

DISSERTATION

THE POPULATION ECOLOGY OF FATHEAD MINNOWS (*PIMEPHALES PROMELAS*) IN
ESTROGEN CONTAMINATED ENVIRONMENTS

Submitted by

Adam Richard Schwindt

Department of Fish, Wildlife, and Conservation Biology

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Fort Collins, Colorado

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Doctoral Committee:

Advisor: Dana L. Winkelman

William Clements

Chris Myrick

Howard Ramsdell

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ABSTRACT

THE POPULATION ECOLOGY OF FATHEAD MINNOWS (*PIMEPHALES PROMELAS*) IN ESTROGEN CONTAMINATED ENVIRONMENTS

Urban freshwater streams and rivers in arid climates are ecosystems dominated by wastewater effluent. Effluent contains a suite of bioactive chemicals including steroid and non-steroid estrogens that have been shown to disrupt vertebrate endocrine systems in laboratory studies. One of these steroid estrogens is 17 α -ethinylestradiol (EE2), a synthetic steroid estrogen used in human oral contraceptives. EE2 enters waterways after incomplete removal during wastewater treatment and can disrupt reproduction in fishes. However, little understanding exists of the ecological consequences of reproductive disruption.

My studies were initiated to evaluate how xenoestrogens might influence the population ecology of aquatic vertebrates. Specifically, I assessed the population ecology of fathead minnows (*Pimephales promelas*), a short-lived fish, that were exposed to early-life, life-time, and adult exposures of EE2. While assessment at the population-level was the goal, an understanding of environmental chemistry and ecotoxicology was needed to fully characterize the effects and consequences of EE2 and this is reflected in my dissertation that is organized into four chapters. Chapter 1, "Influence of community productivity on an estrogen added to aquatic mesocosms" identified chlorophyll a and nitrate as explanatory factors associated with the partitioning of EE2 to organic matter. In addition, it details the development of a high performance liquid chromatography tandem mass spectrometry method capable of quantifying EE2 at sub nanogram per liter concentrations. The method afforded the ability to accurately

measure EE2 concentrations during the experiments. Chapter 2, "Linking multiple biomarkers and varying exposure history in estrogen contaminated environments: is a comprehensive profile of fish health possible?" concludes that EE2 induces many physiological changes in fish at multiple levels of biological organization. Our results suggest that, depending on the timing of exposure (early-life, life-time or exposure as an adult), linking effects between biomarkers may be possible. Here we also demonstrate that the EE2 concentrations used in our experiments are environmentally relevant because fish caged below a wastewater treatment plant displayed a similar physiological response as the fish in the experiments. Chapter 3 "Fish population failure caused by an environmental estrogen is long-lasting and regulated by direct and parental effects on survival and fecundity" presents the effects of EE2 on population dynamics with empirically derived results from a one year long series of experiments. This chapter is the first to demonstrate that an early-life EE2 exposure to the parents causes reduced offspring survival despite the offspring never being directly exposed to EE2. Additionally, we found that an early-life exposure to EE2 caused permanent reproductive disruption and life-time exposures caused reproductive failure. Surprisingly, the summer long exposure to adult fish induced significant declines in male survival culminating with 100% mortality at the highest concentrations. In chapter 4, "A stochastic stage-structured modeling approach to evaluate the effects of estrogenic exposure on population growth rate in a short-lived fish" we demonstrate that EE2 can drastically reduce population growth rate (PGR) mediated by reduced reproductive output and juvenile survival. Declines in PGR were evident despite the lack of statistical significance on the effects of EE2 on egg, embryo, and juvenile fish production seen in the experimental data.

Overall my research demonstrates that by taking a holistic approach we can better understand the potential population-level and multigenerational effects of EE2, and the consequences for population growth.

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INTRODUCTION

The U.S. Environmental Protection Agency (EPA) is charged with regulating human made substances released into the environment that may have negative effects on human and wildlife health. Prior to establishing regulations a significant body of information must be presented demonstrating the effects of a chemical or group of chemicals. Specifically, the EPA stipulates that prior to regulation, studies should demonstrate an ecological effect on populations or communities as recommended by the Science Advisory Board (2008). For example, certain compounds are known to feminize male fish, but if there are no population-level consequences from exposure EPA has little basis for regulation. For pharmaceuticals, such as synthetic estrogens present in birth control, there is not enough information on ecological effects to make regulatory decisions.

Pharmaceuticals and personal care products (PPCPs) are released daily into aquatic environments from waste-water treatment plants and leakage from septic tanks (Daughton and Ternes, 1999). One drug of primary concern is the synthetic estrogen 17 α -ethinylestradiol (EE2), the active ingredient in most birth control pills used by humans. EE2 is excreted in the urine and feces and depending on the method of waste-water treatment > 90% of EE2 can be removed prior to discharge (Wise et al. 2010). However, EE2 exhibits biological activity in aquatic vertebrates at extremely low concentrations. Parrott and Blunt (2005) demonstrated reduced hatch success of fathead minnow (*Pimephales promelas*) embryos exposed to 0.32 ng/L EE2. Measured surface water EE2 concentrations range from non-detectable - 11.6 ng/L in studies using state-of-the-art methods (Kostich et al. 2013). Accordingly, environmental concentrations

of EE2 are relevant for reproductive disruption. The work presented herein used measured concentrations ranging from 3.2 - 10.9 ng/L, also environmentally relevant.

The EPA has recommended that ecotoxicological research take a more holistic, systems-level approach to assessing effects of PPCPs (Daughton 2004). My research integrates early-life effects, as well as short and long-term population effects, so that consequences of exposure can be interpreted at a population-level scale. The proposed experiments were conducted in newly constructed aquatic mesocosms as well as in the laboratory. Mesocosms provide a more ecologically relevant experimental system than indoor laboratories, while allowing control over confounding factors and replication of experimental treatments.

Recognizing the need for studies at multiple levels of biological organization, my research is organized into four chapters. In Chapter 1 we develop the EE2 analytical method and investigate the influence of community productivity on the distribution of EE2 in the water. Chapter 2 addresses the changes to the physiology, endocrinology, and morphology of individual fish. Chapter 3 addresses the effects of EE2 on multiple life-stages of fathead minnows, the influence of parental exposures on offspring survival, as well as the effects on short-term population dynamics in the mesocosms. Chapter 4 is a series of population models that address long-term effects on population growth rate (PGR) as well as the sensitivity of PGR to stochastic vital rates. These approaches yield valuable information to the scientific community and to policy makers charged with regulating PPCPs such as EE2.

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CHAPTER 1

INFLUENCE OF COMMUNITY PRODUCTIVITY ON AN ESTROGEN ADDED TO AQUATIC MESOCOSMS

SYNOPSIS

Ethinylestradiol, the synthetic estrogen in human oral contraceptives, enters waterways after incomplete removal during wastewater treatment and can disrupt reproduction in fishes. Due to concern associated with exposure of fishes to synthetic estrogens, we evaluated environmental factors associated with the distribution of estrogen in the water that could influence bioavailability. We constructed outdoor aquatic mesocosms that were supplied with water from a nearby impoundment. The mesocosms were allowed to colonize naturally with algae and invertebrates transported in the lake water and were stocked with fathead minnows (*Pimephales promelas*) to approximate a complete aquatic community. 17 α -ethinylestradiol (EE2) was added daily for 16 weeks in a randomized complete block design. Treatments consisted of EE2 at nominal concentrations of 0, 5, 10, 20 ng/L. We measured primary productivity, chlorophyll a, dissolved O₂, pH, conductivity, nitrate, and water temperature semi-weekly. Whole-water and 0.45 μ m filtered EE2 concentrations were analyzed using high performance liquid chromatography tandem mass spectrometry on a semi-weekly basis for eight weeks and measured concentrations ranged from 20 - 187% of nominal. The fraction of EE2 adsorbed to particulate matter ranged from 5 - 80%. We used mixed regression models and Akaike's Information Criteria to estimate the relative contributions of primary productivity, chlorophyll a, nitrate, and dissolved O₂ to EE2 in the aqueous phase. Results suggest that as

chlorophyll a and nitrate increased the concentration of EE2 in the aqueous phase decreased. The results suggest that more productive ecosystems may have less dissolved EE2 compared to that that is adsorbed or otherwise degraded. In natural ecosystems we propose that monitoring chlorophyll a, and nitrate can inform to what degree estrogens leave the aqueous phase.

INTRODUCTION

Estrogens can enter aquatic ecosystems from wastewater treatment plants or leakage from septic systems (Daughton 2002). The synthetic estrogen in human oral contraceptives, 17 α -ethinylestradiol (EE2), is of concern because exposure alters reproductive success in female fishes (Van den Belt et al. 2003) and feminizes male fishes (Lange et al. 2001). At higher levels of biological organization, reproductive disruption (Lange et al. 2001, Lange et al. 2009) and population declines (Kidd et al. 2007) have also resulted from EE2 exposure.

The occurrence, persistence, and distribution of EE2 in the environment have been reviewed (Ying et al. 2002). Numerous studies have described the adsorption of steroidal estrogens and other compounds to sewage sludge and removal from the water during wastewater treatment processes (Liu et al. 2009). Recent research describes the removal of EE2 from the water via the sequestration of EE2 by benthic biofilms (Writer et al. 2011) and sediments (Lai et al. 2000, Ying et al. 2003). The bioconcentration and biotransformation of EE2 by microbial and algal communities has also been demonstrated (Lai et al. 2002). However, less is known about the short-term fate of EE2 in the water prior to its incorporation into benthic organic matrices, such as periphyton, biofilm, or sediment. The aqueous and particulate distribution of EE2 in the water has implications for routes of exposure for pelagic fishes that are not benthic oriented – yet this has not been adequately evaluated.

Recent advances in multiple regression techniques permit ecologists and environmental scientists to identify and estimate parameters believed to control biotic and abiotic processes (Johnson & Omland 2004). Multi-model inference using Akaike's Information Criteria (AIC) permits the scientist to evaluate many models simultaneously and without the confines of arbitrary alpha values (Burnham & Anderson 2002). We used AIC to estimate the contribution of community productivity to the loss of EE2 from the aqueous phase to the organic phase given that EE2 is relatively hydrophobic. The use of AIC is common in the ecological literature, but is largely lacking in the environmental sciences. In this study, we demonstrate the utility of multi-model inference based on AIC and show that implementation is reasonably straightforward using a common statistical package and spreadsheets.

Analysis of steroidal estrogens is difficult because of their low concentrations in the environment and because they do not easily ionize during liquid chromatography mass spectrometry (LC/MS). For gas chromatography mass spectrometry methods, large sample volumes (1-2 L) must be extracted to obtain adequate sensitivity. One solution is to derivatize EE2 to a compound that more readily ionizes, which is the method employed in this study. The resulting high performance liquid chromatography tandem mass spectrometry (LC/MS/MS) method was highly sensitive, specific, and rapid. We measured the concentrations of EE2 in whole water and 0.45 μm filtered water added to an array of outdoor aquatic mesocosms. We used mesocosms as our experimental system because they approximate real ecosystems (Odum 1984) and treatment replication and randomized designs are possible, allowing stronger statistical inference. Specifically our objectives were to: 1) Develop a rapid and sensitive analytical method for measuring EE2. 2) Evaluate EE2 distribution in the water using pulsed additions over an

entire season. 3) Develop statistical models to evaluate the relationships between productivity, water quality parameters, and EE2 in the filtered water.

MATERIALS AND METHODS

Mesocosms

The mesocosms were 28 polyethylene tanks (Rubbermaid Corp., Winchester, VA, 2m in diameter, 0.66m deep, 1100 L) supplied with water from College Lake (Fort Collins, Colorado, USA). In the Foothills Fisheries Laboratory (FFL), Colorado State University, the lake water was mechanically filtered and irradiated with ultraviolet light and then pumped into a head tank that supplied water to the mesocosms. Water flow to the mesocosms was maintained to provide sufficient water quality for fathead minnows (*Pimephales promelas*) that were stocked in the mesocosms to approximate a natural aquatic community. Fish were treated in accordance with Institutional Animal Care and Use Committee protocol number 10-1685A at CSU. Water flow to the mesocosms was set by drilling a hole in the inflow pipe calibrated to 1 L/min. Maximum daily water temperatures increased to 28 °C six times and flow rates were increased to 2 L/min during that time. Water volume in the mesocosms averaged 1056 ± 4.4 L (SEM) and the mesocosms were flow-through during the day and static at night, as described further below. The mesocosms were aerated with ambient air at all times, were covered with 6.25 cm² netting (Memphis Net and Twine, Memphis, TN), and one third of the surface area of each mesocosm was shaded with landscaping fabric. The mesocosms colonized naturally with algae and invertebrates.

Chemicals

EE2 and labeled EE2 ($^{13}\text{C}_2$ -EE2) reference standards (> 98% purity) were purchased from Cambridge Isotopes Laboratories, Inc., (Andover, MA). Acetonitrile and methanol were LC/MS grade and were purchased from J.T. Baker Company (Phillipsburg, NJ). Formic acid (> 99% purity) was purchased from Acros Organics (Morris Plains, NJ). Toluene was high performance liquid chromatography grade purchased from Fisher Scientific (Fairlawn, NJ). High purity water (dd- H_2O , > 18 ohm) was obtained from a Barnstead nanopure water system (Dubuque, IA). Analytical grade sodium bicarbonate was purchased from J.T. Baker (Phillipsburg, NJ). Dansyl chloride (> 98% purity) was purchased from Sigma-Aldrich (St. Louis, MO).

EE2 spiking and sample collection

Mesocosms were spiked with EE2 daily. Concentrated stock solutions of EE2 dissolved in methanol were prepared monthly and stored at -20 °C. Working solutions were prepared daily by serial dilution of the stock solution using methanol rinsed glass serological pipets. The pipets were also used to deliver the EE2 (1 ml volume) at the nominal concentrations of 0, 5, 10, and 20 ng/L with seven mesocosms per concentration ($n = 7$). The 0 ng/L treatment served as the solvent control corresponding to approximately 1 mg/L methanol in the mesocosms. Water controls were not used due the low concentration of methanol.

EE2 was added to the mesocosms between 16:00 and 17:00 h beginning in April, 2012. The water to the mesocosms was turned off, and the appropriate volume of working solution was added to the plume of air bubbles in the middle of the mesocosm. We gently mixed the mesocosms with boat paddles, one for each treatment. The following day we turned the water on

between 08:00 and 09:00 h and the water remained on until the next EE2 addition. On one occasion a control (0 ng/L) mesocosm was accidentally spiked with the 10 ng/L solution.

We began adding EE2 into the mesocosms eight weeks before our first EE2 sampling to allow the EE2 to adsorb to the mesocosms. Following the first eight weeks we sampled water from the mesocosms for EE2 on a semi-weekly basis for the next eight weeks. After spiking and mixing with the paddles, we collected four 200 ml samples from different areas in the tank, composited the samples, and then sub-sampled 200 ml of the composite into clean 250 ml amber glass jars (Environmental Sampling Supply, Oakland, CA). Water samples were collected within 5 cm of the water surface and within 30 min of spiking. The samples were stored on ice and transported to the US EPA Region 8 Laboratory, Golden, CO, within 2-h. The samples were transferred to 4 °C and analyzed by LC/MS/MS within 8-d. On one occasion we evaluated the change in EE2 concentrations over a 24-h period. We collected water samples 3× over the course of 24-h consisting of: 1) a pre-spike sample; after the mesocosms had been in flow-through during the day, 2) a post-spike sample; 30 min after spiking and, 3) a static sample collected the following morning, before turning on the water. For all samples we report on the EE2 concentrations from whole water samples and from 0.45 µm filtrate.

Extraction of EE2 and derivatization to dansyl chloride

We prepared the water samples to enhance ionization during mass spectrometry and to ensure detection limits in the low ng/L range. Preparatory steps included: 1) extraction of EE2 with toluene, 2) dry down under N₂ gas, and 3) derivatization to dansyl chloride. Before extraction, samples were removed from 4 °C and allowed to warm to room temperature (22 °C). Each sample was shaken by hand to mix and then a new sterile disposable syringe (BD

Bioscience, Franklin Lakes, NJ) was rinsed in the sample water and then used to transfer 25 ml to a new 60 ml glass septa vial (I Chem, Rockwood, TN). To obtain filtered samples, we added 0.45 μm GHP (hydrophilic polypropylene) leuer lock filters (Pall Life Science, Port Washington, NY) to the tips of the syringes and slowly pushed the water through syringe and filter by hand. We added $^{13}\text{C}_2$ -labelled EE2 at 20 ng/L to each sample as an internal standard (ISTD). We extracted EE2 with 5 ml of toluene (Zhang et al. 2004), vortexed the samples at high speed for 10-s and placed them on a wrist action shaker for 10 min. We removed 2 ml of the toluene extract with a micropipette fitted with a sterile disposable aerosol resistant tip and transferred to new 15 ml glass conical centrifuge tubes (VWR, Radnor, PA). The extracts were capped and stored at -20 °C or immediately blown to dryness under a gentle stream of N_2 in a turbo vap (Caliper Life Sciences, Hopkinton, MA) set at 60 °C. For derivatization, we added 100 μL of freshly prepared dansyl chloride at 3 mg/ml in acetone and 100 μL of 100 mM NaHCO_3 pH 10.5 to the extract, vortexed at high speed for 1 min, incubated for 10 min in a 60 °C water bath, and transferred to high recovery amber silanized 2 ml vials (200 μL total volume). We then immediately commenced LC/MS/MS. Variations on the extraction and derivatization procedures can be found in several publications (e.g. Zhang et al. 2004, Lin et al. 2007, Lien et al. 2009, Yu et al. 2011).

