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An environmental oestrogen disrupts fish population dynamics through direct and transgenerational effects on survival and fecundity

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Summary

1. Increased need for water and projected declines in precipitation due to climate change could leave waterways increasingly dominated by wastewater effluent. Understanding how components of wastewater influence fish populations is necessary for effective conservation and management. Despite research demonstrating effects of oestrogens, such as 17α-ethynylestradiol (EE2), on fish physiology and population failure, the generality of population responses is uncertain and the underlying mechanisms affecting population declines are unknown. EE2 is the steroid oestrogen in human contraceptive pills and has been measured up to 11 ng L^{-1} in the environment.

2. We identify disrupted population dynamics due to direct and transgenerational effects on survival and fecundity. We conducted a year-long study on three generations of fathead minnows *Pimephales promelas* Rafinesque in aquatic mesocosms and laboratory aquaria. We added environmentally relevant concentrations of EE2 daily using a static renewal, which approximates a pulsed exposure that fish experience in natural systems.

3. EE2 (3·2 ng L^{-1}) reduced F0 male survival to 17% (48% lower than controls) and juvenile production by 40% compared to controls. F1 fish continuously exposed to EE2 failed to reproduce, and reproduction of the F1 transferred to clean water was 70–99% less than controls.

4. F2 larval survival, exposed only as germ cells in their parents, was reduced by 51–97% compared to controls. The indirect effect on F2 survival suggests the possibility of transgenerational effects of EE2.

5. *Synthesis and applications*. Our results suggest that fish populations exposed to environmentally relevant 17α-ethynylestradiol (EE2) concentrations may not recover from exposure. Management of short-lived highly fecund fishes should be prioritized to protect fish from the embryo through gonadal differentiation. Reducing effluent will not be possible in many situations; hence, conservation of breeding and rearing habitat in unpolluted tributaries or reaches is needed. Additionally, resource managers could enhance habitat connectivity in rivers to facilitate immigration. Finally, investment in advanced wastewater processing technology should improve removal of bioactive chemicals such as EE2. Our results provide a baseline for regulatory agencies to consider when assessing the ecological effects of environmental oestrogens, and our approach to evaluating population-level effects could be widely applied to other contaminants.

Key-words: 17α-ethynylestradiol, endocrine disruption, fish, freshwater, mesocosm, North America, *Pimephales promelas*, population ecology, transgenerational

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Introduction

Freshwater ecosystems contain approximately 40% of global fish diversity (Dudgeon *et al*. 2006); freshwater ecosystems are highly threatened, and faunal extinction rates (4%) are currently much higher than in the past (Ricciardi & Rasmussen 1999). The effects on freshwater ecosystems stem largely from increased water consumption and the pollution associated with expanding human populations (Sala *et al*. 2000; Malmqvist & Rundle 2002; Dudgeon *et al*. 2006). Semi-arid ecosystems, such as the Great Plains, United States of America (USA), contain freshwater streams and rivers frequently dominated by wastewater effluent (Brooks, Riley & Taylor 2006). For example, flow in the South Platte River downstream of the Denver, Colorado, USA metropolitan area ranges from 69–100% sewage effluent depending on the time of year (Dennehy *et al*. 1993; Strange, Fausch & Covich 1999). Similarly, in 285 of 582 permitted wastewater discharges in Texas, Oklahoma, New Mexico, Arkansas and Louisiana, wastewater effluent comprises over 90% of the stream flow (Brooks, Riley & Taylor 2006). Exacerbating the problem is a projected decrease in stream flow in the inner-mountain and south-western USA resulting from climate change (Barnett, Adam & Lettenmaier 2005). Given current climate projections in the Western USA, stream flow can be expected to become increasingly effluent-dominated, and most fish populations will be exposed to wastewater effluents (Karl, Melillo & Peterson 2009). Understanding how fish populations respond to effluent exposure is critical to management and conservation.

Wastewater effluent may contain a complex chemical mixture of compounds that can influence vertebrate neural, immune and endocrine systems (Daughton & Ternes 1999; Vajda & Norris 2011). These compounds include many pharmaceuticals that enter waterways after incomplete removal during wastewater treatment (Ternes, Joss & Siegrist 2004). Steroidal oestrogens, commonly found in wastewater effluents, negatively affect fish reproduction in laboratory studies (e.g. Länge *et al*. 2001; Nash *et al*. 2004; Fenske *et al*. 2005; McGree *et al*. 2010). Most studies on effects of oestrogens have focused on physiological endpoints and use inferences based on these observations to speculate on population effects (Forbes, Calow & Sibly 2008). Direct evaluation of population-level effects will yield a more precise ecological measure of exposure than biochemical endpoints and be more relevant to management and conservation (Forbes, Calow & Sibly 2008).

Research by Kidd *et al*. (2007) provided evidence that oestrogens dramatically reduced fish abundance. However, their experiment was conducted in oligotrophic boreal lakes, as opposed to more nutrient rich urban streams, and was not designed to identify mechanisms underlying observed population decreases. Histological evidence suggested that observed declines in fathead minnow *Pimephales promelas* Rafinesque abundance were at least partly due to reproductive disruption (Kidd *et al*. 2007). However, mechanisms other than direct effects on adult reproduction could also influence population dynamics. For instance, oestrogen exposure might also reduce adult survival (Thorpe *et al*. 2007) or survival of other life stages (Länge *et al*. 2001), thereby reducing abundance. Additionally, lifetime exposure to oestrogenic compounds may have greater impacts on survival and reproduction than acute exposures (Nash *et al*. 2004; Fenske *et al*. 2005). Most studies do not assess the transgenerational effects that a compound may have on populations, perhaps owing to logistical challenges associated with long experiments. However, in most effluent-dominated systems, organisms could experience transient to lifetime exposures and effects on reproduction and survival may persist even if exposure is reduced or stopped. It is necessary to begin understanding how lifetime exposure affects population dynamics, how such exposure might differ from early-life exposures, whether animals can recover reproductive function following transfer to clean environments, and whether there are transgenerational effects. We define transgenerational as an altered phenotype in the offspring resulting from parental exposure.

Understanding the population consequences of oestrogen exposure requires realistic experiments conducted over appropriate time intervals. We used fathead minnows in our experiments because they are ideal model organisms for population-level studies. They are indigenous throughout much of temperate North America, reach sexual maturity rapidly, reproduce throughout the summer months and are sexually dimorphic (Ankley & Villeneuve 2006). Additionally, their physiological responses to oestrogens are well characterized (Ankley & Villeneuve 2006). We exposed fathead minnows to 17α-ethynylestradiol (EE2), the synthetic oestrogen used in human birth control. EE2 is a potent oestrogen in fish and a common contaminant in municipal wastewater effluents (Kostich, Flick & Martinson 2013). We used outdoor mesocosms because they allowed fathead minnows to be exposed to natural environmental variation, such as photoperiod, water temperature, productivity and nutrients while allowing replication across a range of EE2 concentrations. We also conducted a laboratory experiment using fathead minnows hatched in the mesocosms to evaluate early-life versus lifetime effects on survival and reproduction. We conducted these studies to test the following hypotheses: (i) EE2 reduces survival in multiple life stages, and (ii) EE2 reduces reproductive output over multiple generations. The outcomes of these studies will point to sensitive life stages and mechanisms that appear critical for population sustainability.

