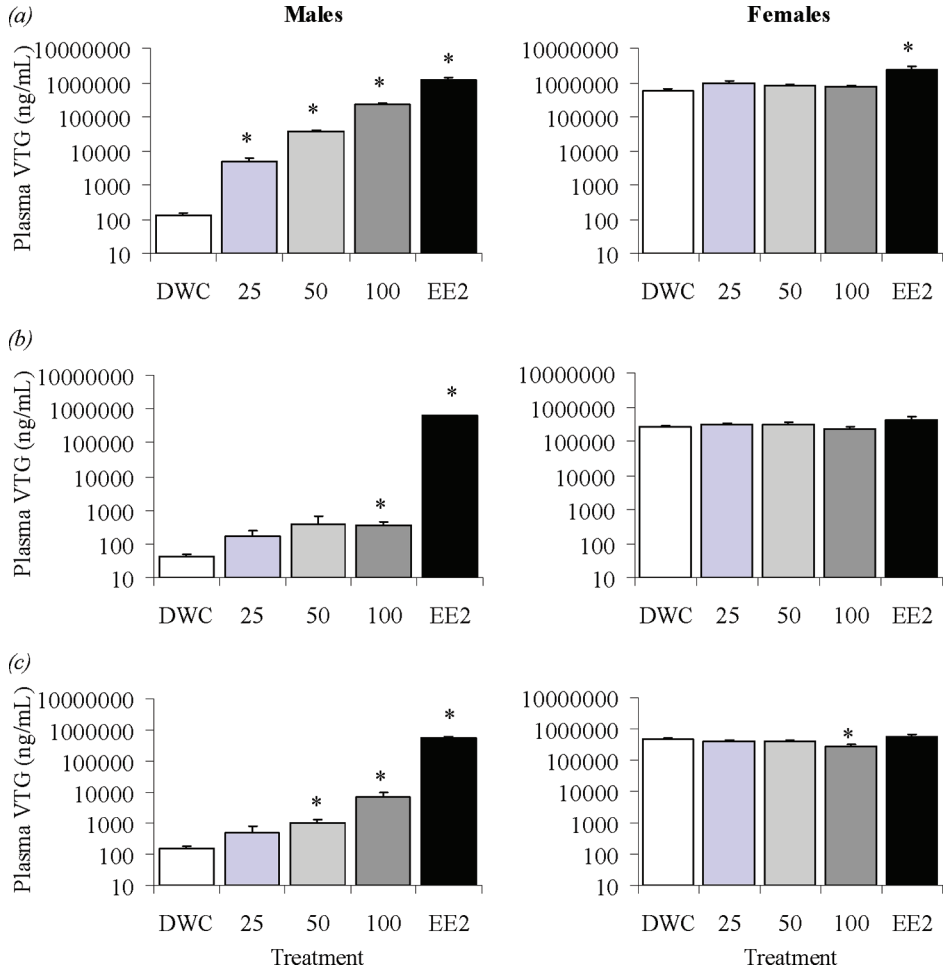

FIGURE 3. Plasma vitellogenin (VTG) concentrations in male (left) and female (right) fathead minnow exposed to graded concentrations (0, 25, 50, and 100% effluent) of effluents (a) I, (b) II, and (c) III and to ethinylestradiol (EE2). Each column represents the mean ± standard error of the mean (SEM). Significant differences between the control and exposure groups are denoted as *$P <$ 0.05.

study (*23*). Given that concentrations of E1 and E2 were comparable between the two experiments for this effluent, this would imply that other chemicals present within this effluent may have contributed to the estrogenic potency of this effluent in the earlier experiment. Large seasonal variations in the estrogenic effluent potency of effluents have been demonstrated previously (*27, 28*) and so were not surprising given that the experiments reported here were conducted many months after the earlier series of experiments on nonbreeding fish (*23*). This does, however, illustrate some of the difficulties faced in assessing the potential impact of effluents on the receiving environment and the need to evaluate the potency (here estrogenic) for every exposure undertaken.