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 mesocosms to help control for any matrix effects. Calibration standards were 1, 5, 10, 25, 50, and 100 ng/L. The quality control samples included continuing calibration blanks (CCB), prep blanks (PB), blank spikes (BS), and duplicate matrix spikes (MS) after every CC, every 20

samples, and at the conclusion of every run. The CCB was dd-H₂O, the PB was dd-H₂O spiked with ISTD, the BS was dd-H₂O spiked with 25 ng/L EE2 and ISTD, and the MS was same mesocosm water used for the CC spiked with 25 ng/L EE2 and ISTD. All quality control samples were extracted and derivatized 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. We calculated the method detection limit (MDL) and limit of quantification (LOQ) by spiking seven replicate 25 ml lake-water samples with 1 ng/L EE2 and then extracted, derivatized, and analyzed by LC/MS/MS. The mean and standard deviation (SD) of the EE2 concentrations in the replicate samples were calculated. The MDL = Student's $t_{(n-1, 1-\alpha = 0.99)} \times \text{SD}$. The LOQ = $3 \times \text{MDL}$.

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. The retention time for EE2 was 2.4 min 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. Samples were separated in reverse phase LC/MS/MS with an Agilent 1290 separation module (Agilent Technologies, Palo Alto, CA, USA) and quantified on an Agilent 6460 triple quadrupole mass spectrometer and Jet Stream™ electrospray interface. An Acquity UPLC HSS T3 column (100 $\times$ 2.1mm, 1.8 μm) separated the analytes (Waters, Milford, MA, 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; nebulizer pressure, 40 psi; and delta EMV, 500. The

sheath gas temperature and flow were 400 °C and 12 L/min, respectively. Nebulization and desolvation gas were provided by a high purity nitrogen generator NM 32LA 230 V (Peak Scientific Instrument Ltd., UK) and the collision gas was > 99.9% N₂ (Airgas, Denver, CO, USA) at 25 psi. Fragmentation and collision energy was set individually for each compound. Multiple reaction monitoring for dansyl-EE2 and labeled dansyl-EE2 (¹³C₂-EE2) was implemented with Agilent Optimizer software. The dansyl-EE2 transition 530.1 amu→171.1 amu and ¹³C₂-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 ddH₂O as the matrix. We observed interfering peaks with the same retention time as EE2 equivalent to 1-3 ng/L in the prep blanks (PB) (Table 1.2) 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 $\times$ 100 mm; 4) Waters Acquity UPLC BEH C18, 1.7 μm , 2.1 $\times$ 100 mm; 5) Waters Acquity HSS T3 C18, 1.8 μm , 2.1 $\times$ 100 mm, before resolving that the Waters Acquity HSS T3 column gave good peak separation.

Water quality and productivity parameters

We chose commonly used community productivity and water quality parameters that are easy to estimate with standard laboratory and field equipment. For chlorophyll a (ChlA) measurements we followed EPA method 445 (USEPA 1997). Water from the mesocosms was collected in high density polyethylene amber bottles transported immediately into the adjacent FFL and 350 - 650 ml was filtered under vacuum through 0.45 μm glass fiber filters (Whatman, Piscataway, NJ). The filters were stored on dry ice and transported to the US EPA Region 8 laboratory, Golden, CO, and stored at -80 °C. Water temperature, dissolved O₂ (DO), pH, conductivity, and nitrate were measured with a YSI meter (YSI, Inc. Yellow Springs, OH). Net primary productivity (NPP) was estimated using the oxygen consumption method in "light bottle - dark bottle" experiments (Lenore et al. 1998) with an YSI meter and BOD bottle O₂ probe (YSI, Inc. Yellow Springs, OH). All samples were collected on a semi-weekly basis.

Data

Unless otherwise specified data are mean $\pm$ SEM. The fraction of EE2 adsorbed to suspended particles $> 0.45 \mu\text{m}$ was calculated as: $\text{EE2}_{\text{adsorbed}} = (\text{EE2}_{\text{ww}} - \text{EE2}_{\text{filtered}}) / \text{EE2}_{\text{ww}}$. Data were inspected for equal variance and normality by inspection of residuals versus predicted, and quantile-quantile plots respectively. All data were analyzed and are presented untransformed.

Ecological modeling

We used mixed regression models to identify the contribution of selected predictor variables to changes in EE2 concentrations in filtered water, relative to the EE2 in other phases, such as adsorbed to suspended particulate matter. The process we employed included the following steps: 1) Identify candidate predictor variables, 2) Reduce the number of variables based on correlation to other predictor variables, 3) Construct linear models of EE2 versus all possible combinations of predictor variables, and 4) Assess the contribution of the predictor variables to EE2 concentrations in the water using Akaike's Information Criteria (Burnham & Anderson 2002).

The candidate predictor variables were treatment (nominal EE2 concentrations), sampling timepoint, DO, pH, conductivity, nitrate, NPP, and ChlA. Because of the large number of mesocosms and the time required to collect water quality and primary productivity data, we were not able to collect all of the samples on the same day. Therefore, we had to interpolate between sample time-points to estimate the quantity of each variable on the date we collected the EE2 samples. We used Proc EXPAND in SAS version 9.3 (© 2012 SAS Institute Inc., Cary, NC) for the linear interpolation. Using the Proc CORR function in SAS we identified Pearson r correlations among the water quality variables. Conductivity and pH were highly correlated ($p < 0.05$) to the other water quality variables, and were not suspected to affect EE2 distribution in the water (within the measured range), so they were not included in the linear modeling. Linear models using all possible combinations of the selected predictor variables (DO, NPP, ChlA, and nitrate) as well as treatment and sampling timepoint were assembled in Proc MIXED (SAS) with 0.45 μm filtered EE2 concentrations as the response variable. The residuals and mesocosms were initially included as random effects. However, the mesocosms did not explain any variance so all

uncertainty was pooled in the residual in the final model. All other effects were fixed effects. A total of 64 models were developed including an "intercept only" model. We modeled filtered water because our objective was to evaluate the environmental parameters associated with variability in the aqueous EE2 concentrations.

For multi-model inference, we used Akaike's Information Criteria (AIC_c), corrected for small sample size (subscripted c) (Burnham & Anderson 2002). Multi-model inference based on AIC_c is not confined to arbitrary alpha values (like step-wise selection) or model-fit based on the adjusted r^2 for each combination of variables. Rather, it balances model fit (bias) and the number of parameters in the model (parsimony), using maximum likelihood estimation. The AIC_c value is default output in Proc Mixed and the rest of the calculations were made in Microsoft Excel spreadsheets (Microsoft Corp, Seattle, WA. The formulas are published elsewhere (Burnham & Anderson 2002, Johnson & Omland 2004) however a brief description of the calculations is warranted. The AIC_c were sorted from smallest to largest and the delta AIC_c was calculated as the difference between the model with the lowest AIC_c and every other model. The relative likelihood of each model was calculated as well as the Akaike weight. The relative likelihood is calculated as $e^{-0.5 \times \Delta AIC_c}$. The Akaike weight is found by dividing the relative likelihood for a given model by the sum of the relative likelihood for all models. The sum of the Akaike weights for models that contain a given predictor variable is the relative importance weight. The relative importance weight is the comparative strength of a given predictor variable relative to the other predictor variables.

Akaike's Information Criteria allows estimating model parameters or effect sizes that are unconditional on any particular model in the set through model averaging (Burnham & Anderson 2002). The result of model averaging is an estimated effect size (the slope in a linear model) of

each predictor variable based on all of the models in the analysis. To calculate the model averaged effect sizes we summed the Akaike weighted parameter estimates for all models. Taking ChlA as an example, we calculated: $\beta_{\text{chlorophyll a}} \times \text{Akaike weight} = \text{the Akaike weighted estimate for ChlA for a given model}$. Then we summed all the weighted parameter estimates to arrive at the average effect size. The parameter estimates (β 's) come from the default output of the Proc Mixed procedure in SAS using the “/solution” command after the model statement. The unconditional standard error (SE) for each effect is described by Johnson and Omland (2004). By "unconditional," we mean not conditional on any given model. The unconditional SE was calculated as: $SE_{\text{uncond}} = \text{Akaike weight} \times [(SE_{\beta})^2 + (\beta - \text{model averaged parameter estimate})^2]$. The SE_{β} is the standard error of the estimated parameter given in the default output from the SAS Proc Mixed procedure. Further quantitative details and theory behind Akaike Information Criteria and multi-model inference are presented by Burnham and Anderson (2002). See Johnson and Omland (2004) for a concise review of advancements in model selection in ecology.

RESULTS

LC/MS/MS Method

We developed a LC/MS/MS method for quantifying trace concentrations EE2 in whole lake-water and 0.45 μm filtered lake-water. The MDL was 0.1 ng/L and the LOQ was 0.3 ng/L. Each peak was examined and integrated manually if needed. Peaks and concentrations were evaluated individually and accepted if the ratio between the qualifier and quantification ions fell within 20% of the expected ratio based on ratios in the calibration curve. If a calculated concentration was below the MDL we report a concentration of one half the MDL. A lower LOQ

is likely possible by simply injecting a greater volume into the LC/MS/MS, or re-suspending the extracted EE2 in a smaller volume of the dansyl-chloride and NaHCO_3 solution. We did not observe matrix effects because the ISTD and preparation of calibration standards in lake water corrected for any effects of the lake water. Recoveries of EE2 from MS samples in whole water were 103% and 97% for the filtered water (Table 1.1). Data for the quality control samples, CCB and PB, are in Table 1.2. Ethinylestradiol added to dd- H_2O and then filtered was not found to bind appreciably to the 0.45 μm GHP filters.

EE2 concentrations in the mesocosms

Over the course of the eight week sampling period in 0.45 μm filtered water, measured concentrations ranged from 20% - 129% of nominal for the 5 ng/L treatment, 43 - 73% of nominal for the 10 ng/L treatment, and 41 - 62% of nominal for the 20 ng/L treatment (Figure 1.1A and Table 1.3). In the whole-water samples, measured concentrations ranged from 40 - 187% of nominal for the 5 ng/L, 96 - 168% of nominal for the 10 ng/L, and 112 - 166% of nominal for the 20 ng/L treatment (Figure 1.1B and Table 1.3). The detection of EE2 in the controls (0ng/L) (Figure 1.1 and Table 1.3) 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. An additional source of EE2 is the one-time accidental addition of EE2 to a 0 ng/L mesocosm.

We investigated the possibility that EE2 was accumulating in the mesocosms (Figure 1.1B) by collecting three samples over the course of 24-h. In the 10 and 20 ng/L treatments EE2 concentrations were lowest in the pre-spike samples. These samples were collected after the mesocosms were flow-through for 15-h. The concentrations more than doubled post-spike, and

then declined slightly in the samples collected after the mesocosms were left static overnight (Figure 1.1 and Table 1.3). A similar pattern was evident in the 5 ng/L treatment in the pre- and post-spike samples; however, the concentrations continued to increase overnight while the mesocosms were static.

Following analysis of the whole water and filtered water samples we observed that whole water EE2 concentrations were noticeably greater than the filtered water (Figure 1.1). Therefore, we estimated the fraction of EE2 sorbed to suspended particulate matter > 0.45 μm (filter pore size). In the 5 ng/L treatment 5% to 79% of the EE2 in the mesocosm was adsorbed to particulate matter (Figure 1.2). Likewise, 24% to 79% was adsorbed in the 10 ng/L treatment and in the 20 ng/L samples 17% to 80% was sorbed to suspended particulate matter.

Ecological modeling

Mean water temperature, DO, conductivity, nitrate, pH, ChlA, and NPP are shown in Table 1.4. Model averaged effect sizes, their unconditional standard errors, the relative weights, and the percent variance explained by each predictor variable are presented in Table 1.5. Because we did not transform the data our effect sizes are on the same scale as the response variable. Therefore, effect sizes can be used as multipliers to predict the change in EE2 per unit change in the predictor variable. For example, over the course of the summer the range in ChlA was 2.4 - 19.5 $\mu\text{g/L}$. A 17 $\mu\text{g/L}$ increase in ChlA corresponds to a 5.47 ng/L drop in EE2 in filtered water ($17 \times -0.321 = -5.47$) where -0.321 is the effect size for ChlA in Table 1.5. The standard errors for the modeled effect sizes are very large, especially for NPP, DO, and nitrate (Table 1.5). The uncertainty in the effect sizes should be considered when interpreting these results.

Examination of the relative importance weights indicates that treatment best predicts the distribution of EE2 in the water (Table 1.5). This is expected because the nominal concentration of EE2 is part of the experimental design. As far as the environmental predictor variables, ChlA was weighted highly compared to the other predictors (Table 1.5, 1.6). Next was primary productivity and nitrate, while DO and sampling time-point were comparatively un-influential relative to the other predictors (Table 1.5, 6). That ChlA is the best environmental predictor is further supported by the fact that models containing NPP, DO, and nitrate only rank highly (based on AIC_c values) when coupled with ChlA (Table 1.6). The percent variance explained by each predictor variable was also calculated (Table 1.5). ChlA and nitrate (in addition to treatment which was expected) explained the most variance in models when they were the only predictor variable. The results of estimating model averaged effect sizes and their relationship to aqueous EE2 in the mesocosms are summarized in Figure 1.3.

No model yielded an Akaike weight ≥ 0.9 (Table 1.6) indicating that there was support for numerous models as explanatory factors for the variation in aqueous EE2 concentrations (Burnham & Anderson 2002). This suggests that the contribution of each explanatory variable should be averaged over all models (Burnham & Anderson 2002), as described in the previous paragraphs. The result was a model averaged effect size and measure of uncertainty for each predictor variable. For example a negative effect would indicate that as a given predictor increases, EE2 in the aqueous phase would be expected to decrease.

DISCUSSION

We initiated our study to evaluate the distribution and behavior of EE2 in the water of outdoor mesocosms. Our investigation focuses on EE2 behavior in the water, as opposed to

sediments, plant matter, or other matrix, because of the lack of published data on this process and its implications for exposed fishes. To our understanding this is the only study that experimentally manipulated EE2 concentrations, estimated the fraction adsorbed to suspended particulate matter, and evaluated the influence of community productivity on the behavior of EE2 in the water in a randomized and replicated design. The success of our work depended largely on the development of a sensitive and rapid LC/MS/MS method for quantifying EE2, given the duration of the experiment and the large number of samples analyzed. Results from our study suggest that EE2 binds to suspended particulate matter in the water and that process is associated with increased ChlA and nitrate. In other words, ChlA and nitrate are inversely related to aqueous EE2.

Whole water EE2 concentrations were always higher than the 0.45 μm filtered water suggesting that the EE2 was binding to suspended particulate matter. Our results do not suggest however that the particulate matter is becoming saturated with EE2 because the fraction of particulate bound EE2 is the same among treatments in our 24-h static sample. If the particulates were saturated, the fraction bound would decline as EE2 increased. To a limited extent, elevated whole water EE2 concentrations relative to the filtered water could also be due to enhancement in the whole water or suppression in the filtered water. Recovery of EE2 from matrix spikes averaged 103% in the whole water and 97% in the filtered water, a 6% difference between the matrices. However, increasing the filtered sample concentration by 6%, to account for enhancement and suppression, does little to explain the difference in whole water and filtered water EE2 concentrations. This indicates that the LC/MS/MS method did not artificially create the differences in EE2 concentrations between the whole and filtered water. Our results are consistent with previously published work reporting that EE2 appears to move from the aqueous

phase to suspended particulates, or that EE2 degrades (Lai et al. 2000, Holthaus et al. 2002). Lai et al (2000) demonstrated in a laboratory study that suspended river sediments adsorbed EE2 within 30 min then desorbed over the next 5-h. Lai et al (2000) used EE2 concentrations in the parts per million range (ppm), well above what is found in wastewater effluent (Heberer et al. 2002). Similarly, in the study by Holthaus et al. (2002) 80% of parts per billion concentrations (ppb) of EE2 adsorbed within 24-h to particulate matter. Writer et al. (2011) demonstrated that river biofilms adsorb EE2 spiked concentrations of 1 - 100 µg/L (ppb) in the field. In our study we spiked the mesocosms with EE2 in the low parts per trillion (ppt) range that is more indicative of environmental concentrations. EE2 in surface waters ranges from non-detectable to 6 ng/L in Europe (Belfroid et al. 1999), and to 273 ng/L in the USA (Kolpin et al. 2002), although the validity of the latter value has been questioned (Ericson et al. 2002). Given the orders of magnitude difference between the concentrations used in this study (ppt) and those cited above (ppm or ppb), we expect different sorption kinetics controlled EE2 partitioning in our study, although this was not explicitly tested.

The observed drop in EE2 overnight was likely due to a combination of microbial degradation, or sorption to algae, sediments, and to the polyethylene sides of the mesocosms. However we tried to minimize the effect of EE2 binding to the mesocosms by spiking for eight weeks before collecting samples. Photo-degradation could have contributed because up to 4-h of daylight remained following spiking. Several studies demonstrated that EE2 is removed from the water by adsorption to various substrates such as river sediments (Lai et al. 2000, Holthaus et al. 2002), biofilms (Writer et al. 2011), and algal cultures (Lai et al. 2002, Shi et al. 2010) as well as by biodegradation (Jürgens et al. 2002, Lai et al. 2002) and photodegradation (Jürgens et al. 2002, Mazellier et al. 2008). The estimated log K_{ow} for EE2 ranges from 3.67 (Lai et al. 2000) to

4.15 (Yoon et al. 2007) and suggests that EE2 readily leaves the water for organic matrices because it is relatively hydrophobic. We did not quantify K_{ow} , sorption kinetics, or a sorption isotherm for this study because the systems were not at equilibrium due to the daily additions of EE2. Further, good estimates of the aforementioned quantities are already available from the literature. The fraction of EE2 adsorbed to particulate matter in the water varied among EE2 treatments and over the course of the experiment, indicating that our systems were not at equilibrium. That the EE2 is not completely flushed out of, degraded, or bio-transformed in the mesocosms before the next addition explains why the measured concentrations are greater than nominal. Additionally, periodic small errors in pipetting the EE2 into the mesocosms could also explain why the measured concentrations were sometimes higher than nominal.