Materials and methods

We conducted two experiments over one year on three generations of fathead minnows in either outdoor aquatic mesocosms or glass aquaria (Fig. 1). All fish were treated in accordance with Institutional Animal Care and Use Committee Protocol No. 10-1685A at Colorado State University, Fort Collins, Colorado.

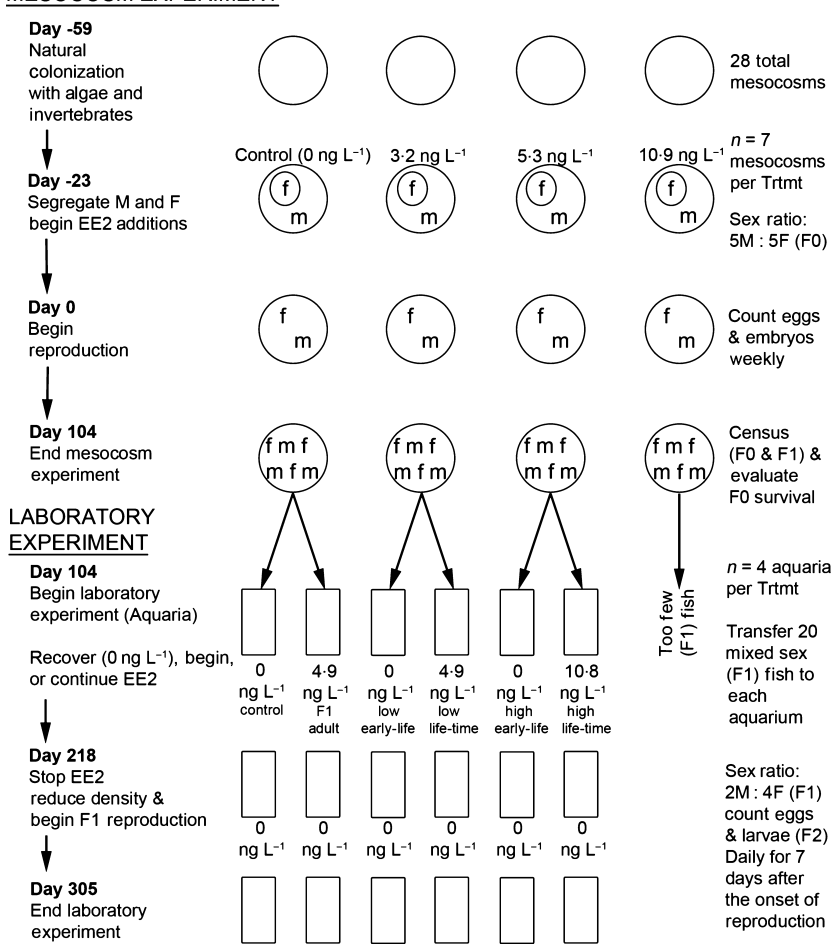

Fig. 1. Outline of experiments evaluating effects of 17α-ethynylestradiol (EE2) on three generations of fathead minnows *Pimephales promelas*. For each treatment, only one mesocosm or aquarium is shown but $n = 7$ mesocosms and $n = 4$ aquaria per treatment. In the laboratory experiment, the low early-life and high early-life treatments are the fish exposed to EE2 from spawning through gonadal differentiation. In the laboratory experiment, 'low' and 'high' are relative to the EE2 concentrations.

FISH EXPOSURES – MESOCOSM EXPERIMENT

The mesocosms were 28 polyethylene tanks (Rubbermaid Corp., Winchester, Virginia, 2 m in diameter, 0·66 m deep, 1100 L) supplied with water from College Lake (Fort Collins, Colorado). Lake water was mechanically filtered to remove large debris (>100 μm), irradiated with ultraviolet light and distributed to the mesocosms. Water flow was set by drilling a hole in the inflow pipe calibrated to 1–2 L min^{-1}. Water quality is presented in Table S1 in Supporting Information. When full, the volume of the mesocosms averaged 1056 ± 4·4 L (SEM). Mesocosms were aerated with ambient air continuously and covered with 6·25-cm^2 netting (Memphis Net and Twine, Memphis, Tennessee), and 30% of the surface area was shaded with landscaping fabric. The mesocosms colonized naturally with algae and invertebrates for 59 days prior to adding fish (Fig. 1).

On 6 May 2011, 23 days before the start of reproduction, five adult male and five adult female fathead minnows (from Aquatic Biosystems, Inc. Fort Collins, Colorado or the US EPA Cincinnati, Ohio) were stocked randomly into the mesocosms (F0 generation) (Fig. 1). We did not use wild-caught fish to ensure that adequate numbers would be available and they had not been exposed to oestrogens. The F0 generation was coded with elastomer tags (Northwest Marine, Shaw Island, Washington) by subcutaneous injection left of the dorsal fin, one colour for each sex. Initial handling mortalities were replaced for 48 h. Females were confined to perforated 189-l polyethylene containers (Rubbermaid Corp.) (Fig. 1). We fed the fish commercial brine shrimp flake (Argent Labs, Redmond, Washington) at 1 gm $tank^{-1}$, and feeding was observed within three days of stocking. Fish also fed on algae growing in the mesocosms. Also on 6 May 2011, treatments were arranged in a randomized complete block design (Fig. 1). On a daily basis, water flow was suspended at 17:00 h, and 99% pure 17α-ethynylestradiol (Sigma Aldrich, St. Louis, Missouri) (EE2) dissolved in methanol was pipetted into the plume of air bubbles in the middle of each mesocosm ($n = 7$ replicates per treatment) at 0, 5, 10, 20 ng L^{-1} (nominal). The control (0 ng L^{-1}) mesocosms received 1 mL of methanol. A water control was not used because of the low methanol concentration. We gently mixed each mesocosm with a boat paddle. The following morning at 09:00 h, water flow was resumed. On one occasion, a control (0 ng L^{-1}) mesocosm was accidentally spiked with the 10 ng L^{-1} solution. We chose static renewal for the EE2 additions because it approximates a pulsed addition to the mesocosm. Pulsed additions are similar to what fish encounter below wastewater treatment plants (Nelson *et al.* 2010).