In the current investigations, for the effluents generally there were good relationships observed between the chemical measurements for the individual steroidal estrogens, the measured estrogenic activity in the rYES and the levels of VTG induction. Furthermore, the observed estrogenic potencies of the effluents, based on these measures, were proportional to the observed effects on reproduction; the effluent with the highest estrogenic content/activity (effluent I) had the greatest inhibitory effect on cumulative egg production and the effluent with very weak estrogenic content/activity (effluent II) did not impact on egg production. This would suggest that analytical measures on the content of estrogenic chemicals (E1 and E2) in combination with in vitro and/or in vivo estimates of estrogenic activity could be used to signal for possible effects of a WwTW effluent on fish reproduction. The inclusion of EE2, however, highlighted an unexpected, but important, anomaly in the results that raised questions for us regarding the suitability of estrogen-specific biomarkers, such as VTG, for predicting the reproductive health effects of real world mixtures. The effects of EE2 were consistent with expectation for both the magnitude of the VTG response (*29*) and the lack of any effect on reproduction (that occurs at an EE2 concentration between 10 and 100 ng/L (*30*)). We show, however, that even though each effluent was less potent for VTG induction, when compared with the EE2 treatment, effluents I and III were both more potent than EE2 in inducing a suppressive effect on egg production. The relations between VTG and reproduction are poorly understood, and only two investigations have attempted to study this, at the level of the individual, in estrogen-exposed fish. In both investigations, decreases in egg production, following exposure to a steroidal estrogen, were observed only at concentrations of the estrogen that induced VTG concentrations in males above a threshold of 1 mg/mL (*8, 25*). In the current investigation, however, concentrations of VTG were more than 25-fold and 140-fold lower than this threshold VTG concentration in males exposed to the dilutions of effluent I and III, respectively, that suppressed egg production.

To help explain the apparent mismatch between the magnitude of the vitellogenic response and effects on reproduction in effluent exposed fish, when compared with responses for exposures to EE2 alone, it should be realized that the signaling mechanisms underlying the neuroendo-

crine regulation of gametogenesis and ovulation are far more complex than for induction of VTG. For example, estrogen signaling controlling reproduction may operate through three estrogen receptor subtypes in fish (*31*) potentially include pathways via membrane estrogen receptors, as well as nuclear ones (*32*), and have activational or suppressive effects at various points in the hypothalamic-pituitary-goandal axis (*33*). In contrast, for VTG induction, this operates through the activation of the estrogen receptor alpha in the liver (*34*). Furthermore, different steroidal estrogens within a mixture such as occur in a WwTW effluent can have different potencies and biological effects via the different estrogen receptor subtypes (*34*). Thus, although the findings from this study imply a different sensitivity for VTG induction and reproductive output for individual estrogens (EE2) when compared with their sensitivity to "real-world" complex estrogenic mixtures, in reality this is an oversimplification as the effluent will contain a complex mixture of estrogens that can interact at many parts of the hypothalamic−pituitary−gonadal axis to have suppressive or stimulatory effects on reproduction. What is clear, however, is that reliance on measures of VTG induction alone could result in an underestimation of the potential reproductive health effects posed by WwTW effluents for wild fish populations.

Effluents are highly variable in their chemical composition and physical parameters and given the complexity of the signaling mechanisms underlying the regulation of fecundity, it is also possible that factors additional to their estrogenic content contributed to their effects on reproduction. For example, exposures to effluent I have previously been associated with wider adverse health effects, including genotoxic damage, immnosuppression and nephrotoxicity (*35*) that may in turn lead to indirect affects on reproduction. Additionally chemicals that target other pathways within the reproductive axis, such as through affecting the activity of the aromatase enzymes (*8, 36*), have also been shown to reduce egg production, and these could be present within the test effluents. Indeed, in the exposure to effluent III, a significant concentration-related decrease in plasma VTG concentrations was observed in the females, which is consistent with exposure to both antiestrogenic chemicals (*37*) and aromatase inhibitors (*8, 36*). Further, it has recently been demonstrated that many estrogenic effluents also possess antiandrogenic activity (*38*) and laboratory exposures have further confirmed that exposure of reproductively active fish to antiandrogens can similarly inhibit egg production (*20*).

These data further demonstrate the health implications of WwTW effluents for fish populations and reveal for the first time that exposure to estrogenic WwTW effluents can result in a reduced reproductive output in fish. Crucially, we have demonstrated that although estrogenicity is a contributing factor to the suppressive effects of WwTW effluents for fish reproduction, simple analytical methods and bioassays designed to measure individual estrogens and to quantify their effects on estrogen receptor-mediated pathways alone do not provide a holistic understanding of the reproductive health effects of complex real-world mixtures. This has far-reaching implications for the use of estrogen-specific biomarkers, such as vitellogenin, to predict the wider reproductive health effects of environmental mixtures. Our data support the need for integrative test methods that can assess effects on the entire reproductive axis to develop a true understanding on the long-term health effects of effluent discharges.

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