We evaluated the relationship between water quality and productivity to changes in EE2 concentrations using multi-model inference from AIC (Burnham & Anderson 2002). Increased ChlA, and nitrate were associated with a loss of EE2 from the aqueous phase. Chlorophyll a provides a measure of the organic matter in the water, nitrate in the water could fertilize the phytoplankton, yielding increased organic matter. EE2 is a relatively hydrophobic steroid (log K_{ow} of approximately 4), so its physical-chemical properties indicate an affinity for organic material. Therefore, as the organic matter increases (indicated by increased ChlA and nitrate) EE2 leaves the dissolved phase for the organic phase. Similar patterns were observed by Lai et al. (2000) where adsorption of EE2 to the sediment was correlated to increased total organic carbon. Likewise, Johnson et al. (1998) in their investigations of octylphenol, a weak xenoestrogen, found that sediments high in organic carbon more readily bound octylphenol than sediments lacking organic carbon. Sorption of the organic pesticide atrazine to pond sediments is also positively related to organic carbon (Gao et al. 1998a, b). ChlA is a photosynthetic pigment

in plants necessary for the transfer of energy that governs the overall process of synthesizing new organic material and is expressed a *quantity*. Conversely, net primary productivity is the *rate* of organic carbon fixation resulting from the combined processes of photosynthesis and community respiration (Bott 2006). NPP was only moderately related to EE2, indicated by the comparatively small effect size. The small effect of NPP suggests that the rate of carbon fixation is not as important as the standing crop of photosynthetic material, represented by ChlA, for the loss of EE2 from the aqueous phase. While neither ChlA nor NPP are direct measures of organic carbon, the use of ChlA and NPP as a surrogate analysis appears to be suitable given that estrogens were found to move from the water to organic matrices in all studies we found. Further, estimation of ChlA and NPP is cost effective, easily accomplished with standard limnology equipment, and integrates multiple levels of biological organization. The effects of nutrients represented by nitrate on EE2 follows a similar pattern to ChlA. That is, as nitrates increase, EE2 in the aqueous phase decreases. Taking the effects of ChlA and nitrate together we conclude that waters rich in nutrients and phytoplankton are associated with low dissolved EE2 relative to ecosystems that are nutrient and plankton poor. An alternative explanation is that the EE2 is altering the microbial community that in turns affects community productivity. This could also explain the association between dissolved EE2 with ChlA and nitrate. Regardless of the mechanisms regulating community productivity, the partitioning of EE2 to suspended particulates occurs before the EE2 adsorbs to sediments, biofilms, or plant matter although we did not test this explicitly. Conceivably, the loss of EE2 from the water is the first step for overall sequestration into other matrices.

In natural ecosystems we propose that monitoring ChlA and nitrate can inform to what degree estrogens leave the aqueous phase. We do not claim that these are the only important

parameters involved. And our results do not provide cause and effect relationships because we did not attempt to isolate the ultimate physical and chemical drivers that control EE2 partitioning. Collectively, the parameters we chose as predictor variables are likely indirect indicators of community photosynthesis, respiration, and nutrient input or overall community productivity. Regardless, our results suggest that as community productivity increases, EE2 moves from the aqueous phase to some other matrix, such as suspended particulate matter, or is subject to some form of degradation. Our modeling results also suggest that ChlA appears to be most important because it retains the highest relative importance weight, explains the most variance in the models, and holds the greatest effect size. These facts suggest that NPP and DO exhibit a degree of non-importance relative to ChlA. The whole water samples were always higher than the 0.45 μm filtered concentrations that suggests that EE2 is at least in part moving into the suspended particulate matter. The results discussed herein may be more applicable to estrogen impacted surface waters because effluent dominated systems have different water quality characteristics than non-effluent dominated systems (Brooks et al. 2006).

Very little is known about routes of exposure of steroidal estrogens for fishes. Presumably fishes are exposed over the gills during ventilation and possibly through the diet given that estrogens sorb to organic material. Results from this study suggest that, if aqueous exposures are more important than dietary, the more productive ecosystems could reduce the bioavailability of EE2 to fishes. For future work, we propose that indicators of community productivity be experimentally manipulated (in contrast to our study) while changes in EE2 are monitored. A mechanistic understanding of the effects of community productivity on estrogen partitioning will provide clues as to what types of ecosystems are at more or less risk of estrogen contamination.

Biotic and abiotic interactions in real ecosystems are constantly in flux. These interactions affect the distribution of EE2 in an ecosystem and are likely never at equilibrium. The literature to date, of which the citations in this paper are a good example, on the fate of EE2 in the environment is confined to laboratory studies using either simulated matrices, cultures, or using materials collected from rivers, aquifers, and holding ponds (Ying et al. 2002, Liu et al. 2009). We do not view this as a limitation; rather, these studies support the patterns observed in our study. Our objective was to use the mesocosms as surrogate functioning ecosystems and evaluate the chemical behavior of EE2 *in situ* using pulsed exposures. In aquatic environments receiving wastewater effluent, chemicals reach streams in a pulsed nature (Martinović et al. 2008, Nelson et al. 2010). We implemented a pulsed pattern with daily EE2 spikes and then identified ecosystem characteristics that explained, to some degree, the variability in the EE2 concentrations. While the mesocosms are human constructs and not natural ecosystems, Odum (1984) argued that they are effective tools to bridge the gap between nature and the laboratory. As such we advocate the use of mesocosms and further research is needed to better evaluate routes of exposure, and the effects of complex aquatic communities on the fate and transport of estrogens and xenoestrogens in the environment.

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Table 1.1. Recovery of 17 α -Ethinylestradiol from Matrix Spikes in 0.45 μ m Filtered Water (FW) and Whole Water (WW).

Sample ¹	Nominal (ng/L)	Measured Range (ng/L)	Range % Recovery	Mean $\pm$ SEM (ng/L)	Mean % Recovery
FW ($n = 35$)	25	21.95 - 27.33	88 - 109%	24.33 $\pm$ 0.28	97%
WW ($n = 26$)	25	23.14 - 30.29	92 - 121%	25.87 $\pm$ 0.32	103%

Table 1.2. Analysis of 17 α -Ethinylestradiol in Continuing Calibration Blanks (CCB) and Preparatory Blanks (PB) in 0.45 μ m Filtered (FW) and Whole Water (WW).

Matrix	Sample ID	Average (ng/L)	Standard Deviation	Minimum (ng/L)	Maximum (ng/L)
FW	CCB ($n = 13$)	0	0	0	0
	PB ($n = 18$)	0.31	0.75	0	2.45
WW	CCB ($n = 13$)	0	0	0	0
	PB ($n = 21$)	0.69	1.30	0	3.64

Table 1.3. Mean $\pm$ SEM 17 α -ethinylestradiol Concentrations in 0.45 μ m Filtered Water (FW) and Whole Water (WW) Over the Entire Season and Over 24-h.

Sampling Timepoint	0 ng/L	5 ng/L	10 ng/L	20 ng/L
Entire Season (Range, 4 Timepoints)	^{FW} 0.13 $\pm$ 0.13	^{FW} 0.98 $\pm$ 0.11	^{FW} 4.30 $\pm$ 0.49	^{FW} 8.15 $\pm$ 0.55
	to	to	to	to
	0.54 $\pm$ 0.12	6.47 $\pm$ 1.19	7.33 $\pm$ 0.33	12.41 $\pm$ 0.80
	^{WW} 0	^{WW} 2.02 $\pm$ 0.15	^{WW} 9.61 $\pm$ 1.47	^{WW} 22.36 $\pm$ 0.89
Pre-spike (24-h)	to	to	to	to
	1.17 $\pm$ 0.32	9.38 $\pm$ 0.44	16.84 $\pm$ 0.81	33.35 $\pm$ 2.55
	^{FW} 0	^{FW} 0.48 $\pm$ 0.17	^{FW} 1.38 $\pm$ 0.45	^{FW} 3.26 $\pm$ 0.13
	^{WW} 0.04 $\pm$ 0.03	^{WW} 1.98 $\pm$ 0.07	^{WW} 4.17 $\pm$ 0.45	^{WW} 8.63 $\pm$ 0.58
Post-spike (24-h)	^{FW} 0.13 $\pm$ 0.13	^{FW} 0.98 $\pm$ 0.11	^{FW} 4.30 $\pm$ 0.49	^{FW} 10.57 $\pm$ 0.65
	^{WW} 1.16 $\pm$ 1.16	^{WW} 2.02 $\pm$ 0.15	^{WW} 9.81 $\pm$ 0.75	^{WW} 22.36 $\pm$ 0.89
Static (24-h)	^{FW} 0	^{FW} 1.56 $\pm$ 0.10	^{FW} 3.34 $\pm$ 0.28	^{FW} 7.58 $\pm$ 0.68
	^{WW} 0.11 $\pm$ 0.10	^{WW} 4.07 $\pm$ 0.19	^{WW} 8.40 $\pm$ 0.85	^{WW} 19.13 $\pm$ 1.13

Table 1.4. Mean (Range) Water Quality and Primary Productivity Indicators.

EE2 Treatment	0 ng/L	5 ng/L	10 ng/L	20 ng/L
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)
DO ¹ (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)
Cond ² (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)
ChlA ³ (µg/L)	7.9 (3.4-14.2)	6.8 (2.4 - 19.5)	6.1 (3 - 10.6)	6.0 (3.2 - 12.8)
NPP ⁴ (mgC/m ³ /hr)	32.5 (6.7 - 111.4)	18.3 (0 - 129.4)	17.4 (0 - 42.7)	11.7 (0 - 36)

¹DO = Dissolved O₂²Cond = Conductivity³ChlA = Chlorophyll a⁴NPP = Net Primary Productivity

Table 1.5. Model Averaged Effect Sizes $\pm$ Unconditional SE, Relative Importance Weights, and Percent Variance Explained by Predictor Variables on 17 α -Ethinylestradiol from Filtered Water.

Explanatory Variables	Treatment	Sampling Period	ChlA ¹	DO ²	NPP ³	Nitrate
Effect Size	1.8 $\pm$ 1.315	0.072 $\pm$ 0.211	-0.321 $\pm$ 0.118	0.072 $\pm$ 0.64	0.017 $\pm$ 0.019	-0.134 $\pm$ 1.089
Weight	1.000	0.296	0.972	0.251	0.585	0.209
% Variance	75.71%	0.54%	16.37%	0.13%	11.31%	16.20%

¹ChlA = Chlorophyll a

²DO = Dissolved O₂

³NPP = Net Primary Productivity

Table 1.6. Results From Multi-Model Inference Using Akaike's Information Criteria.

Model ¹	Rel Wt ²	K ³	-2log L ⁴	AIC _c ⁵	Δ AIC _c ⁶	Rel L ⁷	Akaike Wt ⁸	Evidence Ratio ⁹
Trtmt + ChlA		4	342.8	351.3	0.9	0.64	0.15	1.57
Trtmt+Period+NPP+ChlA		6	339.3	352.4	2	0.37	0.09	2.72
Trtmt+ChlA+NPP+NO3		6	339.5	352.6	2.2	0.33	0.08	3.00
Trtmt+DO+ChlA+NPP		6	339.5	352.6	2.2	0.33	0.08	3.00
Trtmt+Period+ChlA		5	342	352.8	2.4	0.30	0.07	3.32
Trtmt+ChlA+NO3		5	342.6	353.4	3	0.22	0.05	4.48
Trtmt+DO+ChlA		5	342.6	353.4	3	0.22	0.05	4.48
Trtmt+Period+NO3+NPP+ChlA		7	338.6	354.1	3.7	0.16	0.04	6.36
Trtmt+DO+ChlA+NPP+NO3		7	339.2	354.7	4.3	0.12	0.03	8.58
Trtmt+Period+DO+NPP+ChlA		7	339.3	354.8	4.4	0.11	0.03	9.03
Trtmt+Period+DO+ChlA		6	341.9	355	4.6	0.10	0.02	9.97
Trtmt+Period+NO3+ChlA		6	342	355.1	4.7	0.10	0.02	10.49
Trtmt+DO+ChlA+NO3		6	342.5	355.6	5.2	0.07	0.02	13.46
Trtmt+Period+DO+NO3+NPP+ChlA		8	338.6	356.6	6.2	0.05	0.01	22.20
Trtmt+Period+DO+NO3+ChlA		6	341.3	357.4	7	0.03	0.01	33.12

Table 1.6. Continued

Model ¹	Rel Wt ²	K ³	-2log L ⁴	AIC _c ⁵	Δ AIC _c ⁶	Rel L ⁷	Akaike Wt ⁸	Evidence Ratio ⁹
Trtmt	1.000	3	351.8	358.1	7.7	0.02	0.00	46.99
Trtmt+Period		4	349.9	358.5	8.1	0.02	0.00	57.40
Trtmt+DO		4	350.6	359.2	8.8	0.01	0.00	81.45
Trtmt+Period+NO3		5	348.7	359.5	9.1	0.01	0.00	94.63
Trtmt+NPP		4	351.4	359.9	9.5	0.01	0.00	115.58
Trtmt+NO3		4	351.7	360.2	9.8	0.01	0.00	134.29
Trtmt+Period+NO3+NPP		6	347.3	360.5	10.1	0.01	0.00	156.02
Trtmt+Period+NPP		5	349.7	360.5	10.1	0.01	0.00	156.02
Trtmt+Period+DO		5	349.9	360.7	10.3	0.01	0.00	172.43
Trtmt+DO+NPP		5	350.2	361	10.6	0.00	0.00	200.34
Trtmt+DO+NO3		5	350.3	361.1	10.7	0.00	0.00	210.61
Trtmt+NPP+NO3		5	351	361.8	11.4	0.00	0.00	298.87
Trtmt+Period+DO+NO3		6	348.7	361.8	11.4	0.00	0.00	298.87
Trtmt+Period+DO+NPP		6	349.7	362.8	12.4	0.00	0.00	492.75
Period+DO+ChlA+NO3		6	438.8	451.9	101.5	0.00	0.00	>10 ⁵

Table 1.6. Continued

Model ¹	Rel Wt ²	K ³	-2log L ⁴	AIC _c ⁵	Δ AIC _c ⁶	Rel L ⁷	Akaike Wt ⁸	Evidence Ratio ⁹
Period+ChlA+NPP+NO3		6	439.9	453	102.6	0.00	0.00	>10 ⁵
NO3+ChlA		4	444.6	453.1	102.7	0.00	0.00	>10 ⁵
Period + NO3		4	442.3	453.1	102.7	0.00	0.00	>10 ⁵
Period+DO+ChlA+NPP+NO3		7	438.7	454.2	103.8	0.00	0.00	>10 ⁵
DO+NO3+ChlA		5	444	454.8	104.4	0.00	0.00	>10 ⁵
NO3+NPP+ChlA		5	444.3	455.1	104.7	0.00	0.00	>10 ⁵
Period+NPP+NO3		5	442.1	455.2	104.8	0.00	0.00	>10 ⁵
Period+DO+NO3		5	442.1	455.2	104.8	0.00	0.00	>10 ⁵
Period+DO+NPP+NO3		6	442.6	455.8	105.4	0.00	0.00	>10 ⁵
NPP+ChlA		4	449.6	458.1	107.7	0.00	0.00	>10 ⁵
ChlA	0.972	3	453.1	459.4	109	0.00	0.00	>10 ⁵
NO3	0.209	3	453.3	459.6	109.2	0.00	0.00	>10 ⁵
DO+NO3		4	449.1	459.9	109.5	0.00	0.00	>10 ⁵
NO3+NPP		4	451.6	460.1	109.7	0.00	0.00	>10 ⁵
Period+ChlA+NPP		5	449.4	460.2	109.8	0.00	0.00	>10 ⁵

Table 1.6. Continued

Model ¹	Rel Wt ²	K ³	-2log L ⁴	AIC _c ⁵	Δ AIC _c ⁶	Rel L ⁷	Akaike Wt ⁸	Evidence Ratio ⁹
DO+NPP+ChlA		5	449.5	460.3	109.9	0.00	0.00	$>10^5$
DO+ChlA		4	453	461.5	111.1	0.00	0.00	$>10^5$
Period+DO+ChlA+NPP		6	448.5	461.6	111.2	0.00	0.00	$>10^5$
DO+NO3+NPP		5	448.6	461.7	111.3	0.00	0.00	$>10^5$
Period+ChlA		4	453.1	461.7	111.3	0.00	0.00	$>10^5$
Period+DO+ChlA		5	452.6	463.4	113	0.00	0.00	$>10^5$
NPP	0.585	3	458	464.3	113.9	0.00	0.00	$>10^5$
Period+NPP		4	456.8	465.4	115	0.00	0.00	$>10^5$
DO+NPP		4	457.9	466.4	116	0.00	0.00	$>10^5$
Period+DO+NPP		5	456.2	467	116.6	0.00	0.00	$>10^5$
Intercept		2	467.8	472	121.6	0.00	0.00	$>10^5$
Period	0.296	3	467.4	473.7	123.3	0.00	0.00	$>10^5$
DO	0.251	3	467.7	474	123.6	0.00	0.00	$>10^5$
Period + DO		4	467.3	475.8	125.4	0.00	0.00	$>10^5$

¹Model: describes which parameters are included as explanatory variables in the Proc Mixed "Model" statement in SAS. The response variable is always EE2 in the filtered water. Trtmnt = nominal EE2 concentration added to each mesocosm (the experimental treatment), Period = sampling date, DO = dissolved oxygen, NO3 = nitrates, NPP = net primary productivity, ChlA = chlorophyll a

²Relative Weight (Rel Wt) = Sum of the Akaike Weights for each model that contains the parameter of interest.

³K = numbers of parameters estimated. All models contain the intercept, σ^2 , and at least one other β (except the intercept only model).

⁴-2log L and AIC_c are default output from Proc MIXED in SAS.