On 29 May 2011 (day 0), we combined the males and females in the mesocosms, and fish were allowed to behave naturally for 102 days (Fig. 1) during which time reproductive output was assessed weekly. This experimental design resulted in direct waterborne exposures to the F0 and F1 generations (Fig. 1). While fish were reproducing, we fed concentrated newly hatched *Artemia* sp. nauplii at 2 mL day^{-1}. We produced *Artemia* nauplii in a conical hatchery (Aquatic Ecosystems, Apopka, FL, USA) by adding eggs at 1 gm L^{-1} in 25 parts per thousand constantly aerated sea water (Instant Ocean, Blacksburg, Virginia) incubated at 26–28 °C for 24 h.

FISH EXPOSURES – LABORATORY EXPERIMENT

On day 104, we randomly selected four mesocosms each from the 0, 5 and 10 ng L^{-1} treatments for inclusion in the laboratory study. Mesocosms exposed to 20 ng L^{-1} produced too few fish. The laboratory treatments ($n = 4$ replicates) were arranged in a randomized complete block design. From each selected mesocosm, we transferred mixed-sex F1 juvenile fathead minnows into two 60-l glass aquaria, ≤20 fish to each aquarium. One aquarium continued the same exposure as in the mesocosm (lifetime exposure), and the other aquarium was not exposed (early-life exposure) (Fig. 1). This design resulted in F1 fish that received a direct early-life EE2 exposure (from spawning through gonadal differentiation) in the mesocosm water (Fig. 1). In this early-life treatment, the F2 generation was exposed through germ cells of the F1 parents; thus, effects on F2 survival are considered transgenerational. The design also resulted in F1 fish that received a lifetime exposure (Fig. 1). Control (0 ng L^{-1}) F1 fish were also split into two aquaria. The laboratory control continued at 0 ng L^{-1}, and the other aquarium was exposed at 5 ng L^{-1} and is referred to as the F1 adult exposure (Fig. 1). Control F1 fish from the mesocosms were not exposed at 10 ng L^{-1} in the laboratory because of too few aquaria. EE2 (5 or 10 ng L^{-1} nominal) dissolved in 1 mL of ethanol was added daily to the appropriate aquaria as a static renewal by micropipettor. Water flow (0·5 L min^{-1}) was shut off at 17:00 h, EE2 was added and flow resumed at 08:00 h. The control aquaria received 1 mL of ethanol. Aquaria were aerated constantly with ambient air, fish were held on a 12:12 h light/dark cycle and fed commercial brine shrimp flake *ad libitum*.

From day 167 until the end of the experiment on day 305 (30 March 2012), we mixed heated and ambient lake water in a head tank before distributing to the aquaria (18–27 °C). On day 218, we ceased EE2 exposures because temperatures dropped below 20 °C when the aquaria were static. We also reduced the density of the F1 fish to stimulate reproduction (Fig. 1). We selected two visibly distinct males and four presumed females, based on no dorsal spot and relatively smaller size, an optimal ratio for reproduction (Denny 1987). The fish matured for another three weeks, water temperatures were maintained between 20 and 25 °C, and a 16:8 h light/dark cycle was implemented to stimulate reproduction (Denny 1987). All fish were unexposed for at least 53 days prior to the start of reproduction.

POPULATION ENDPOINTS

Spawning substrate was provided consisting of 15-cm-diameter hemispheres of polyvinyl chloride (PVC) pipe (Denny 1987) on the bottom of the mesocosms. One substrate was provided for each pair of fish. In the laboratory, we provided 7·5-cm-diameter PVC hemispheres, one for each male. Eggs were first observed on 1 June 2011, and reproduction was complete by 17 August 2011 after 21 days of no egg production. We checked each substrate weekly for eggs and embryos to minimize disturbance and the risk of multiple counts of the same eggs or embryos. Egg and embryo data may be underestimated because fathead minnows hatch in less than seven days (Denny 1987). Each substrate was photographed and returned to the mesocosms for continued development. Eggs and embryos on the digital photographs were counted using ImageJ (NIH, Bethesda, Maryland).

We stopped the mesocosm experiment on 9 September 2011 (day 104), and over two days drained each mesocosm and netted all fish into aerated buckets. We did not expose fish during this final sampling. Each labelled bucket was photographed, and fish were counted using ImageJ (National Institutes of Health, Bethesda, MD, USA). The F0 generation, identified by elastomer tags, was sampled for physiological endpoints. F1 juveniles were selected based on relatively large size for stocking the laboratory aquaria.

F1 reproduction began on 17 February 2012 (day 162) in the laboratory. We ceased feeding in tanks with spawning fish; adults spawned for seven days after which they were sampled for physiological endpoints. We counted eggs daily by image analysis or by hand. The eggs were incubated in beakers with 25 °C, aerated well-water and 0·1 mg mL^{-1} formalin to prevent fungal growth. Most eggs hatched within four days, and F2 larvae were counted; newly hatched larvae were also counted the following day. We checked substrates and counted eggs and larvae for seven days. We allowed reproduction to continue in all aquaria, while control (0 ng L^{-1}) fish continued to spawn. After control fish ceased spawning, any aquarium that had not spawned was sampled for physiological endpoints. Eight aquaria spawning concurrently with the last control tank continued spawning until the end of the seven-day reproductive period.

PHYSIOLOGICAL ENDPOINTS

We measured hepatic vitellogenin (VTG) mRNA expression in male fish. VTG is an egg-yolk precursor protein normally found in female fish, but in males, VTG is indicative of oestrogen exposure (Schwindt, Feist & Schreck 2007). Fish were euthanized in 250 mg L^{-1} tricaine methanesulfonate, livers were extracted aseptically, snap frozen on dry ice and then stored at −80 °C in the laboratory. Total RNA was extracted, and VTG mRNA was assessed using quantitative real-ime PCR following Biales *et al*. (2007).

WATER SAMPLES

We measured EE2 twice per week during the final eight weeks of the mesocosm experiment. Thirty minutes after spiking, we collected four 200-mL samples from different areas of the mesocosm within 5 cm of the surface, composited the samples and subsampled 200 mL into clean 250-mL amber glass jars (Environmental Sampling Supply, Oakland, California). We also collected three samples over a 24-h period, 30 min, 16 and 23 h after spiking with EE2. EE2 in the laboratory was assessed monthly in single grab samples. We transported the samples to the US EPA Region 8 Laboratory, Golden, Colorado, on ice, then transferred to 4 °C within two hours. EE2 in whole water or 0·45-μm filtrate was analysed by high-performance liquid chromatography tandem mass spectrometry (LC/MS/MS) within eight days (Appendix S1, Supporting information). Water temperature, dissolved O_2, pH, conductivity and nitrate were measured every other week with a YSI meter (YSI Inc., Yellow Springs, OH, USA) (Table S1, Supporting information).