⁵ $\Delta AIC_c = AIC_c - AIC_{min}$ where AIC_{min} comes from the model with the smallest AIC_c value.

⁶Relative Likelihood (Rel L) = $e^{-0.5 \times \Delta AIC_c}$

⁷Akaike weight (Akaike wt) = Rel L for a given model / Σ Rel L for all models

⁸Evidence Ratio = Akaike wt for a given model / Akaike wt for the best model. The best model has the lowest AIC_c .

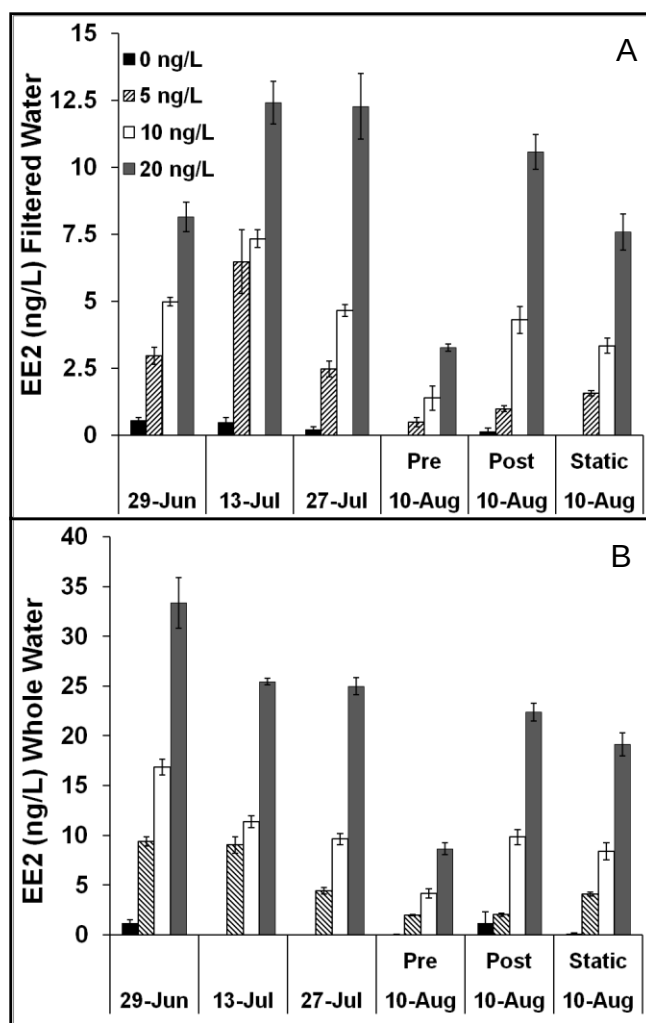


Figure 1.1. Measured 17 α -ethinylestradiol concentrations in 0.45 μ m filtered water (A) and whole water (B) over an eight week time course. Nominal concentrations are indicated by the pattern in the bars, black = 0 ng/L, striped = 5 ng/L, white = 10 ng/L, grey = 20 ng/L. Three samples were collected during 10-Aug sampling event. "Pre" refers to samples collected after 15-h of flow-through, "Post" is 30 min after spiking, and "Static" samples were collected after the water flow was turned off for 9-h. Bars are mean $\pm$ SEM.

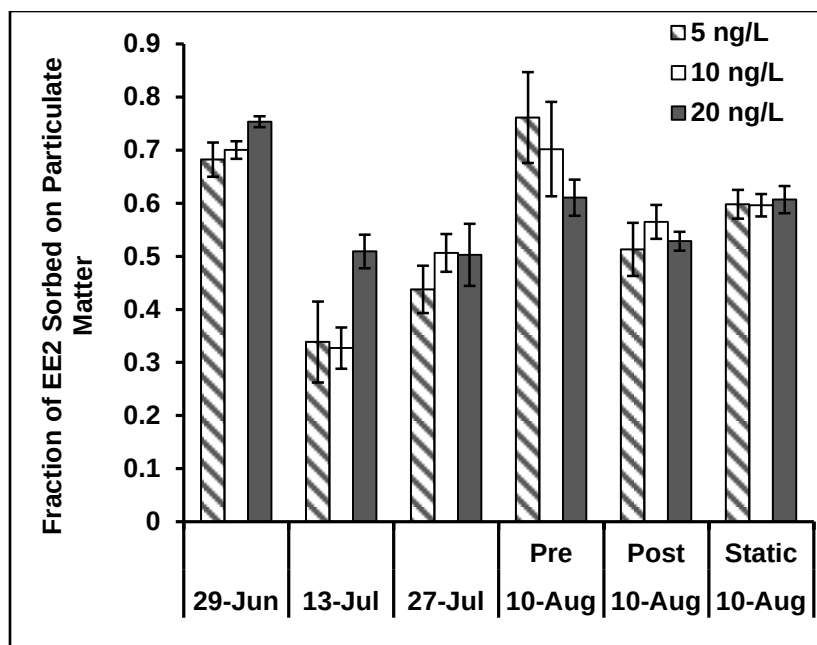


Figure 1.2. Fraction of 17 α -ethinylestradiol sorbed to suspended particulate matter. Particle size is greater than 0.45 μ m. Nominal concentrations are indicated by the pattern in the bars, striped = 5 ng/L, white = 10 ng/L, grey = 20 ng/L. Three samples were collected during 10-Aug sampling event. "Pre" refers to samples collected after 15-h of flow-through, "Post" is 30 min after spiking, and "Static" samples were collected after the water flow was turned off for 9-h. Bars are mean $\pm$ SEM.

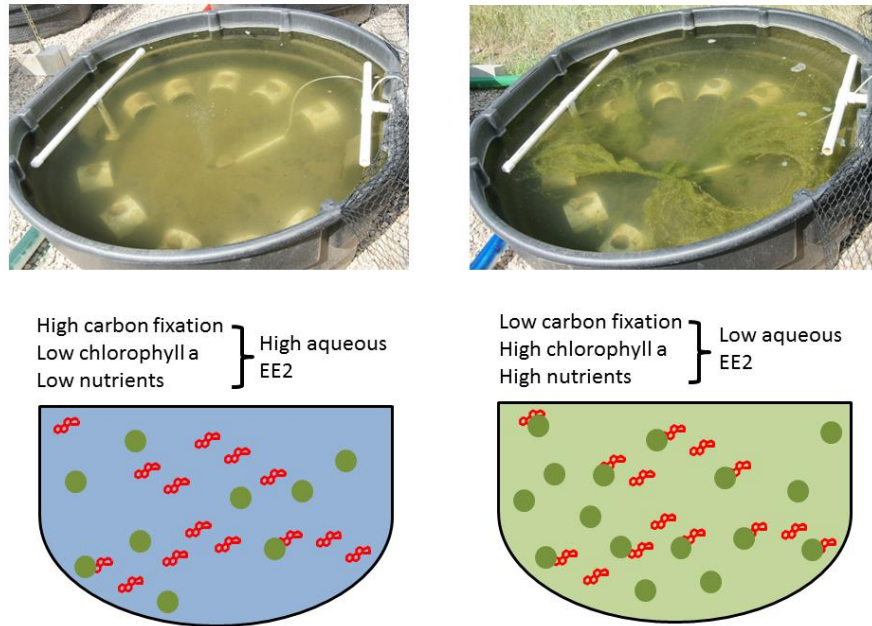


Figure 1.3. Conceptual diagram of the distribution of EE2 in the mesocosms in relation to primary productivity (carbon fixation), chlorophyll a, and nitrates (nutrients).

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CHAPTER 2

LINKING MULTIPLE BIOMARKERS AND VARYING EXPOSURE HISTORY IN ESTROGEN CONTAMINATED ENVIRONMENTS: IS A COMPREHENSIVE PROFILE OF FISH HEALTH POSSIBLE?

SYNOPSIS

Research on the physiological or endocrine effects of pharmaceuticals and personal care products rarely assess impacts at increasing levels of biological organization. To provide a more comprehensive picture of fish health in estrogen contaminated environments, we present findings from mesocosm, laboratory, and field studies on early-life, life-time, and short-term effects on the metabolome, gene expression, organ systems, and whole-fish morphology. 17α -ethinylestradiol (EE2) changed the external appearance of male fish to resemble females and induced ovipositors in juvenile fathead minnows (*Pimephales promelas*). Testicular ova were induced in male fish exposed to EE2 early in life or exposed for their entire life, but not in sexually mature males. Vitellogenin induction was similar in male fathead minnows exposed to either wastewater effluent or 3.2 ng/L of EE2 and significantly greater than fish at the reference site or not exposed to EE2. Effects of EE2 on the hepatic metabolome differed temporally. A 7-d EE2 exposure induced amino acid production and reduced abundance of metabolites associated with energy production. At 102-d EE2 generally induced energy metabolites and amino acids, although fewer metabolites were different than control compared to the 7-d exposure. These results indicate that EE2, a potent estrogen receptor agonist, induces many changes in fish at

multiple levels of biological organization. Our results suggest that, depending on the exposure window (early-life, life-time or adult), linking effects between biomarkers may be possible.

INTRODUCTION

Estrogens can enter aquatic ecosystems from wastewater treatment plants or leakage from septic systems and exhibit pseudo-persistence because they are continually discharged (Daughton 2002). The synthetic estrogen in human oral contraceptives, 17 α -ethinylestradiol (EE2), is of concern because exposure alters reproductive success in female fishes (Van den Belt et al. 2003) and feminizes male fishes (Lange et al. 2009). At higher levels of biological organization, reproductive disruption (Lange et al. 2001, Lange et al. 2009) and population declines (Kidd et al. 2007) have also resulted from EE2 exposure.

The effects of EE2 on fish vary widely from changes in metabolite profiles (Ekman et al. 2008, Ekman et al. 2009) to complete reproductive failure as described in Chapter 3. The severity of effects depends on the duration and window of exposure (Fenske et al. 2005, Nash et al. 2004). The use of experiments capable of identifying effects at multiple levels of biological organization can help link biochemical or organ level responses to individual changes that affect reproductive success (Ankley et al. 2010). However, few examples exist in the literature linking EE2 induced changes in subcellular metabolic networks to changes at the cellular level in gene expression to effects on organ systems and secondary sex characters. In this study we integrate the effects of EE2 on hepatic metabolite profiles, gene expression, reproductive development, and secondary sex characters using three different experimental systems and two generations of fish.

The objectives of our study were to: 1) evaluate the effects of early-life, life-time, and adult EE2 exposures on gonad microstructure, 2) assess feminization in immature fish and in adult males, 3) evaluate effects on the hepatic metabolome, and 4) link effects of feminization in the lab to fish exposed in the field.

MATERIALS AND METHODS

Experiments

Two experiments were conducted over the course of one year on two generations of fathead minnows. The experimental design, exposure history of each generation of fish, and population-level effects are described in detail in Chapter 3 of this document. Briefly, adult fathead minnows (F0 generation) were exposed daily for 102-d in a static renewal to EE2 (0, 3.2, 5.3, 10.9 ng/L measured) in outdoor aquatic mesocosms ($n = 7$) in a randomized complete block design. F1 offspring produced by the F0 generation were also exposed in the mesocosms. During the course of the mesocosm experiment, five additional adult males were caged *in situ* in the mesocosms for 7-d to evaluate the short-term effects of EE2 on the fish. The fish were caged in 15 cm diameter $\times$ 30 cm PVC pipes with 2 mm mesh fastened to the ends of the pipes with zip ties and the cages were placed on the bottom of the mesocosm. Following the mesocosm experiment, the F1 generation was transferred to the laboratory where randomly chosen mesocosms from the 0, 3.2, 5.3 ng/L treatments were split into a recovery group and a continuous exposure group ($n = 4$). Fish from the 0 ng/L treatment in the mesocosms either continued at 0 ng/L or were exposed to 4.9 ng/L measured EE2 in the laboratory. This design

resulted in a treatment structure that included an early-life exposure in fish allowed to recover from the EE2 and a life-time exposure for fish continually exposed (Table 2.1).

We also conducted a field study to evaluate the effects of potentially estrogenic wastewater effluent on male fish. Two wastewater treatment plants (WWTP) in the Denver, CO metropolitan area volunteered to participate in the study; a reference site was chosen on Clear Creek, a tributary of the South Platte River, near the mouth of Clear Creek Canyon in Golden, CO that was not directly subjected to wastewater effluent. Both WWTP1 and WWTP2 have primary and secondary (activated sludge) treatment, solids removal, and disinfection with chlorine. WWTP1 receives approximately 83×10^6 L/d and has a tertiary step including nitrifying trickle filters and a de-nitrification process for nutrient removal. WWTP2 receives approximately 454×10^6 L/d and has two treatment complexes (South and North). The tertiary treatment (biological nutrient removal) currently only occurs on the WWTP2's North complex. There is no nutrient removal on wastewater treated in the South complex.

Fifteen adult male fathead minnows were caged in the river within 50 m of the WWTP discharge in 15 cm $\times$ 150 cm PVC pipes that were capped on either end. Sections of PVC were removed and replaced with stainless steel mesh to allow water flow. Cages were anchored in a slow moving section of the stream using fence posts and bailing wire. This design is limited by pseudo-replication given that we have one cage per site, only one stream, and that inference is based on using individual fish as the experimental unit. Limitations in the number of fish available prevented the use of multiple cages per site. We deployed the fish on a Friday and retrieved them the following Tuesday (5-d exposure). Fish were euthanized and necropsies were performed as described below. All fish were treated in accordance with Institutional Animal Care and Use Committee protocol number 10-1685A at CSU.

Fish Necropsy

Following completion of the experiments, fish were euthanized in 250 mg/L tricaine methane sulfonate and livers were removed and preserved. Hepatic vitellogenin (VTG) mRNA was assessed following Biales et al. (2007). While processing the livers for VTG analysis, a portion of each liver was removed for metabolite profiling. Gonads were removed, placed in tissue cassettes, fixed for 24- to 48-h in Davidson's solution (Fournie et al. 2000), and then transferred to 10% buffered formalin. Male and female gonads were kept in separate vials and segregated by EE2 treatment.

Effects of EE2 on the Gonads

The gonads were processed for routine histological examination and stained with hematoxylin and eosin using standard procedures (Humason 1972). To evaluate the induction of testis-ova (predominantly testis with ova intermingled) in males, gonad sections (5 µm) were examined under 100× total magnification using transects of each section on the microscope slide with a Leica DM2500 compound light microscope outfitted with a Spot Idea camera and Spot image capture software version 4.6. When oocytes were found, their presence was confirmed on the adjacent section. We also evaluated the spermatogenic capacity of the males by subjectively scoring each testis based on a 5-point scale. A "0" indicated no sperm, "1" indicated < 10% of the testis had sperm, "2" indicated 11 - 30%, "3" indicated 31 - 50%, "4" indicated 51 - 70%, and "5" indicated 71 - 90%. No testis contained 100% sperm.

Induction of Ovipositors

We evaluated the size of ovipositors on juvenile fathead minnows from the F1 generation following the mesocosm experiment. Juvenile fish from each mesocosm that were not included in the laboratory study were euthanized in 250 mg/L tricaine methane sulfonate, and placed in 70% ethanol. All fish were immature as indicated by the absence of secondary sex characters. In the lab, ovipositor size (mm²) was determined for 10 fish from each mesocosm. Ovipositors were measured using ImagePro Express version 4.0.5 (Media Cybernetics, Inc. Rockville, MD) on digital images of fish captured with an Olympus SZX7-dissecting microscope (Olympus, Corp. Japan) outfitted with a Spot Insight camera (Diagnostic Instruments, Inc. Sterling Heights, MI) and Spot image capture software version 4.0.5 at 20× total magnification.

Liver metabolite extraction and ¹H-NMR

Adult fish from the F0 generation exposed for 7-d or 102-d were evaluated for changes in hepatic metabolites. F1 fish were not evaluated because liver masses were too small to allow extraction and analysis. Polar and lipophilic phases were extracted from frozen (-80 °C) liver using a biphasic procedure adopted from Viant et al. (2007). Briefly, individual liver samples were extracted in 1.5 mL microcentrifuge tubes using a dual-phase extraction process. The polar and lipophilic phases were removed by micropipette and transferred to 1.5 mL microcentrifuge tubes or 1.5 mL clear glass vials, respectively, and placed at 4 °C. Prior to NMR analysis, the polar and lipophilic samples were dried under nitrogen gas at 20 °C using a N-EVAP 111 (Organomation Associates, Inc.). The polar phase was reconstituted in 600 uL of 0.1 M sodium phosphate buffered deuterium oxide (pH 7.4) containing 50 µM sodium 3-(trimethylsilyl) propionate-2,2,3,3-d4 (TSP). The lipophilic phase was reconstituted in 600 uL of

CDCL₃:CD₃OD (2:1) containing 1.47 mM tetramethylsilane (TMS). Each supernatant was transferred to a 5 mm NMR tube. ¹H-NMR spectra from both phases were acquired at 21.5 °C on a 500 MHz JEOL Eclipse+ spectrometer (500.16 MHz, ¹H) equipped with a 5 mm triple resonance probe. Spectra from the polar phase was collected using a 1D-NOESY pulse sequence with 2.7-s acquisition time, 16,000 data points, 2048 scans per sample, and spectral width at 6,000 Hz. Spectra from the lipophilic phase were collected using a standard pre-saturation pulse sequence with 2.7-s acquisition time, 16,000 data points, 512 scans per sample, and spectral width at 6,000 Hz. A 2-s pre-saturation delay at a field strength of 40 Hz was employed to reduce the intensity of the residual water resonance. Note that while NMR spectra for both polar and lipophilic fractions were obtained, our results will focus on the polar fraction.