STATISTICAL ANALYSIS

The response variables were F1 and F2 eggs, F1 juveniles, F0 adult survival, F2 larvae, F2 embryo survival (ratio of larvae to eggs) and VTG. Measured EE2 was the explanatory variable. Egg, embryo and larvae counts were summed over the experiments and averaged across treatments. We tested the hypotheses

that EE2 reduces numbers of eggs, embryos, larvae and juvenile fish and reduces survival relative to controls with generalized linear mixed models. Pairwise comparisons were made between EE2 treatments and controls for each experiment. Experiment-wise error was not considered because of the small number of comparisons. To assess the transgenerational effect of EE2, we compared F2 embryo survival (exposed as germ cells in their parents) to controls with an *a priori* linear contrast. Log-transformed VTG (because of increasing variance) in EE2-treated fish was compared to the control using a mixed linear model.

The binomial distribution (logit link) was specified for survival data, and the negative binomial distribution (because of count data with high variance) (log link) was specified for count data. All models were fit using Proc GLIMMIX (SAS software v9.3, ©2012, SAS Institute Inc. Cary, NC, USA) (or Proc MIXED for the VTG) with residuals and block as random effects and the EE2 treatment as the fixed effect. A block × treatment interaction was included as a random effect in the F2 embryo survival test to correct overdispersion. The F1 juvenile and egg models would not converge so block was included as a fixed effect. In aquaria where no F1 reproduction was observed, we substituted a '1' for the '0' in the egg and F2 larvae data so that parameters could be estimated and statistics calculated. An odds ratio for death of F0 adult males per ng L^{-1} increase in EE2 was assessed by logistic regression (Proc LOGISTIC). All data were analysed untransformed (except for VTG) and plotted against the measured EE2 concentrations. Residuals versus predicted plots and q–q plots, respectively, were used to assess equal variance and normality. Type 1 error rate was $\alpha = 0{\cdot}05$. Data are mean ± SEM.

Results

WATER CHEMISTRY

The EE2 detection limit was 0·1 ng L^{-1}, and the limit of quantification was 0·3 ng L^{-1}. Recovery of EE2 from matrix spikes and quality assurance data are given in Tables S2 and S3 (Supporting information), respectively. The concentrations in the 0, 5, 10 and 20 ng L^{-1} mesocosm treatments were 0·34 ± 0·04, 3·22 ± 0·64, 5·32 ± 0·19 and 10·85 ± 0·39 ng L^{-1}, respectively (Table 1). Concentrations were less than nominal because EE2 is hydrophobic and binds to organic matter. Measured concentrations in the 0, 5 and 10 ng L^{-1} laboratory treatments were 0·17 ± 0·1, 4·95 ± 0·55 and 10·75 ± 1·77 ng L^{-1}, respectively. Over the course of 24 h in the mesocosms, EE2 attenuated following the spike (Table S4, Supporting information) indicating a pulsed exposure. Detection of EE2 in the controls (0 ng L^{-1}) (Table 1, Table S4, Supporting information) was surprising. The water for these experiments was pumped from a reservoir subject to extensive human recreation that may be the source of trace EE2 in the control mesocosms. Hereafter, the population results will be based on the mean measured concentrations.

MESOCOSM EXPERIMENT

EE2 exposure significantly reduced F0 male survival ($F_{2,18} = 11{\cdot}58$, $P = 0{\cdot}0006$, Fig. 2). Survival in control males averaged 66 ± 8% and male survival significantly declined at 3·2 ng L^{-1} to 17 ± 9% ($t_{15} = 3{\cdot}89$, $P = 0{\cdot}001$) and 5·3 ng L^{-1} to 14 ± 6% ($t_{15} = 4{\cdot}07$, $P = 0{\cdot}0007$). No males survived in mesocosms exposed at 10·9 ng L^{-1} for 126 days (Fig. 2). EE2 significantly increased the odds of male death by a factor of 1·7 × (1·3–2·2; 95% C.I.) for every 1 ng L^{-1} increase in EE2. Survival in control females averaged 66 ± 7% and declined to 45 ± 8% in females exposed to 10·9 ng L^{-1} (Fig. 2).

EE2 exposure significantly decreased F0 reproductive output in all life stages ($F_{3,23{\cdot}74} = 20{\cdot}53$, $P < 0{\cdot}0001$ eggs; $F_{3,21{\cdot}19} = 23{\cdot}62$, $P < 0{\cdot}0001$ embryos; $F_{3,18} = 16{\cdot}29$, $P < 0{\cdot}0001$ juveniles; Fig. 3a,b). Blocking was not

Table 1. 17α-ethynylestradiol (EE2) in the mesocosms ($n = 7$ replicates) or laboratory aquaria ($n = 4$ replicates) and hepatic vitellogenin (VTG) mRNA expression in male fathead minnows *Pimephales promelas*. In the laboratory experiment, the first concentration is EE2 in the mesocosm, the second is EE2 in the aquaria. Data are mean ± SEM

Treatment	Nominal EE2 (ng L^{-1})	Measured EE2 (ng L^{-1}) (range)	Relative VTG:18S expression
Mesocosm experiment (5M:5F per mesocosm)			
Control	0	0·34 ± 0·04*(0–1·3)	0·006 ± 0·004
Low	5	3·22 ± 0·64*(0·38–12·8)	1·95 ± 0·74‡
Medium	10	5·32 ± 0·19*(2·76–8·11)	4·89 ± 2·14¶
High	20	10·85 ± 0·39*(6·84–19·11)	No Fish
F1 Laboratory experiment (20 fish per aquarium; reduced to 2M:4F for spawning trials)			
Control	0, 0	0·34 ± 0·04* 0·17 ± 0·1† (0–1·3) (0–0·39)	0·002 ± 0·0007
F1 adult exposure	0, 5	0·34 ± 0·04* 4·95 ± 0·55† (0–1·3) (3·8–6·4)	0·003 ± 0·001
Low early-life exposure	5, 0	3·22 ± 0·64* 0·17 ± 0·1† (0·38–12·8) (0–0·39)	0·006 ± 0·0003
High early-life exposure	10, 0	5·32 ± 0·19* 0·17 ± 0·1† (2·76–8·11) (0–0·39)	0·004 ± 0·0005
Low lifetime exposure	5, 5	3·22 ± 0·64* 4·95 ± 0·55† (0·38–12·8) (3·8–6·4)	0·01 ± 0·001
High lifetime exposure	10, 10	5·32 ± 0·19* 10·75 ± 1·77† (2·76–8·11) (6·66–13·13)	0·01 ± 0·01

*EE2 concentrations–0·45-μm-filtered water.
†EE2 concentrations–whole water.
‡Least-squares mean is statistically different from 0 ng L^{-1} ($t_5 = 6{\cdot}87$, $P = 0{\cdot}0003$).
¶Least-squares mean is statistically different from 0 ng L^{-1} ($t_5 = 8{\cdot}50$, $P = 0{\cdot}0002$).

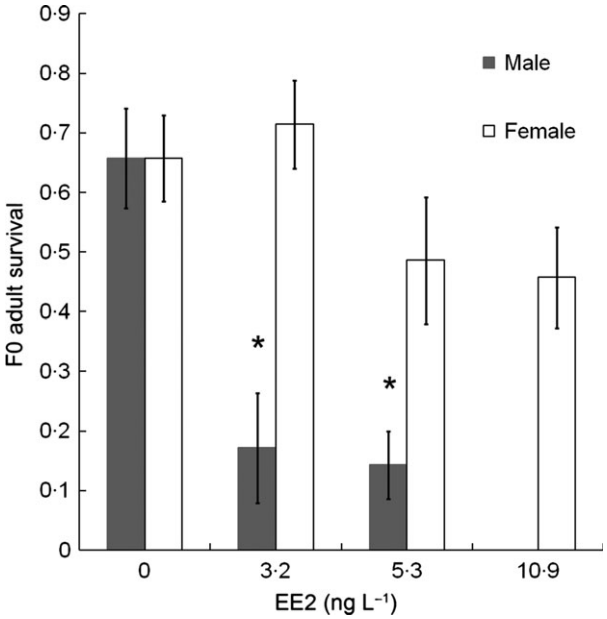

Fig. 2. Effect of 17α-ethynylestradiol on fathead minnow *Pimephales promelas* survival in the F0 generation. The (*) indicates significant difference from controls (0 ng L^{-1}) based on least-squares means ($0{\cdot}002 < P < 0{\cdot}003$) within sex ($n = 7$ replicates). Bars are mean±SEM.

significant ($P = 0{\cdot}5$). At 10·9 ng L^{-1}, F0 reproductive output was significantly lower than controls and was limited to an average of 125 ± 60·2 eggs ($t_{23{\cdot}88} = 7{\cdot}11$, $P < 0{\cdot}0001$), 4 ± 3·4 embryos ($t_{23{\cdot}1} = 7{\cdot}78$, $P < 0{\cdot}0001$) and 0·86 ± 0·86 fish ($t_{18} = 6{\cdot}90$, $P < 0{\cdot}0001$). Egg, embryo and juvenile production in the controls were not statistically different compared to the, 3·2 or 5·3 ng L^{-1} exposures ($0{\cdot}3787 < P < 0{\cdot}1451$) (Fig. 3a,b).

LABORATORY EXPERIMENT

EE2 exposure reduced F1 fathead minnow egg production ($F_{5,15} = 19{\cdot}37$, $P < 0{\cdot}0001$) and numbers of F2 larvae ($F_{5,15} = 36{\cdot}73$, $P < 0{\cdot}0001$; Fig. 3c). Blocking was not significant ($P = 0{\cdot}09$). One tank of lifetime-exposed F1 fish (3·2 ng L^{-1} in mesocosms; 4·9 ng L^{-1} in laboratory, Table 1) produced 1488 eggs, none of which were viable; the remaining tanks in this treatment did not produce eggs. Mean egg output from early-life-exposed F1 parents (3·2 ng L^{-1}) was significantly less (478 ± 256) than control fish (1581 ± 309) ($t_{15} = 2{\cdot}65$, $P = 0{\cdot}02$). Likewise, numbers of F2 larvae (236 ± 187) from early-life-exposed parents (3·2 ng L^{-1}) were reduced from controls (1195 ± 205) ($t_{15} = 3{\cdot}04$, $P = 0{\cdot}008$). In parents exposed to 5·3 ng L^{-1} early in life, egg production (5·7 ± 4·7) and F2 larvae (0·33 ± 0·33) were significantly reduced compared to the controls ($t_{15} = 7{\cdot}19$ and 8·85, respectively, $P < 0{\cdot}0001$).

EE2 exposure to the F1 generation suggests reduced F2 embryo survival ($F_{3,8{\cdot}8} = 3{\cdot}19$, $P < 0{\cdot}08$). However, reduced F2 survival between the average of the early-life-exposed parents (19 ± 10%) and the control (70 ± 5%) was significant ($F_{1,\ 9} = 8{\cdot}32$, $P = 0{\cdot}02$, *a priori* linear contrast) (Fig. 3d). Survival of the F2 embryos exposed to EE2 (3·2 ng L^{-1}) only through the parental germ cells (Fig. 1) was half (35 ± 17%) that of the controls. In F2 embryos whose parents were exposed to 5·3 ng L^{-1} early in life, survival averaged only 2·2 ± 2·2%. In F1 fish subjected to lifetime EE2 exposure, no F2 embryos survived (Fig. 3d).

VTG EXPRESSION

In the mesocosm experiment, VTG significantly increased in a concentration-dependent manner ($F_{2,6{\cdot}58} = 45{\cdot}81$, $P = 0{\cdot}0001$) (Table 1). VTG expression was significantly increased in the 3·2 ng L^{-1} (1·95 ± 0·74) and 5·3 ng L^{-1} (4·89 ± 2·14) groups compared to the controls (0·006 ± 0·004) ($t_5 = 6{\cdot}87$, $P = 0{\cdot}0003$; and $t_5 = 8{\cdot}50$, $P = 0{\cdot}0002$, respectively). VTG expression in the laboratory experiment was negligible (Table 1) because we stopped EE2 exposures to facilitate reproduction on day 218 (Fig. 1), giving at least 53 days of no EE2 exposure prior assessing VTG expression. Interassay coefficient of variation (CV) for VTG was 2·25% and 1·86% for the 18S rRNA (reference gene). Intra-assay CV for both VTG and 18S was <7%. The absence of VTG expression in control fish (Table 1) indicates that background EE2 concentrations have little, if any, effect on the fish in either experiment.

Discussion

We assessed reproductive performance and survival in three generations of fathead minnow populations exposed to trace levels EE2, the oestrogen in most human contraceptive pills. Exposure of F0 adults during the reproductive season significantly reduced male survival. Survival of F2 embryos exposed only as germ cells in their sexually immature parents was significantly reduced from controls, suggesting a transgenerational effect. Exposure of F0 adults during the reproductive season reduced eggs, embryos and F1 juvenile production. Lifetime exposure of F1 fish resulted in reduced F2 eggs and no F2 larvae. Interestingly, individuals exposed early in life and transferred to clean water produced significantly fewer eggs and F2 larvae, suggesting population recovery is not possible within the given time frame and experimental conditions. These results indicate that multiple mechanisms, including reduced reproductive output and survival, disrupt population dynamics in short-lived fishes from oestrogen-contaminated environments. EE2 concentrations range from 0·4 to 11·6 ng L^{-1} in aquatic environments (Kostich, Flick & Martinson 2013), indicating that the concentrations we used (3·2–10·9 ng L^{-1}) are environmentally relevant.