Ovipositor, VTG, and sperm abundance data analysis

Plots of residual versus predicted values and q-q plots were used to assess normality and equal variance. We used ANCOVA to evaluate the effects of EE2 on the size of ovipositors in the F1 generation with fish total length as a covariate. Models were fit using Proc GLM in SAS software version 9.3 (©2012, SAS Institute Inc., Cary, NC). Due to increasing variance, ovipositor data were log transformed. Due to a significant interaction between ovipositor size and total length we could not test for differences among intercepts at all lengths. Therefore, differences in ovipositor size were assessed at totals length of 20, 30, 40, and 50 mm using least squares means (adjusted means). A Tukey adjustment was included to control experiment-wise error rate. The hepatic VTG mRNA expression data were log transformed because of increasing variance. Differences in sperm abundance and VTG levels were assessed using linear mixed models with experimental block included as random effect using Proc MIXED in SAS. Pairwise

differences were assessed by least squares means with a Tukey adjustment. Type I error was arbitrarily set at $\alpha = 0.05$. Unless otherwise specified data are reported as mean $\pm$ SEM.

NMR data processing and analysis

Acquired spectra were processed using ACD/1D NMR Manager (Advanced Chemistry Development, Toronto, Canada). Samples were zero-filled to 32,768 points, exponentially line broadened to 0.3 Hz, and Fourier transformed. Spectra were then phase-corrected, baseline-corrected, and referenced to TSP. Subsequently, all spectra were binned (0.01 ppm wide bins) and exported to Microsoft Excel (Microsoft, Corp. Seattle, WA) spreadsheets. Two regions of the binned data were excluded to eliminate residual water (4.75 - 4.95 ppm) and methanol peaks (3.35 - 3.37 ppm). The remaining bins for each spectrum were normalized to unit total intensity to account for differences in processed tissue masses.

To identify metabolites that were significantly different between exposure classes (class being defined by both exposure duration and concentration), we generated ‘*t*-test filtered difference spectra’ based on the binned, edited, and normalized spectra. Previous studies have demonstrated the effectiveness and appropriateness of this method for assessing metabolites that are significantly altered by contaminant exposure (Collette et al. 2010, Ekman et al. 2012). These difference spectra were generated by first calculating an average class spectrum for each of the exposure classes. We then subtracted the average spectra of the control fish (0 ng/L) (e.g., 7-d controls) from the average spectra of the corresponding exposure class (e.g. 3.2 ng/L EE2). This was done separately for each sampling period (7-d and 102-d). In parallel with this subtraction of the average spectra, we applied a *t*-test to each spectral bin that made pairwise comparisons between exposed males and control males. This allowed us to assess if average differences

within a particular bin were significantly different between exposed and control fish. If the t -test for a particular bin comparison was not significant ($p \geq 0.05$), we set the average difference to 0, otherwise we reported the average difference. Differences that were > 0 indicate metabolites that were greater in the exposed males relative to the controls, and peaks with magnitudes of < 0 represented metabolites that were lower in the exposed males. For those bins that were found to be significantly different based on the t -test, we identified metabolite peaks using Chenomx NMR Suite 7.0 (Chenomx, Inc. Edmonton, Canada) and previously published spectra standards (Ekman et al. 2008, Wishart et al. 2009, Teng et al. 2009). The difference spectra presented in Appendix III, Figure AIII.1.

RESULTS

EE2 concentrations are presented in detail in Chapters 1 and 3; the results below are based on measured concentrations collected during the experiments. Because of the complex treatment structure during the laboratory study, EE2 concentrations and treatment structure are presented in Table 2.1 to aid in interpretation.

Effect of EE2 and wastewater effluent on VTG

EE2 significantly increased VTG mRNA in male fathead minnows exposed for either 7-d ($F_{3,24}=38.74$, $p<0.0001$) or 102-d ($F_{2,6.58}=45.81$, $p<0.0001$) (Figure 2.1). After 7-d of *in situ* cage exposure in the mesocosms VTG expression increased significantly at 3.2 ng/L (0.92 ± 0.13) ($t_{24}=7.33$, $p<0.0001$), 5.3 ng/L (1.39 ± 0.44) ($t_{24}=9.04$, $p<0.0001$), and 10.9 ng/L (2.04 ± 0.25) ($t_{24}=9.85$, $p<0.0001$) compared to control (0 ng/L) (0.003 ± 0.0008) (Figure 2.1A). In the 102-d EE2 exposure VTG expression was significantly greater than controls (0 ng/L) (0.006 ± 0.004) at

both 3.2 ng/L (1.92 ± 0.74) ($t_{6.75}=6.87$, $p=0.0008$) and 5.3 ng/L (4.90 ± 2.14) ($t_{5.85}=8.50$, $p=0.0002$) (Figure 2.1B). No male fish survived the 102-d exposure at 10.9 ng/L. VTG was also significantly different depending on field sites subjected to wastewater effluent or reference site water ($F_{2,39}=84.40$, $p<0.0001$). VTG expression in male fish at WWTP2 (0.88 ± 0.25) was elevated compared to both the reference site (0.0007 ± 0.0004) ($t_{39}=19.83$, $p<0.0001$) and WWTP1 (0.004 ± 0.003) ($t_{39}=13.82$, $p<0.0001$) (Figure 2.1A). VTG production was negligible in the laboratory experiment because all fish were unexposed for 53-d prior to sampling (see Chapter 3 for details on the experimental timeline). Males exposed to 3.2 ng/L for 7-d produced similar VTG expression levels as fish exposed for 5-d at WWTP2 (Figure 2.1A).

Effect of EE2 on Gonads

Analysis of male gonads from the laboratory experiment (effects on the F1 generation, see Chapter 3 for details) revealed abnormalities consistent with reproductive failure. Testis-ova were observed in 50% of the life-time exposed F1 male fish and in 14% of the early-life exposed males as well as in breeding F1 males exposed only as adults (Table 2.1, Figure 2.2A,B). Testis-ova were not found in the control (0 ng/L) fish. No males were found in two tanks subjected to continuous EE2 exposure (one at the low and one at the high dose). No testis-ova were found in the F0 adult male fish. EE2 reduced the spermatogenic capacity of the fathead minnows in the F1 generation ($F_{5,12}=6.10$, $p=0.005$). Relative amounts of sperm in control fish (3.5 ± 0.35) were significantly greater than in life-time exposed fish (1.63 ± 0.63) at the highest EE2 concentrations (5.3ng/L in the mesocosms, 10.8 ng/L in the laboratory) ($t_{12}=4.52$, $p=0.007$) (Table 2.1).

Effect of EE2 on Secondary Sex Characteristics

EE2 changed the external appearance of male fish in the F1 generation so that life-time EE2 exposed male fish at the highest EE2 concentrations (5.3 ng/L in the mesocosms, 10.8 ng/L in the laboratory) were nearly indistinguishable from females (Figure 2.2C-F). Male appearance was not affected in the adult F0 males or in the adult F1 males that were exposed only in the laboratory. EE2 also induced premature development of ovipositors on juvenile F1 fathead minnows. An interactive effect of EE2 treatment and total length was found ($F_{2,5}=235.76$, $p<0.0001$) so effects of EE2 on ovipositor size required evaluation at specific total lengths of the fish. EE2 significantly increased ovipositor size among all treatments and at all lengths tested ($0.0001<p<0.006$) (Figure 2.3). Ovipositors were not found on adult male fish from the F0 generation.

Effect of EE2 on hepatic metabolites

EE2 induced or suppressed hepatic metabolites in a temporally explicit manner but no consistent patterns emerged in relation to a concentration response within a given timepoint (Table 2.2). Metabolites associated with energy production were generally down-regulated at 7-d but up-regulated after 102-d exposure. The amino acid taurine was down-regulated at 7-d but was not different than controls at 102-d. The amino acids isoleucine, leucine, and valine were all up-regulated at 7-d but were not different than control after the 102-d EE2 exposure. The fatty acid biosynthesis intermediates, phosphocholine and N-methylnicotinamide, were up-regulated at 102-d. The magnitude of differences in metabolites between the control and each treatment are shown in Appendix III, Figure AIII.1.

DISCUSSION

In our study, we integrated the effects of EE2 on fathead minnows at multiple levels of biological organization. Exposure timing and duration appear to be key factors in determining response to EE2 exposure. At 102-d exposure, F0 adults, with no previous exposure history, exhibited VTG production in males, but no effects on gonad structure, external appearance, or ovipositor development, and reproductive output was not significantly affected (reproductive data are in Chapter 3). Additionally, VTG expression after 102-d was coincident with reduced male survival (survival data are in Chapter 3). A short-term 7-d EE2 exposure to adult males also induced VTG mRNA expression. In contrast to acute adult exposures, early-life and life-time EE2 exposures to the F1 generation induced durable effects on the male reproductive traits such as testis-ova in the gonads, reduced sperm production, and induced ovipositor development. These physiological changes were coincident with reproductive failure (Chapter 3), but not VTG production.

Up to 50% of the male fish in the F1 generation exposed to EE2 early in life or throughout their life-time developed the testis-ova condition. For the early-life exposure, the persistence of testis-ova in the gonads as adults suggests a developmental reorganization that permanently alters testicular structure (Guillette et al. 1995). Testis-ova were also induced in F1 males exposed only as adults demonstrating the plasticity of the fathead minnow gonad. It is not known if the testis-ova condition in F1 adult males is permanent, as in the early-life exposed fish, but the condition persisted at least 53-d in which fish were not exposed to EE2. Iteroparous fish retain primordial germ cells throughout their life suggesting exposures to steroid estrogens after normal differentiation could lead to testis-ova (Devlin & Nagahama 2002). It was interesting that the F0 adult males did not develop the testis-ova condition despite being exposed to similar EE2

concentrations for roughly the same period of time as the F1 generation. At the beginning of the mesocosm experiment the F0 males were sexually mature. The F1 males exposed only as adults were 4-6 months old and had undergone differentiation but had not yet reached sexual maturity. This suggests that the male gonad is still susceptible to estrogens even after differentiation; whereas, sexually mature male gonads are more resistant. This observation reinforces the significance of the exposure window. Early-life and life-time exposures are more costly to the fathead minnow than exposures occurring at sexual maturity alone. The testis-ova condition has been observed in numerous cases of endocrine disruption in the lab (e.g. Lange et al. 2001, Nash et al. 2004, Fenske et al. 2005) and the field (e.g. Woodling et al. 2006, Vajda et al. 2008, Schwindt et al. 2009) and has been implicated in reduced male fertility (Jobling et al. 2002).

Mature female fathead minnows deposit eggs using an ovipositor. Ovipositors have been found on male and immature fish exposed to steroid estrogens (Parrott & Blunt 2005) and, hence, are biomarkers of exposure. While we detected an interaction between fish length and ovipositor size, ovipositors were different among treatments at all lengths tested (20 - 50 mm total length). This suggests that ovipositor induction is a robust indicator of chronic estrogen exposures in juvenile fish of all lengths in the study. We do not know if the presence of an ovipositor in males interferes with reproduction. However, the induction of ovipositors in the EE2 exposed F1 fish is concerning from an energetic standpoint. The unnecessary growth of ovipositors in male fish or immature female fish could represent an energy loss that may be used for other needs, such as somatic and gonadal growth.

Secondary sex characteristics are important for queuing reproduction. Life-time and early-life exposures to EE2 changed the external appearance of the F1 males so that they resembled females. Previous work in our lab on the co-familial red shiner (*Cyprinella lutrensis*)

indicates this change is associated with disrupted reproductive behaviors (McGree et al. 2010). Other evidence exists that males feminized by EE2 can actually disrupt normal reproduction between unexposed fish (Nash et al. 2004). While we did not quantify reproductive behaviors in this study, the reduced appearance of masculine characteristics in the EE2 exposed F1 males was coincident with reduced reproductive success (Chapter 3). As in the gonads, we did not observe effects of EE2 on the already sexually mature F0 males. Perhaps elevated testosterone in sexually mature fish, prior to estrogen exposures, is somewhat protective of effects at the organ and whole-fish levels of biological organization.

In our data, VTG expression in male fathead minnows exposed to 3.2 ng/L was nearly identical to VTG expression in males caged below a WWTP in the Denver, CO metropolitan area. This suggests that the EE2 concentrations used in our study are environmentally relevant. Wastewater effluent is a complex chemical mixture and the VTG expression in those males caged in the field likely results from exposure to multiple estrogens. This suggests that the total estrogenicity of the effluent was comparable to 3.2ng/L EE2. Despite the extensive work measuring steroid estrogens in environmental samples it is not clear what represents an ecologically realistic concentration. In surface waters measured EE2 concentrations often exceed predicted concentrations derived from mass balance calculations (Anderson et al 2004). Recent efforts using a modeling framework estimated the extent of pollution by steroidal estrogens in US surface waters. Kostich et al. (2013) estimated that 6 ng/L EE2 was the maximum predicted concentration, falling near the median of observed concentrations that ranged from non-detectable to 11.6 ng/L for surface waters. Despite the disagreement between modeled and measured concentrations, we used EE2 concentrations within the range of measured surface water concentrations summarized by Kostich et al. (2013).

The effects of EE2 on hepatic metabolite profiles varied temporally but showed little effect of the actual EE2 concentrations. This could be due to the fact that the measured EE2 concentrations were not that different across treatments (3.2 - 10.9ng/L) compared to other studies where a concentration response was observed with EE2 concentrations at 10 and 100 ng/L (Ekman et al. 2008, Ekman et al. 2009). The estrogen receptor mediated effects (e.g. VTG induction) were much more sensitive to the EE2 concentrations than the metabolite profiles. Still interesting though is that the majority of response from the metabolome takes place at the 7-d timepoint because by 102-d few metabolites were different than the control fish. This suggests the effects EE2 has on liver physiology are attenuated after a long-term exposure, and that the animal has compensated for the effects, or compensatory mechanisms are near exhaustion (Schreck 2000). It is tempting to conclude that, because few metabolites were different than control, the 102-d exposed fish had compensated for the EE2 exposure. However, survival dropped significantly at 3.2 ng/L (Chapter 3) suggesting that hepatic compensatory mechanisms to the EE2 exposure were exhausted. Our understanding of survival in the EE2 treated males facilitates interpretation in this case. That is, because EE2 exposures resulted in reduced male survival suggests that a proportion of fish were not able to compensate. It would be difficult to distinguish between compensation and exhaustion in studies without complimentary information on fish survival.

An effect of exposure duration was found when comparing the VTG and metabolite profiles. A 7-d exposure induced various amino acids, presumably for the synthesis of VTG protein. However, at 102-d VTG mRNA expression was still elevated but amino acids were largely not different than control fish, suggesting, perhaps, that VTG transcription and translation became decoupled. Linking effects of pharmaceuticals at multiple levels of biological

organization is a significant need in ecotoxicology (Boxall et al. 2012) and our results indicate that linkages are context dependent (Table 2.3). For example, observation of VTG in male fathead minnows does not necessarily translate to reproductive failure for the population. Conversely, the absence of VTG is not necessarily indicative of a healthy population. Further research is needed in linking metabolite profiles to specific mechanisms of action and consequences for fish health. Studies implementing a multi-biomarker approach in fish exposed to multiple chemicals with multiple modes action are also needed.

In our study we demonstrated the utility of drawing inferences from multiple biomarkers at increasing levels of biological organization. This provides a more complete picture of the physiological state of the fish but still does not afford the luxury extrapolating to population effects. Our results complement the published literature, and advance our understanding of the linkages between biomarkers of estrogen exposures. Our results suggest that none of the physiological biomarker or endpoints used in our study, and many other studies, qualify as a definitive marker for population-level effects. Multigenerational experiments employing multiple endpoints are necessary to paint a complete picture of individual responses to environmental estrogens.

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Table 2.1. 17 α -Ethinylestradiol (EE2) Treatment Structure, Nominal and Measured Concentrations, Sperm Abundance, and Incidence of Testis-ova in Fathead Minnows (*Pimephales promelas*) in the F1 Generation.

Treatment	Nominal ¹ EE2 (ng/L)	Measured ² EE2 (ng/L) (range)	Sperm Abundance	% of Fish with Testis - Ova
Control	0 , 0	0.34 $\pm$ 0.04 ³ , 0.17 $\pm$ 0.1 ⁴ (0-1.3) , (0-0.39)	3.50 $\pm$ 0.35 ⁵	0
Low	0 , 5	0.34 $\pm$ 0.04 ³ , 4.95 $\pm$ 0.55 ⁴ (0-1.3) , (3.8-6.4)	3.30 $\pm$ 0.12	14.3% ⁶ (2 of 14 fish)
Low Early-Life Exposure	5 , 0	3.22 $\pm$ 0.64 ³ , 0.17 $\pm$ 0.1 ⁴ (0.38-12.8) , (0-0.39)	2.83 $\pm$ 0.17	14.3% (1 of 7 fish)
High Early- Life Exposure	10 , 0	5.32 $\pm$ 0.19 ³ , 0.17 $\pm$ 0.1 ⁴ (2.76-8.11) , (0-0.39)	3.25 $\pm$ 0.14	0
Low Life-Time Exposure	5 , 5	3.22 $\pm$ 0.64 ³ , 4.95 $\pm$ 0.55 ⁴ (0.38-12.8) , (3.8-6.4)	4.00 $\pm$ 0.57	50% (2 of 4 fish)
High Life-Time Exposure	10 , 10	5.32 $\pm$ 0.19 ³ , 10.75 $\pm$ 1.77 ⁴ (2.76-8.11) , (6.66-13.13)	1.63 $\pm$ 0.63*	50% (2 of 4 fish)

¹The first number is the nominal concentration the mesocosms, the second number is the nominal concentration in the laboratory study. The mesocosm study lasted 102-d and the laboratory study was 203-d and studies were conducted sequentially.

²The first set of numbers are the mean $\pm$ SEM of measured EE2 concentrations in the mesocosms, the second set of number are the measured concentrations in the laboratory study

³EE2 concentrations from 0.45 μ m filtered water

⁴EE2 concentrations from whole water

⁵Data are mean $\pm$ SEM

⁶Data are mean %

*Sperm abundance compared to 0 ng/L (t_{12} =4.52, p =0.007)

Table 2.2. Summary of Changes to Liver Metabolites of Fathead Minnows (*Pimephales promelas*) following 7-d or 102-d exposure to 17 α -Ethinylestradiol.