Reproduction was permanently disrupted in the F1 fish spawned in the mesocosms and allowed to recover for an equal period of time in the laboratory. Most dramatically,

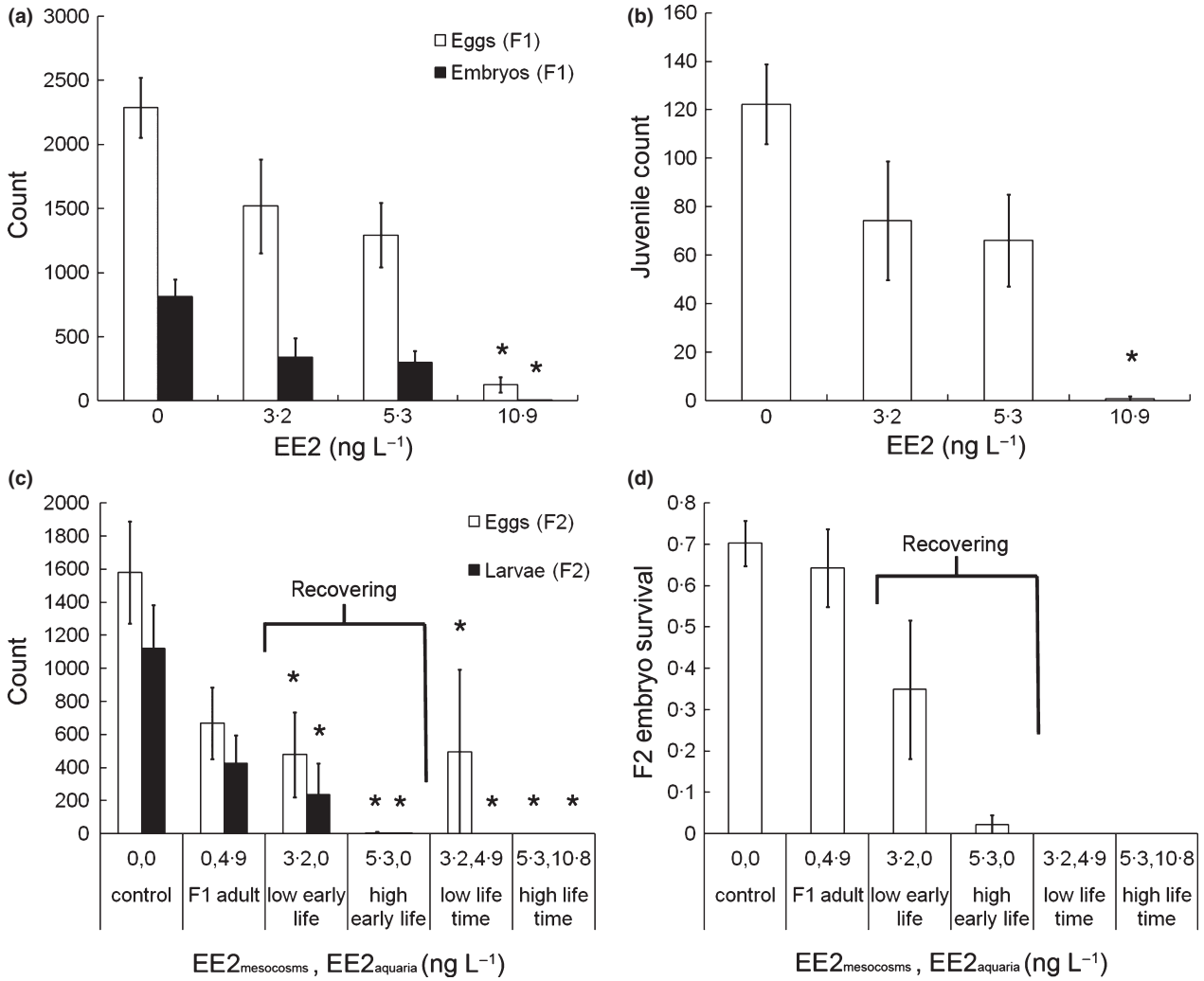

Fig. 3. Effect of 17α-ethynylestradiol (EE2) on F1 eggs, embryos (a), juvenile fathead minnows *Pimephales promelas* (b) (n = 7 replicates, mesocosm experiment), and F2 eggs and larvae (c) and embryo survival (d) (n = 4 replicates, laboratory experiment). On the x-axis in (c & d), the first number is the mesocosm EE2 concentration, and the second is the aquaria EE2 concentration. In (c & d), 'early life' refers to F1 exposure from spawning through gonadal differentiation, these fish are recovering from the EE2 exposure; 'low' and 'high' are relative to the EE2 concentration. The (*) indicates significant differences from controls (0 ng L^{-1}) in that experiment and within life stage based on least-squares means with $0{\cdot}0001 < P < 0{\cdot}02$. In (d), the average of the F1 early-life exposure groups (recovering) was significantly different than F1 control ($F_{1,9} = 8{\cdot}32$, $P = 0{\cdot}02$, *a priori* linear contrast). Bars are mean±SEM.

F1 parents exposed early in life (5·3 ng L^{-1}) produced almost no F2 eggs and zero larvae. Despite recovering for over five months, F1 fish exposed early in life (3·2 ng L^{-1}) produced fewer F2 eggs and larvae than controls. Studies on zebrafish *Danio rerio* Hamilton suggest that partial recovery from EE2 exposure is possible at similar concentrations (Nash *et al.* 2004; Fenske *et al.* 2005), contrasting with our results. A likely mechanism for disruption was reduced male fertility (Nash *et al.* 2004). The mechanisms underlying the ability or inability to recover from exposure to EE2 are unknown, but differences in reproductive development between the species may offer a possible explanation. Both fish are gonochoristic; however, zebrafish pass through an all-female phase before differentiating, while fathead minnows differentiate directly to male or female (Devlin & Nagahama 2002). The direct differentiation of fathead minnows could result in a differential response to early-life EE2 exposure. Despite differences between our study and those cited, EE2 has significant effects on reproductive performance in both species indicating a need for comparative studies testing the relationship between mode of gonad development and reproductive disruption.

Male survival in the F0 generation was significantly reduced by EE2. We know of no other studies reporting significant effects of trace EE2 concentrations on adult survival. The physiological mechanism behind reduced male survival in our study is unknown, but could be due to the inability to metabolize oestrogen-induced proteins, such as VTG. VTG is an egg-yolk protein normally found in females (Schwindt, Feist & Schreck 2007) that can be induced in male fish following oestrogen

exposures (e.g. Länge *et al*. 2001; Nash *et al*. 2004; Schwindt, Feist & Schreck 2007; McGree *et al*. 2010). However, there is limited capacity for males to metabolize VTG resulting in kidney failure (Thorpe *et al*. 2007). Despite the significant decline in male survival, we did not observe statistically significant effects on reproductive output of the F0 generation until our highest EE2 concentration. These results suggest that, even when male survival is low, reproductive output is maintained at levels similar to unexposed fish.