Metabolite	Statistically Significant ¹ Change (Up or Down) Relative to Control				
	7-d EE2			102-d EE2	
	3.2 ng/L	5.3 ng/L	10.9 ng/L	3.2 ng/L	5.3 ng/L
<i>Energy Metabolism</i> ²					
AMP, ADP, ATP ³	Down	Down	Down	Up	Up
Fumarate				Up	
Glucose	Down	Down	Down	Down	
Glycogen			Down		
Lactate				Down	Up
UDP-glucose (TA) ⁴			Up	Up	Up
<i>Amino Acids</i>					
Aspartate		Down			
Glutamate	Down				Up
Histidine	Down				Up
Isoleucine	Up	Up	Up		
Leucine	Up	Up	Up		
Phenylalanine	Up	Up	Up		Up
Taurine	Down	Down	Down		
Tryptophan			Up		
Tyrosine	Up	Up	Up		Up
Valine	Up	Up	Up		
<i>Others</i>					
Acetate				Up	
Creatine				Up	
Creatinine		Up	Up		
Formate			Down		
N-Methylnicotinamide (TA)				Up	
Nicotinate/Niacin (TA)		Down			
Phosphocholine				Up	

¹Determined by *t*-test, *p*<0.05 where direction "up" or "down" is relative to control

²Mathews et al. (2000)

³AMP=Adenosine monophosphate, ADP=Adenosine diphosphate, ATP=Adenosine triphosphate

⁴(TA)=Tentative assignment of the metabolite

Table 2.3. Summary of Physiological, Endocrine, and Whole-fish Effects of 17 α -Ethinylestradiol (EE2) or Wastewater Effluent (WWTP) on Fathead Minnows (*Pimephales promelas*).

Fish	Adult Sentinel	Adult Sentinel	F0 Generation	F1 Generation	F1 Generation	F1 Generation	F1 Generation
Experimental Venue	Field Study	Mesocosms	Mesocosms	Mesocosms	Laboratory	Mesocosms & Laboratory	Mesocosms & Laboratory
Treatment/Duration	WWTP/5-d	EE2/7-d	EE2/102-d	EE2/102-d	EE2/116-d	EE2/Early-Life	EE2/Life-Time
Endpoints ¹							
VTG	Yes	Yes	Yes	---	No	No	No
Testis	---	---	No	---	Yes	Yes	Yes
HM	---	Yes	Yes	---	---	---	---
Ovipositor	---	---	No	Yes	---	---	---
♂ Appearance	No	No	No	---	No	No	Yes

¹Yes = effects observed; No = no effects observed; VTG = vitellogenin in males; HM = hepatic metabolites; ♂ Appearance = external appearance of EE2 treated males compared to controls; "---" = no data.

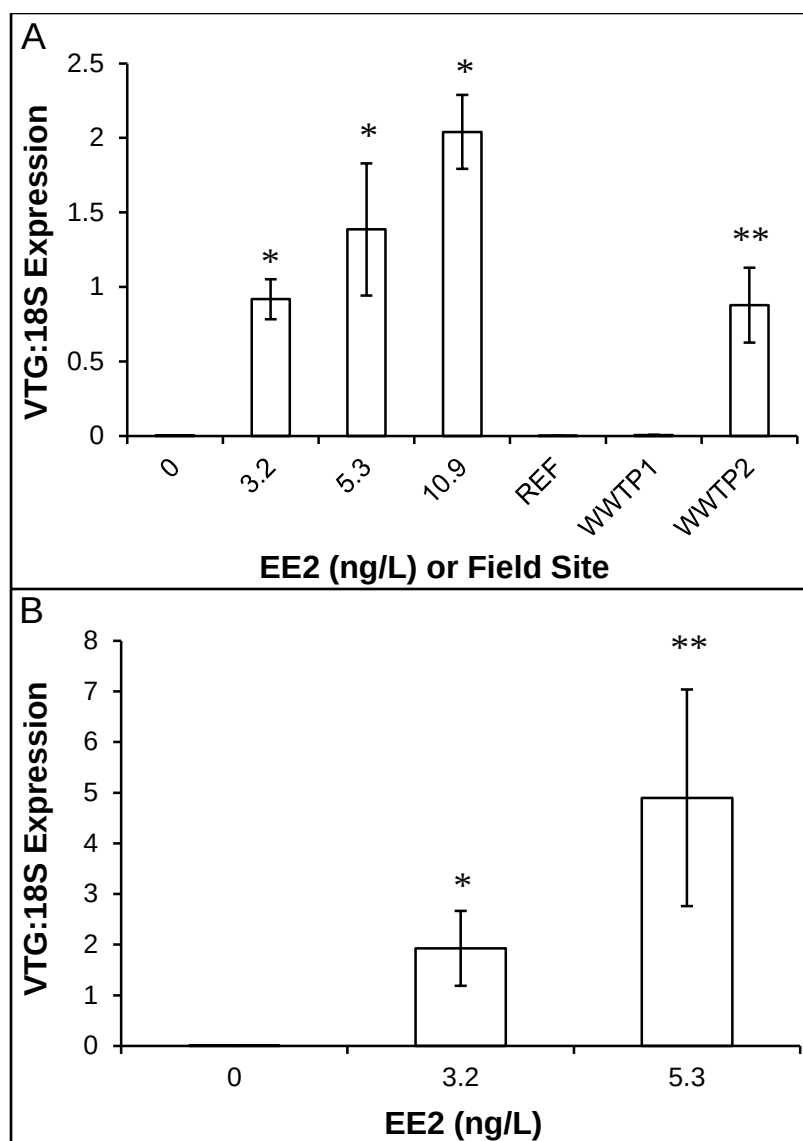


Figure 2.1. Effect of 17 α -ethinylestradiol (EE2) or wastewater effluent on male fathead minnow (*Pimephales promelas*) vitellogenin (VTG) mRNA expression in the liver. A 7d EE2 exposure (3.2 ,5.3, 10.9 ng/L) significantly induced VTG compared to control (0 ng/L) $^{*}(7.33 < t_{24} < 9.85, p < 0.0001)$ (A). VTG increased significantly in fish deployed below WWTP2 in relation to the reference site (REF) as well as compared to WWTP1 $^{**}(9.2 < t_{39} < 12.53, p < 0.0001)$ (A). Fish exposed for 102-d to EE2 significantly increased VTG in a concentration response manner compared to control $^{*}(t_{6.75} = 6.87, p = 0.0003)$; $^{**}(t_{5.85} = 8.5, p = 0.0002)$ (B).

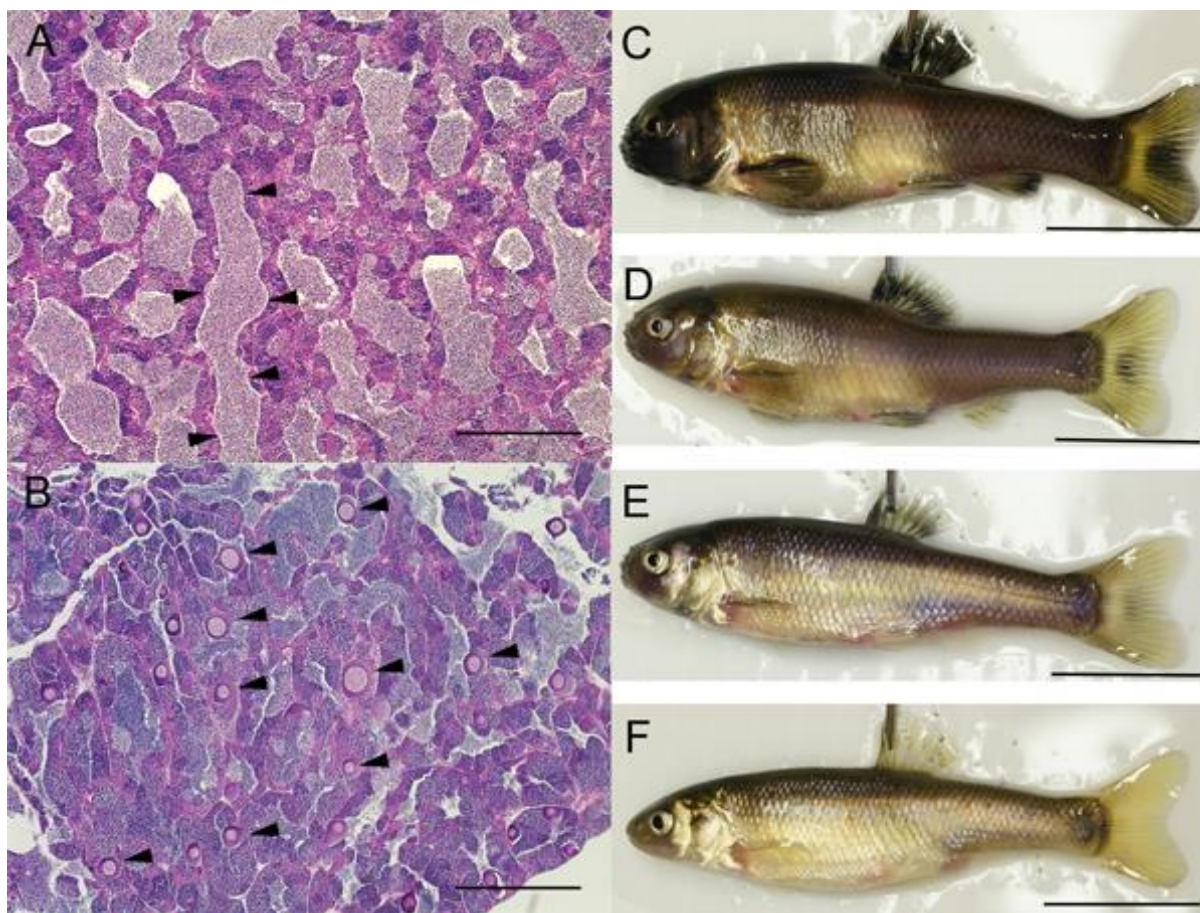


Figure 2.2. Effect of 17 α -Ethinylestradiol (EE2) on the male fathead minnow (*Pimephales promelas*) gonad and effects on secondary sex characteristics. In the control testis (A) arrows outline spermatozoa in the sperm duct indicating normal development. However, life-time exposure to 5.3 - 10.8ng/L EE2 (B) induced the abnormal testis-ova condition (arrows). Normal secondary sex characteristics are represented in the control fish (C), altered sex characteristics are shown in males exposed to 3.2 - 5.3ng/L EE2 (D) and 5.3 - 10.8ng/L EE2 (E). Shown for comparison is a control female (F). Pictured fish are representative of all fish in the treatment (C-F). Hematoxylin and Eosin, bars = 250 μ m in A&B. Bars = 1 cm in C-F.

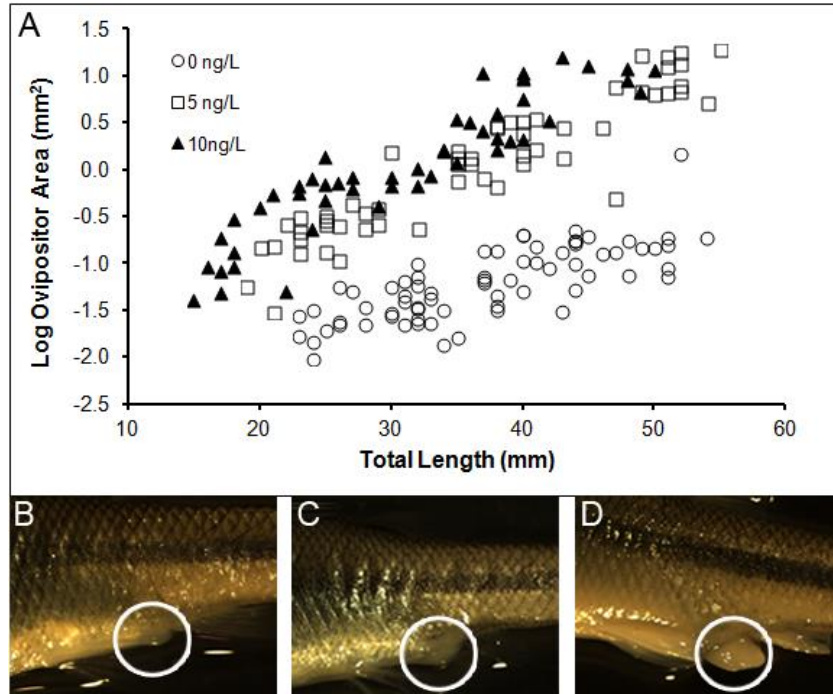


Figure 2.3. Effect of 17α -Ethinylestradiol (EE2) on the premature development of ovipositors in the juvenile fathead minnow (*Pimephales promelas*). An interaction between ovipositor area and total length (A) was found ($F_{3,5}=235.73$, $p<0.0001$) so statistical differences in ovipositor area were estimated at a total length of 20, 30, 40, and 50mm and all groups differed significantly from one another ($0.0001<p<0.006$). Circles in B-D show range of sizes of ovipositors observed from control (B) to EE2 treated fish (C & D).

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CHAPTER 3

FISH POPULATION FAILURE CAUSED BY AN ENVIRONMENTAL ESTROGEN IS LONG LASTING AND REGULATED BY DIRECT AND PARENTAL EFFECTS ON SURVIVAL AND FECUNDITY

SYNOPSIS

Despite significant research demonstrating effects of estrogens such as 17 α -ethinylestradiol (EE2) on fish, the underlying mechanisms regulating population failure are unknown. Projected water shortages could leave waterways increasingly dominated by wastewater effluent and understanding mechanisms is necessary for conservation and management. Here we identify mechanisms of population failure in three generations of fathead minnows including direct and parental effects on survival and fecundity. EE2 concentrations, as low as 3.2 ng/L, reduced F0 male survival to 17% and juvenile production by 40%. F1 offspring continuously exposed to EE2 failed to reproduce and offspring transferred to clean water reproduced 70 - 99% less than controls. Furthermore, survival of F2s was reduced 51% - 97% compared to controls, despite the absence of direct embryonic exposure. The indirect effect on F2 survival suggests the possibility of transgenerational effects of EE2. Our results suggest that chronically exposed populations may not be able to recover in the absence of immigration.

INTRODUCTION

Freshwater ecosystems contain approximately 40% of global fish diversity; freshwater ecosystems are highly threatened and faunal extinction rates (4%) are currently much higher than

in the past (Ricciardi & Rasmussen 1999; Dudgeon et al. 2006). 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 et al. 2006). For example, flow in the South Platte River downstream of the Denver, Colorado, metropolitan area ranges from 69 - 100% sewage effluent depending on the time of year (Strange et al. 1995, Dennehy et al. 1998). Similarly, in 285 of 582 permitted wastewater discharges in Texas, Oklahoma, New Mexico, Arkansas, and Louisiana sewage effluent comprises over 90% of the stream flow (Brooks et al. 2006). Exacerbating the problem is a projected decrease in stream flow in the inner-mountain and southwestern USA resulting from climate change (Barnett et al. 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 et al. 2009). Understanding how fish populations respond to effluent exposure is critical to management and conservation.

Wastewater effluent is 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, excreted by humans, that enter waterways after incomplete removal during wastewater treatment (Ternes et al. 2004). Steroidal estrogens, a class of compounds commonly found in wastewater effluents, negatively affect fish reproduction in laboratory studies (*e.g.* Lange et al. 2001, Nash et al. 2004, Fenske et al. 2005, McGree et al. 2009). Most studies on effects of estrogens have focused on physiological endpoints and use inferences based on these observations to speculate on population effects

(Relyea & Hoverman 2006; Forbes et al. 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 et al. 2008).

Research by Kidd et al. (2007) provided compelling evidence that estrogens 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 the observed population decreases. Histological evidence suggested that the observed declines in fathead minnow 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, estrogen exposure might also reduce adult survival (Thorpe et al. 2007) or survival of other life stages (Lange et al. 2001) thereby reducing abundance. Additionally, life-time exposure to estrogenic compounds may have greater impacts on survival and reproduction than acute exposures (Nash et al. 2004, Fenske et al. 2005, Zha et al. 2008). Most studies do not assess the parental effects (exposure to offspring through the parents) that a compound may have on populations, perhaps owing to the logistical challenges associated with long experiments. However, in most effluent-dominated systems, organisms could experience transient to life-time exposures and the effects on reproduction and survival may persist even if exposure is reduced or stopped. It is necessary to begin understanding how life-time exposure affects population dynamics, how such exposure might differ from early-life exposures, if animals can recover reproductive function following transfer to clean environments, and if there are any parental effects.

Understanding the population consequences of estrogen exposure requires realistic experiments conducted over an appropriate time interval. We used fathead minnows (*Pimephales*

promelas) 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, fathead minnows have been widely used in laboratory studies and their physiological responses to estrogens are well known (Ankley & Villeneuve 2006). We exposed fathead minnows to 17 α -ethinylestradiol (EE2), the synthetic estrogen in human birth control. EE2 is a potent estrogen in fish and a common contaminant in wastewater effluents (Kostich et al. 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 (Caquet et al. 2000). We also conducted a laboratory experiment using the fathead minnows hatched in the mesocosms to estimate the effects of early-life versus life-time exposure on survival and reproduction. We conducted these studies to test the following hypotheses: 1) EE2 reduces survival in multiple life stages, and 2) EE2 reduces reproductive output over multiple generations. The outcomes of these tests will point to sensitive life-stages and mechanisms that appear critical for population sustainability.