Male and female survival in control mesocosms suggests a base level of mortality not associated with EE2. Possible stressors that may induce mortality include competition for food and mates, as well as competition with other species. EE2 is an additional stressor that may induce mortality because of the inability to physiologically compensate for exposure to multiple stressors (Schreck 2000). If this is the case, it is especially concerning because fish in natural environments are exposed to multiple environmental stressors (Schreck 2000) including oestrogens (e.g. Vajda *et al*. 2008; Schwindt *et al*. 2009). A better understanding of the factors causing adult mortality in unexposed and exposed fish is clearly needed.

Although male survival did not affect reproductive output (<10·9 ng L^{-1}), increased male mortality could have effects beyond reproductive disruption, such as inbreeding depression or directional selection. If reproducing males were resistant to EE2 and resistance is heritable, oestrogen-resistant populations could evolve. We did not evaluate survival during the reproductive season and do not know if most males reproduced then died, or if the few surviving males were those that reproduced. The relatively high variance in F0 reproductive output of exposed fish suggests that some males are more resistant than others. The potential for selection should be addressed by genotyping parents and offspring in future studies. Fewer males reproducing could also cause inbreeding depression. However, inbreeding depression in wild fathead minnows is unlikely because the species evolved in environments that caused large periodic reductions in population size (Danylchuk & Tonn 2003). Undertaking research to evaluate the evolutionary responses to oestrogen exposure is an obvious extension of our work.

Survival of F2 embryos whose parents were exposed early in life was significantly reduced despite F2 embryos never being exposed to waterborne EE2. The F2 were exposed as primordial germ cells or sperm and eggs in early stages of development in the parental gonad. Thus, reduced F2 survival suggests a transgenerational effect that could result in directional selection if the potentially epigenetic changes are heritable. Alternatively, a transient maternal effect that is not heritable may explain our observations. However, reduced F2 survival was not likely due to residual EE2 in the parental bloodstream because VTG mRNA was not detected.

Our experiments could not evaluate the genetic consequences of exposure; however, other research exposing zebrafish to EE2-induced DNA methylation suggests epigenetic effects (Strömqvist, Took & Brunström 2010). The investigators did not link methylation states to changes across generations (Strömqvist, Took & Brunström 2010). Only further research including more generations can resolve these issues (Skinner 2008) and describing the extent of transgenerational effects on future generations will have critical implications for the management of fish populations.

Importantly, we used laboratory-cultured fathead minnows and those fish may not represent wild populations. Locally adapted wild populations may be naturally resistant to oestrogen through exposure over numerous generations. Future research should address the potential for selection in wild populations with common-garden experiments where populations from polluted and pristine rivers are compared to evaluate oestrogen resistance. Laboratory cultures are unlikely to represent the genetic and life-history variation present in natural populations (Coe *et al*. 2009). Because fathead minnows are used extensively in ecotoxicology (Ankley & Villeneuve 2006), it is critical that we understand the extent of clinal variation in wild populations and if that variation is represented in laboratory cultures.

Managing fish populations in effluent-dominated systems will be challenging. However, our results suggest that protecting short-lived highly fecund fishes from the embryo through to gonadal differentiation is most critical for population sustainability. Reducing effluent will not be possible in many situations; therefore, conservation of breeding and rearing habitat in unpolluted tributaries or reaches is needed. Additionally, resource managers could enhance habitat connectivity in rivers to facilitate immigration. In zebrafish, reproduction was recovered with addition of unexposed males (Nash *et al*. 2004), so reproductively viable immigrants may be important for population sustainability. Finally, recent work on a municipal wastewater effluent-dominated river indicated that oestrogenic pollutants can be removed following upgrades to WWTPs and implies that investment in infrastructure can lead to ecological benefits (Barber *et al*. 2012).

Empirical studies documenting effects of oestrogens on population endpoints are lacking. The diversity of effects presented herein suggests multiple mechanisms contribute to disrupted population dynamics including: (i) reduced male survival, (ii) reproductive failure, assumed to be caused by developmental reprogramming (Van Aerle *et al*. 2002) and (iii) transgenerational effects that limit offspring survival. Our study demonstrates that recovery of populations from oestrogen exposures may not be possible, at least under these experimental conditions. That is, even if fish migrate away from contaminated environments, population effects may persist given the effects of early-life F1 exposures on the F2 generation. Our results provide a baseline for regulatory agencies to consider when assessing the ecological effects of environmental

oestrogens. Our approach to evaluating population-level effects could be widely applied to other contaminants. Future research should assess other population effects in a modelling framework.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. LC/MS/MS methods.

Table S1. Mean (range) water quality parameters in the mesocosms.

Table S2. Recovery of 17α-ethynylestradiol from matrix spikes.

Table S3. Continuing calibration blank and preparatory blank data.

Table S4. Measured 17α-ethynylestradiol concentrations.

Appendix S1. LC/MS/MS methods

Chemicals. 17α-ethynylestradiol (EE2) and labeled EE2 ($^{13}C_2$-EE2) reference standards were >98% purity (Cambridge Isotopes Laboratories, Inc., Andover, Massachusetts). Acetonitrile and methanol were LC/MS grade (J.T. Baker Company, Phillipsburg, New Jersey). Formic acid was >99% purityas (Acros Organics, Morris Plains, New Jersey). Toluene was HPLC grade (Fisher Scientific, Fairlawn, New Jersey). Double deionized H_2O (dd-H_2O) was obtained from a Barnstead system (Dubuque, Iowa). Sodium bicarbonate was ACS grade (J.T. Baker, Phillipsburg, New Jersey). Dansyl chloride was >98% purity (Sigma-Aldrich, St. Louis, Missouri).

Extraction of EE2 and derivatization to dansyl chloride. The water samples were prepared for LC/MS/MS after Zhang *et al.* (2004) with the following exceptions: 1) $^{13}C_2$-labelled EE2 at 20 ng L^{-1} was the internal standard (ISTD); 2) the EE2 extraction included 10 min on a wrist action shaker; 3) dansyl chloride concentration was increased to 3 mg ml^{-1}; 4) the dansyl chloride reaction was increased to 10 min and; 5) lake-water used as the matrix. Filtered samples were obtained with 0.45 µm GHP (hydrophilic polypropylene) leur lock filters (Pall Life Science, Port Washington, New York) added to the tips of the syringes and slowly pushed the water through syringe and filter by hand.

Calibration curve and quality control samples. Calibration curves (CC) were prepared for each run. For the CCs we used water from the 0 ng L^{-1} mesocosms and standards were 1, 5, 10, 25, 50, and 100 ng L^{-1}. Continuing calibration blanks (CCB), prep blanks (PB), blank spikes (BS), and duplicate matrix spikes (MS) were included after every CC, every 20 samples, and at the conclusion of every run. The CCB was dd-H_2O, the PB was dd-H_2O spiked with ISTD, the BS was dd-H_2O spiked with 25 ng L^{-1} EE2 and ISTD, and the MS was the same mesocosm water

used for the CC spiked with 25 ng L^{-1} EE2 and ISTD. All quality control samples were prepared side-by-side with the mesocosm samples. The CC was fit to a quadratic model with 1/x weighting and $R^2 > 0.997$ for all assays. The method detection limit (MDL) was estimated as the Student's $t_{(n-1,1-\alpha = 0.99)}$ × standard deviation calculated from seven replicate 25 ml lake-water samples spiked with 1 ng L^{-1} EE2 and analyzed by LC/MS/MS. The limit of quantification was 3 × MDL. The quality control results are in Tables S2 and S3.

Liquid chromatography and mass spectrometry conditions. The solutions of 0.1% formic acid in dd-H2O (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). were pumped at a flow rate of 0.5 mL min^{-1}. The retention time for EE2 was 2.4min using a gradient profile of: 0 min-50% B, 1 min-95% B, 3 min-95% B. For each injection we included four wash steps after 3 min consisting of 2 min cycles alternating between 95% B (5% A) and 5% B (95% A) mobile phases. Total run time was 13 min per 20 μL injection, in the electrospray interface positive mode (ESI+). Samples were quantified in reverse phase LC/MS/MS with an Agilent 1290 separation module (Agilent Technologies, Palo Alto, California, USA) and an Agilent 6460 triple quadrupole mass spectrometer and Jet Stream electrospray interface. An Acquity UPLC HSS T3 column (100×2.1mm, 1.8 μm) separated the analytes (Waters, Milford, Massachusetts, USA). Working conditions were as follows: column temperature, 40 °C; sample temperature, 4 °C; capillary voltage, 3000 V; needle voltage, 0 V; gas temperature, 300 °C; gas flow, 5 L min^{-1}; nebulizer pressure, 40 psi; and delta EMV, 500. The sheath gas temperature and flow were 400°C and 12 L min^{-1}, respectively. Nebulization and desolvation gas were provided by a high purity nitrogen generator NM 32LA 230V (Peak Scientific Instrument Ltd., United Kingdom) and the collision gas was >99.9% nitrogen (Airgas, Denver, Colorado, USA) at 25 psi. Fragmentation and collision energy settings were done individually for each compound. Multiple

reaction monitoring (MRM) for dansyl-EE2 and labeled dansyl-EE2 ($^{13}C_2$-EE2) was implemented with Agilent Optimizer software. The dansyl-EE2 transition 530.1 amu→171.1 amu and $^{13}C_2$-EE2 dansyl-EE2 transition 532.1 amu→171.1 amu were used for quantification, and the confirmation ion was 156.0 amu. Quantification was done by matrix-matched isotope dilution standard calibration. A dwell time of 200 ms per ion pair was used. Agilent MassHunter software was used for data acquisition (version B.04.01) and quantification (version B.05.00).

Preparatory Blanks. During the course of running the mesocosm samples on the LC/MS/MS complications arose that were not present during method development using ddH_2O as the matrix. We observed interfering peaks with the same retention time as EE2 equivalent to 1-3 ng L^{-1} in the prep blanks (PB) (Table S3) on 29 June 2011 whole and filtered water and the 13-July-2011 whole water samples. We believe interference became evident because we were using lake water as the matrix. As such, we anticipated that numerous compounds with a phenol group, or other compounds with a free hydroxyl could be present in the water that would be subjected to derivatization to dansyl chloride. To remedy this we included the post-elution wash steps described above. We also tested a variety of columns to identify one that would give good separation of peaks in the total ion chromatogram. The following columns were tested: 1) Agilent Zorbax Extend-C18, 5 µm, 4.6 × 50 mm; 2) Agilent Zorbax SB-C18, 1.8 µm, 2.1 × 50 mm; 3) Agilent Zorbax Extend-C18, Rapid Resolution HT, 1.8 µm, 2.1 × 100 mm; 4) Waters Acquity UPLC BEH C18, 1.7 µm, 2.1 × 100 mm; 5) Waters Acquity HSS T3 C18, 1.8 µm, 2.1 × 100 mm, before resolving that the Waters Acquity HSS T3 column gave good peak separation.

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Table S1. Mean (Range) water quality parameters in the mesocosms.

[1]EE2 treatment	0 ng L^{-1}	5 ng L^{-1}	10 ng L^{-1}	20 ng L^{-1}
Temp °C	21.1 (18.5 - 23.3)	21.3 (18.9 - 23.5)	21.3 (18.4-23.4)	21.9 (19.5-27.3)
Dissolved O_2 mg/L	7.65 (7.13 - 8.07)	7.67 (7.11 - 8.01)	7.60 (6.62 - 8.03)	7.67 (7.06 - 8.16)
Conductivity µS/cm	322 (256 - 371)	323 (257 - 370)	323 (257 - 379)	326 (258 - 380)
Nitrate mg/L	1.33 (0.33 - 4.31)	1.25 (0.34 - 4.2)	1.05 (0.37 - 3.22)	1.06 (0.38 - 3.21)
pH	8.4 (7.9 - 8.6)	8.5 (8.3 - 8.6)	8.4 (8.3 - 8.7)	8.5 (8.3 - 8.7)

[1]EE2 = 17α-Ethynylestradiol

Table S2. Recovery of 17α-ethynylestradiol from matrix spikes.

Sample	Nominal Spike Concentration ($ng\ L^{-1}$)	Range of Measured Concentrations ($ng\ L^{-1}$)	Range of Recoveries	Average Concentration ± SEM ($ng\ L^{-1}$)	Average Recovery
Whole Water $n = 34$	25	20.8 - 30.29	83 - 121%	24.42 ± 0.41	96.5%
Filtered Water (0.45 µm) $n = 35$	25	21.95 - 27.33	88 - 109%	24.33 ± 0.28	97%

Table S3. Continuing calibration blank (CCB) and preparatory blank data for 17α-ethynylestradiol (ng L^{-1}) in filtered and whole water.

Matrix	Sample ID	Average	Standard Deviation	Minimum	Maximum
Filtered Water	CCB *n* = 13	0	0	0	0
	Prep Blank *n* = 18	0.31	0.75	0	2.45
Whole Water	CCB *n* = 21	0	0	0	0
	Prep Blank *n* = 29	0.5	1.14	0	3.64

Table S4: Measured 17α-ethynylestradiol (EE2) concentrations collected 30 min, 16 h, or 23 h after spiking. Data are mean±SEM and (range).

Nominal EE2 Concentrations	0ng L^{-1}	5ng L^{-1}	10ng L^{-1}	20ng L^{-1}
30 min	0.13±0.13 (0-0.92)	0.98 ± 0.11 (0.38-1.25)	4.30 ± 0.49 (2.76-6.24)	10.57 ± 0.65 (8.08-13.17)
16 h	0	1.56 ± 0.10 (1.45-1.77)	3.34 ± 0.28 (2.34-4.16)	7.58 ± 0.68 (5.70-10.57)
23 h	0	0.48 ± 0.17 (0-1.01)	1.38 ± 0.45 (0-3.19)	3.26 ± 0.13 (2.58-3.77)