MATERIALS AND METHODS

We conducted two experiments over the course of one year on three generations of fathead minnows (*Pimephales promelas*) (Figure 3.1A). The first experiment exposed adult fathead minnows to EE2 in outdoor aquatic mesocosms; we define these adults as the F0 population. The mesocosms were constructed at the Foothills Fisheries Laboratory (FFL), Colorado State University (CSU), Fort Collins, Colorado. The second experiment (F1 - F2

generations) was conducted in the FFL in glass aquaria receiving the same lake water as the mesocosms. The F1 generation consisted of individuals spawned in the mesocosms by the F0 adults and the F2 generation consisted of fish produced by the F1 generation in the lab (see details below). All fish were treated in accordance with Institutional Animal Care and Use Committee protocol number 10-1685A at CSU.

The mesocosms were 28 polyethylene tanks (Rubbermaid Corp., Winchester, VA, 2 m in diameter, 0.66 m deep, 1100 L) supplied with water from College Lake (Fort Collins, Colorado, USA). The lake water was mechanically filtered and irradiated with ultraviolet light. Water flow was maintained to provide sufficient water quality for fathead minnows and was set by drilling a hole in the inflow pipe calibrated to 1 L/min. Maximum daily water temperatures increased to 28 °C six times and flow rates were increased to 2 L/min during that time. Water volume in the mesocosms averaged 1056 ± 4.4 L (SEM). The mesocosms were aerated with ambient air at all times, covered with 6.25 cm² netting (Memphis Net and Twine, Memphis, TN), and one third of the surface area of each mesocosm was shaded with landscaping fabric. The mesocosms colonized naturally with algae and invertebrates for 59-d prior to adding fish (Figure 3.1A).

Fish Exposures - Mesocosm Experiment

17 α -ethinylestradiol (Sigma Aldrich, 99% pure) (EE2) dissolved in methanol was added to the mesocosms daily. Serial dilutions were made using a glass serological pipet from a concentrated stock solution to achieve the nominal concentrations of 0, 5, 10, 20 ng/L in the mesocosms and were delivered in a 1 ml volume. Treatments ($n = 7$) were arranged in a randomized complete block design. The 0 ng/L mesocosm received 1 mg/L of solvent and a water control was not used because of the low solvent concentration. Flow of water was

suspended at 17:00 h and EE2 was pipetted into the plume of air bubbles in the middle of each mesocosm. 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) mesocosm was accidentally spiked with the 10 ng/L solution. We chose a static renewal for the EE2 additions because it approximates a pulsed input into the mesocosm, similar to what fish encounter below wastewater treatment plants (Nelson et al. 2010).

Adult virgin male and female fathead minnows were obtained from Aquatic Biosystems, Inc. (Fort Collins, CO) or the US EPA (Cincinnati, OH) and stocked randomly into the mesocosms (5M:5F) on (6-May-2011). Males and females of the F0 generation were coded with elastomer tags (Northwest Marine, Shaw Island, WA) by subcutaneous injection left of the dorsal fin. Initial handling mortalities were replaced for 48-h. For 23-d, the fish were held separately by confining females to perforated 189 L polyethylene containers (Rubbermaid Corp.) (Figure 3.1A). After the 23-d pre-exposure, we combined the males and females in the mesocosms. The fish were allowed to behave naturally for 102-d (Figure 3.1A) and reproductive output was assessed as described below. This experimental design resulted in direct water-borne exposures to the F0 and F1 generations (Figure 3.1B).

We fed the fish commercial brine shrimp flake (Argent Labs, Redmond, WA) at a rate of 1 gm/tank and active feeding was observed within 3-d of stocking the fish. We also observed the fish feeding on the algae growing on the sides of the mesocosms. While the fish were reproducing we fed 2 ml/d of concentrated newly hatched *Artemia* sp. nauplii. We produced brine shrimp nauplii in a conical hatchery (Aquatic Ecosystems, Apopka, FL) by adding eggs at 1 gm/L in 25 parts per thousand constantly aerated seawater (Instant Ocean, Blacksburg, VA), and incubating at 26 - 28 °C for 24-h.

Fish Exposures - Laboratory Experiment

Following the mesocosm experiment, we transferred up to 20 mixed-sex juvenile fathead minnows from the F1 generation into the 60 L glass aquaria in the laboratory (Figure 3.1). Mesocosms exposed to 20 ng/L produced insufficient numbers of F1 offspring, so they were not included in the laboratory study. For the remaining treatments, due to limited tank space, we could not include every mesocosm. Therefore, mesocosms with sufficient numbers of F1 fish were randomly selected for inclusion in the laboratory study, four mesocosms each ($n = 4$) from the 0, 5, and 10 ng/L treatments. Laboratory treatments were arranged in a randomized complete block design. F1 fish from each randomly selected mesocosm were split into two aquaria; one aquarium continued the same exposure as in the mesocosm and the other aquarium was not exposed. The experimental design resulted in F1 fish that received only an early-life EE2 exposure (from spawning through gonadal differentiation) in the mesocosms. Therefore, the F2 exposure was indirect occurring through the parents (Figure 3.1B). The design also resulted in F1 fish that were continuously exposed throughout their lives (Figure 3.1A). Hereafter, we refer to these treatments as early-life and life-time exposures (Table 3.1). Control (0 ng/L) F1 fish from the mesocosms were split into two aquaria, one continued at 0 ng/L, the other was exposed at 5 ng/L, and this treatment will be referred to as the adult exposure. EE2 dissolved in ethanol was added daily as a static renewal using disposable pipet tips. Flow was shut off at 17:00 h and resumed at 08:00 h.

From 15-October-2011 until the end of the experiment on 28-March-2012 we mixed heated lake water with the ambient lake water in a head tank before distributing to the aquaria at a rate of 0.5 L/min. Water temperatures were maintained between 18 and 27 °C and aquaria were aerated constantly with ambient air. Fish were held on a 12:12 light-dark cycle, and fed

commercial brine shrimp flake *ad libitum*. After 116-d of continuous exposure or recovery we reduced the density of the fish to stimulate F1 reproduction (Figure 3.1A). We selected two visibly distinct males and four presumed females, which we based on the absence of a dorsal spot and relatively smaller size. A 2:1 female to male sex ratio is optimal for fathead minnow breeding (Denny 1987). The fish were allowed to mature for another three weeks and the light-dark cycle was changed to 16:8 to help stimulate reproduction (Denny 1987). We ceased EE2 exposures after the density reduction because we could not maintain water temperatures suitable for reproduction ($>20^{\circ}\text{C}$) during the static exposure. Therefore, all fish were unexposed for 53-d prior to the start of reproduction. Consequently, the lack of exposure during this period should make any observed effects of EE2 exposure conservative.

Population Endpoints

In both the outdoor mesocosms and the laboratory aquaria we provided substrate for spawning (Denny 1987). In the mesocosms the substrate consisted of 15 cm diameter hemispheres of polyvinyl chloride (PVC) pipes that were placed 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 substrate, one for each male. We first observed eggs in the mesocosms on 6-June-2011 and we considered reproduction complete by 17-Aug-2011 after 21-d of no egg production in any treatment. We checked each substrate weekly (as opposed to daily) for eggs and embryos to minimize disturbance and the risk of multiple counts of the same eggs or embryos. We recognize that our egg and embryo data may be somewhat underestimated because fathead minnows hatch in less than 7-d. Eggs and embryos on the substrate were photographed

and then returned to the mesocosms for continued development. Eggs and embryos were counted using ImageJ (NIH, Bethesda, MD).

We stopped the mesocosm experiment on 9-September-2011, and over 2-d drained each mesocosm, and netted all fish into aerated buckets. Each labeled bucket or buckets was photographed and fish were counted using ImageJ. The F0 generation was identified by the elastomer tag and sampled for physiological endpoints. F1 juveniles were selected based on relatively large size for stocking the laboratory aquaria.

F1 reproduction began on 10-March-2012 in the laboratory. We ceased feeding in tanks with spawning fish; adults spawned for 7-d after which they were sampled for physiological endpoints. We counted the eggs daily by image analysis or by hand. The eggs were incubated in beakers with 25 °C moderately hard, aerated well-water and 0.1 mg/mL formalin to prevent fungal growth. Most of the eggs hatched within 4-d and the F2 larvae were counted. We conducted another count of newly hatched larvae the following day. We continued checking the substrates and counting eggs and larvae for 7-d. We allowed reproduction to continue in all tanks as long as there were control (0 ng/L) fish still spawning. After the control fish ceased spawning, any tank that had not spawned was sampled for physiological endpoints. Eight tanks spawning concurrently with the last control tank continued spawning until the end of the 7-d reproductive period.

Physiological Endpoints

We measured vitellogenin (VTG) mRNA expression in the liver of male fish. VTG is an egg-yolk precursor protein normally found in female fish, but in males VTG is indicative of estrogen exposure (Arukwe & Goksoyr 2003). Fish were euthanized in 250 mg/L tricaine

methanesulfonate, livers were extracted aseptically, snap frozen on dry ice, and then transferred to -80 °C in the laboratory. Total RNA was extracted and VTG mRNA was assessed using quantitative real time PCR following Biales et al. (2007).

Water Samples

We measured EE2 semi-weekly during the final eight weeks of the mesocosm experiment and on a monthly basis during laboratory experiment. Methods for analyzing EE2 in the water are presented in Chapter 1.

Statistical analysis

The response variables for the experiments were F1 and F2 egg and embryo counts, F1 juvenile counts, F0 adult survival, F2 embryo survival (ratio of larvae to eggs), and VTG. Measured EE2 was the explanatory variable. Egg and embryo counts were summed over the reproductive period for each mesocosm or tank and then averaged across treatments. Generalized linear mixed models (GLMM) were used to test the alternate hypotheses that EE2 reduces numbers of eggs, embryos, and juvenile fish as well as survival. Pairwise comparisons were made between the EE2 treated groups and the control. Adjustments for experiment-wise error were not included because of the small number of comparisons. We also tested the alternate hypothesis that an early-life EE2 exposure diminishes F2 embryo survival with an *a priori* linear contrast calculated at the average of our EE2 treated groups compared to the control. Log transformed VTG (because of increasing variance) in EE2 treated fish was compared to the control using a mixed linear model.

The binomial distribution with logit link was specified for the survival data and the negative binomial distribution with log link was specified for the count data. Initial models involving count data fit with the Poisson resulted in overdispersion. All models were fit using Proc GLIMMIX in SAS software v9.3 (©2012, SAS Institute Inc. Cary, NC) (or Proc Mixed for the VTG) with residuals and block as random effects and the EE2 treatment as the fixed effect. When no F1 reproduction was observed a "1" was added to the egg and F2 larvae data so that parameters for that tank could be estimated and statistics calculated. The F1 juvenile and F1 egg models would not converge so block was from a random to a fixed effect. A block by treatment interaction was included as a random effect in the F2 embryo survival test to correct overdispersion. An odds ratio for death of F0 adult males per unit increase in EE2 was assessed by logistic regression (Proc LOGISTIC). All data are analyzed and presented untransformed (except for VTG) and plotted against the measured EE2 concentrations. Studentized residuals versus predicted plots and q-q plots were used to assess equal variance and normality of the data. Type 1 error rate was set at $\alpha = 0.05$. Data are reported as mean $\pm$ SEM.

RESULTS

Water chemistry

The concentrations in the 0, 5, 10 and 20 ng/L mesocosm treatments were 0.34 ± 0.04 , 3.22 ± 0.64 , 5.32 ± 0.19 , and 10.85 ± 0.39 ng/L, respectively (Table 3.1). The measured concentrations in the 0, 5, and 10 ng/L laboratory treatments were 0.17 ± 0.1 , 4.95 ± 0.55 , and 10.75 ± 1.77 ng/L, respectively. Over the course of a 24-h period in the mesocosms EE2 attenuated following the spike (Table 3.2) indicating a pulsed exposure. The detection of EE2 in

the controls (0ng/L) (Tables 3.1 & 3.2) 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 ($F_{2,18}=11.58$, $p=0.0006$, Figure 3.2). Survival in control males averaged $66\pm 8\%$ and male survival significantly declined at 3.2 ng/L to $17\pm 9\%$ ($t_{15}=3.89$, $p=0.001$) and 5.3 ng/L to $14\pm 6\%$ ($t_{15}=4.07$, $p=0.0007$). No males survived in mesocosms exposed at 10.9 ng/L for 102-d (Figure 3.2). EE2 significantly increased the odds of male death by a factor of $1.7\times$ (1.3-2.2; 95% C.I.) for every 1 ng/L increase in EE2. Survival in control females averaged $66\pm 7\%$ and declined to $45\pm 8\%$ in females exposed to 10.9 ng/L, an effect that was suggestive of significance ($p=0.1$) (Figure 3.2).

EE2 exposure significantly decreased F0 reproductive output in all life stages ($F_{3,23.74}=20.53$, $p<0.0001$ eggs; $F_{3,21.19}=23.62$, $p<0.0001$ embryos; $F_{3,18}=16.29$, $p<0.0001$ juveniles; Figure 3.3A, B). Blocking was not significant ($p=0.5$). At 10.9 ng/L F0 reproductive output was significantly lower than controls and was limited to an average of 125 ± 60.2 eggs ($t_{23.88}=7.11$, $p<0.0001$), 4 ± 3.4 embryos ($t_{23.1}=7.78$, $p<0.0001$), and 0.86 ± 0.86 fish ($t_{18}=6.90$, $p<0.0001$). Egg, embryo, and juvenile fish production in the controls was not statistically different compared to the, 3.2, or 5.3 ng/L exposures ($0.3787<p<0.1451$); however, a clear trend of reduced reproductive output is evident (Figure 3.3A, B).

Laboratory Experiment

EE2 exposure reduced F1 fathead minnow egg production ($F_{5,15}=19.37$, $p<0.0001$) and numbers of F2 larvae ($F_{5,15}=36.73$, $p<0.0001$; Figure 3.3C). Blocking was not significant ($p=0.09$). One tank of life-time exposed F1 fish (3.2 ng/L mesocosms; 4.9 ng/L laboratory, Table 3.1) 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) was significantly less (478 ± 256) than control fish (1581 ± 309) ($t_{15}=2.65$, $p=0.02$). Likewise, numbers of F2 larvae (236 ± 187) from early-life exposed parents (3.2 ng/L) were reduced from controls (1195 ± 205) ($t_{15}=3.04$, $p=0.008$). In parents exposed to 5.3 ng/L early in life, egg production (5.7 ± 4.7) and F2 larvae (0.33 ± 0.33) were significantly reduced compared to the controls ($t_{15}=7.19$ and 8.85 , respectively, $p<0.0001$).

Parental EE2 exposure reduced F2 embryo survival, an effect that was only suggestive of significance ($F_{3,8.8}=3.19$, $p<0.08$). However, reduced F2 survival between the average of the early-life exposed parents ($19\pm10\%$) and the control ($70\pm5\%$) was significant ($F_{1,9}=8.32$, $p=0.02$, *a priori* linear contrast) (Figure 3.3D). Survival of the F2 embryos exposed to EE2 (3.2ng/L) only through the parents (Figure 3.1B) was half ($35\pm17\%$) that of the controls. And in F2 embryos whose parents were exposed to 5.3ng/L early in life, survival averaged only $2.2\pm2.2\%$. In F1 fish subjected to life-time EE2 exposure no embryos survived (Figure 3.3D).

VTG expression

In the mesocosm experiment VTG significantly increased in response to EE2 in a concentration dependent manner ($F_{2,6.58}=45.81$, $p=0.0001$) (Table 3.1). VTG expression was significantly elevated in both the 3.2 ng/L (1.95 ± 0.74) and the 5.3 ng/L (4.89 ± 2.14) groups

compared to the controls (0.006 ± 0.004) ($t_5=6.87$, $p=0.0003$ and $t_5=8.50$, $p=0.0002$, respectively). VTG expression in the laboratory experiment was negligible (Table 3.1). This is because we stopped EE2 exposures to facilitate reproduction at 218-d (Figure 3.1A), giving 53-d of no EE2 exposure prior assessing VTG expression. Inter-assay 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 the control fish (Table 3.1) indicates that the background EE2 concentrations have little, if any, affect on the fish in either experiment.

DISCUSSION

We assessed the reproductive performance and survival of three generations of fathead minnow populations exposed to trace levels of the estrogen (EE2) used in human contraceptive pills. Exposure of adults during the reproductive season caused dramatic declines in male survival and even reduced female survival. Survival of F2 embryos exposed to EE2 only through the parents was significantly reduced from controls, indicating a potential transgenerational effect. Acute exposure of F0 adults during the reproductive season reduced eggs, embryos, and F1 juvenile fish production. Life-time exposure of the F1 generation resulted in reduced F2 egg production and no F2 larval production. Interestingly, individuals exposed early in life and then transferred to clean water produced significantly fewer eggs and F2 larvae, suggesting population recovery is not possible within the given timeframe. These results indicate that multiple mechanisms, including reduced reproductive output and survival, drive population failure in short-lived fishes from estrogen-contaminated environments. Finally, these effects occur at environmentally relevant EE2 concentrations (3.2 - 10.9 ng/L) as indicated by Kostich et

al. (2013) where maximum predicted EE2 concentrations (6 ng/L) were similar to maximum observed concentrations (non-detectable to 11.6 ng/L) in surface waters.

Reproduction was permanently disrupted in both F1 males and females that were spawned in the mesocosms and allowed to recover for an equal period of time in the laboratory. Most dramatically, F1 parents exposed early in life (5.3 ng/L) produced almost no F2 eggs and zero larvae. Despite not being exposed to EE2 for over five months, F1 fish exposed early in life (3.2 ng/L) produced fewer F2 eggs and larvae than unexposed controls. Studies on zebrafish suggest that partial recovery from EE2 exposures is possible at similar exposure concentrations (Nash et al. 2004, Fenske et al. 2005), contrasting with our results. The physiological 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 (born male or female); 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 the fathead minnow could result in a differential response to an early-life EE2 exposure. Despite some differences between our study and those cited, EE2 has significant effects on reproductive performance in both species and indicates a need for comparative studies that explicitly test the relationship between mode of gonad development and estrogen induced reproductive disruption.

Male survival in the F0 generation was significantly reduced by exposure to EE2. The physiological mechanism behind reduced male survival in our study is unknown, but could be due to the inability to metabolize estrogen-induced proteins, such as VTG. VTG is an egg yolk protein normally found in females (Arukwe & Goksoyr 2003) that can be induced in male fish following estrogen exposures (*e.g.* Lange et al. 2001, Nash et al. 2004, Schwindt et al. 2007,

McGree et al. 2010). However, there is limited capacity for males to metabolize VTG resulting in kidney failure (Herman & Kincaid 1988). 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. Our results suggest that, even when male survival is low, reproductive output is sufficient to sustain at least a fraction of the population that control fish maintain. To our understanding, no other studies report significant effects on adult survival at trace EE2 concentrations. The 33% mortality for both male and female survival in the control mesocosms suggests a base level of mortality not associated with EE2. The EE2 is an additional stressor that may induce mortality because of the inability of the fish 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). Changes in water quality and food abundance, as well as inter- and intra-specific competition, are all potential stressors that fish encounter in natural systems in addition to chemical stressors. A better understanding of the factors causing adult mortality is clearly needed.

Survival of F2 embryos produced by fathead minnows exposed to EE2 early in life was significantly reduced despite the F2 embryos never being directly exposed to water-borne EE2. Exposure could only have occurred through the F1 parents who were exposed as gametes through gonadal differentiation. Our study could not separate maternal from paternal contributions leading to the reduced F2 survival. However, the reduced F2 survival was probably not due to residual EE2 in the bloodstream of the F1 parents as indicated by the absence of VTG mRNA. More likely, reduced survival could be due to poor gamete quality resulting in poor embryo survival, or due to other paternal or maternal effects. The surprising reduction in F2

survival despite the F2 generation never directly experiencing EE2 suggests, at a minimum, parental effects, but could also result from epigenetic effects. The possibility of transgenerational effects of EE2 suggest that further studies are needed to determine if the effects are heritable. Although difficult and time-consuming, identifying the durability of parental effects in the F3 and F4 generations is needed to confirm heritability of the deleterious traits (Skinner 2008).

Empirical studies documenting effects of environmental estrogens on fish populations are lacking; however, the collapse of fathead minnow populations following EE2 exposure provided convincing evidence for the importance of population-level studies (Kidd et al. 2007). Our study confirms that EE2 exposure can have negative consequences for population growth and persistence. We demonstrated that a seasonal EE2 exposure has lasting effects that may affect population sustainability in natural ecosystems. Additionally, our results suggest that even if fish migrate away from contaminated environments, or if contamination is removed, population effects may persist given the effects of early-life exposures. The diversity of effects presented herein suggests multiple mechanisms could contribute to population failure including: 1) reduced male survival, 2) reproductive failure, assumed to be caused by developmental reprogramming (Van Aerle et al. 2002), and 3) transfer of parental traits that limit offspring survival. Our study is the first to demonstrate that recovery of populations from estrogen exposures may not be possible, at least under these experimental conditions. Future research should assess other population effects in a modeling framework. Experiments evaluating the heritability of effects of EE2 on fish reproduction with long-term studies carried into the F4 generation are needed.

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Table 3.1. Concentrations of 17 α -Ethinylestradiol (EE2) in the Mesocosms or Laboratory Tanks and Vitellogenin (VTG) mRNA Expression Relative to 18S rRNA Expression [mean $\pm$ SEM and (range)] in the Livers of Male Fathead Minnows (*Pimephales promelas*).

Treatment	Nominal EE2 (ng/L)	Measured EE2 (ng/L) (range)	Relative VTG:18S Expression
<u>Mesocosm Experiment</u>			
Control	0	0.34 $\pm$ 0.04 ^a (0-1.3)	0.006 $\pm$ 0.004
Low	5	3.22 $\pm$ 0.64 ^a (0.38-12.8)	1.95 $\pm$ 0.74 [*]
Medium	10	5.32 $\pm$ 0.19 ^a (2.76-8.11)	4.89 $\pm$ 2.14 ^{**}
High	20	10.85 $\pm$ 0.39 ^a (6.84-19.11)	No Fish Survived
<u>Laboratory Experiment</u>			
Control	0 , 0 ^c	0.34 $\pm$ 0.04 ^a , 0.17 $\pm$ 0.1 ^b (0-1.3) , (0-0.39)	0.002 $\pm$ 0.0007
Low	0 , 5	0.34 $\pm$ 0.04 ^a , 4.95 $\pm$ 0.55 ^b (0-1.3) , (3.8-6.4)	0.003 $\pm$ 0.001
Low Early-Life Exposure	5 , 0	3.22 $\pm$ 0.64 ^a , 0.17 $\pm$ 0.1 ^b (0.38-12.8) , (0-0.39)	0.006 $\pm$ 0.0003
High Early-Life Exposure	10 , 0	5.32 $\pm$ 0.19 ^a , 0.17 $\pm$ 0.1 ^b (2.76-8.11) , (0-0.39)	0.004 $\pm$ 0.0005
Low Life-Time Exposure	5 , 5	3.22 $\pm$ 0.64 ^a , 4.95 $\pm$ 0.55 ^b (0.38-12.8) , (3.8-6.4)	0.01 $\pm$ 0.001
High Life-Time Exposure	10 , 10	5.32 $\pm$ 0.19 ^a , 10.75 $\pm$ 1.77 ^b (2.76-8.11) , (6.66-13.13)	0.01 $\pm$ 0.01

^aEE2 concentrations from 0.45 μ m filtered water. ^bEE2 concentrations from whole water.

^cThe first concentration is EE2 in the mesocosm, the second is EE2 in the aquaria.

^{*}VTG, compared to 0 ng/L ($t_5=6.87$, $p=0.0003$); ^{**}VTG, compared 0 ng/L ($t_5=8.50$, $p=0.0002$)

Table 3.2. Measured 17 α -Ethinylestradiol (EE2) Concentrations Collected 30 min, 16-h, or 23-h After Spiking. Data are mean $\pm$ SEM and (range).

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

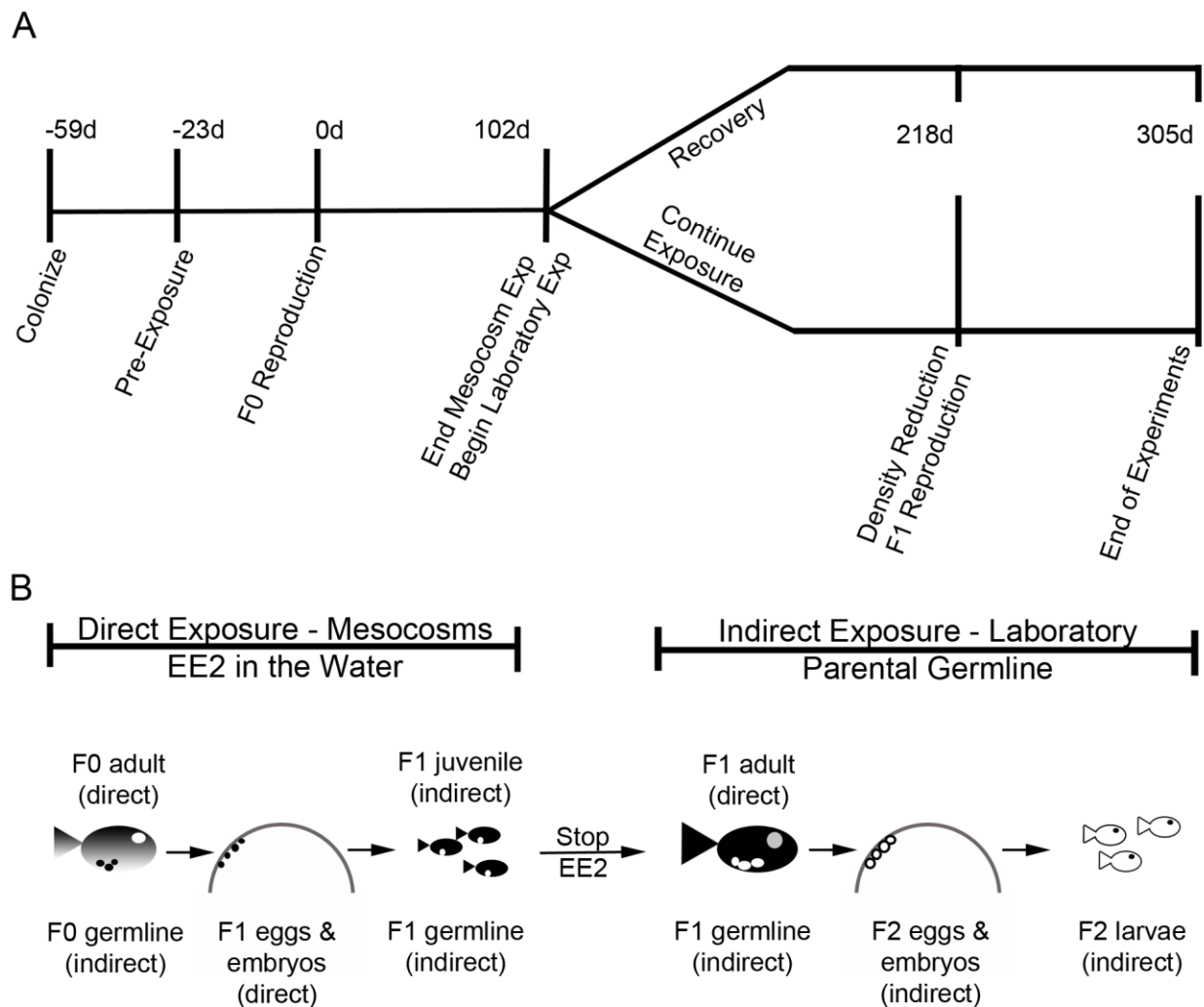


Figure 3.1. Experimental timeline for the mesocosm and laboratory experiments (A) and exposure history of the fish (B). In (A) at -59-d the mesocosms were filled and allowed to colonize with algae and invertebrates. At -23-d pre-exposure of separated male and female fathead minnows (*Pimephales promelas*) began. At 102-d mesocosms were split into a recovery and continued exposure group and moved into the laboratory aquaria. In (B) only the recovery treatments are shown for the laboratory experiment.

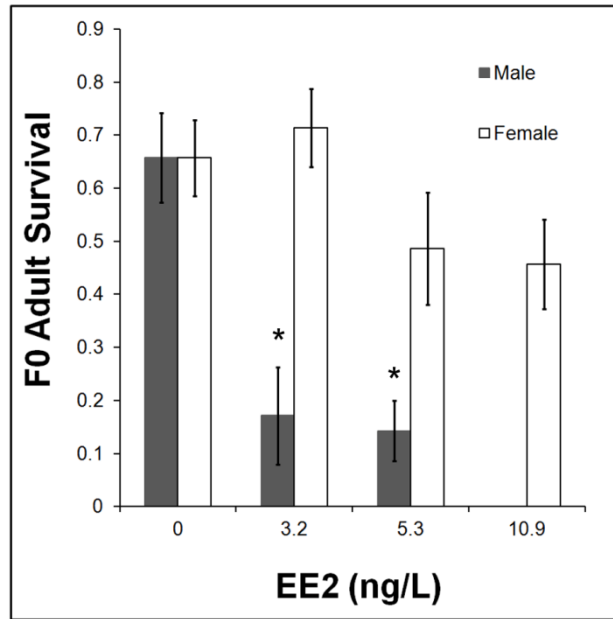


Figure 3.2. Effect of 17 α -ethinylestradiol (EE2) on male and female fathead minnow (*Pimephales promelas*) survival in the F0 generation. EE2 concentrations are measured averages over the 102-d exposure. The (*) indicates significant difference from controls (0 ng/L) based on least squares (adjusted) means ($0.002 < p < 0.003$) within sex. Bars are arithmetic means $\pm$ SEM.

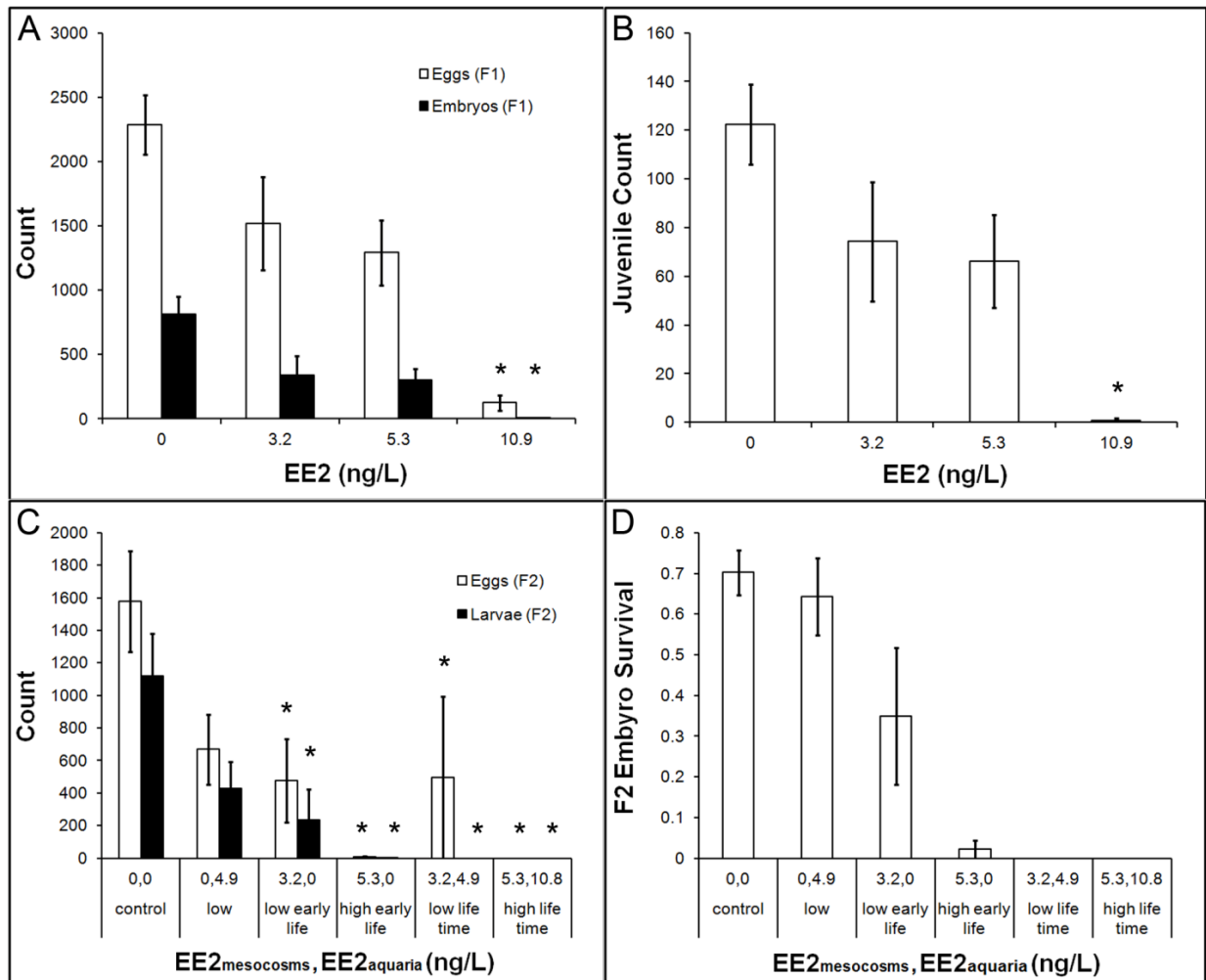


Figure 3.3. Effect of 17 α -ethinylestradiol on F1 egg, embryo (A), juvenile fathead minnow (*Pimephales promelas*) (B) counts in the mesocosms, and F2 egg, larvae (C) counts and embryo survival (D) in the laboratory aquaria. On the x-axis in (C) and (D), the first number is the EE2 concentration in the mesocosm and the second is the EE2 concentration in the laboratory aquaria. The (*) indicates significant differences from controls (0 ng/L) within life-stage based on least squares (adjusted) means with $0.0001 < p < 0.02$. In (D) an *a priori* linear contrast between control and the early-life exposure groups (average of the 3.2,0 ng/L and 5.3,0 ng/L) was significant ($F_{1,9}=8.32$, $p=0.02$). Bars are arithmetic means $\pm$ SEM.

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CHAPTER 4

A STOCHASTIC STAGE-STRUCTURED MODELING APPROACH TO EVALUATE THE EFFECTS OF ESTROGENIC EXPOSURE ON POPULATION GROWTH RATE IN A SHORT-LIVED FISH

SYNOPSIS

Urban freshwater streams and rivers in arid climates are ecosystems where wastewater effluent represents the majority, if not all of the water flow. The effluent contains a suite of bioactive chemicals including steroid and non-steroid estrogens that disrupt vertebrate endocrine systems in laboratory studies. Little understanding exists however of the ecological consequences. Here we integrate empirically generated population-level data into stochastic population models and identify effects of a common environmental estrogen on population growth rate (λ) and estimate sensitivity of the vital rates. Matrix population models were developed for fathead minnows (*Pimephales promelas*), a short-lived seasonally reproducing fish where males spawn with multiple females and care for the eggs. At environmentally relevant concentrations of 17 α -ethinylestradiol, the estrogen in human contraceptive pills, stochastic λ (λ_s) averaged 0.36 (0.05 - 4.42, 95% CI) indicating a rapidly declining population. This effect on λ_s was evident despite statistically insignificant effects on individual vital rates like fecundity and counts of juvenile fish. Stochastic sensitivity analysis indicated the decline in λ_s was mediated firstly by reduced survival of age-0 fish and secondly through reduced egg production. These results suggest that effects on male survival and physiology are relatively unimportant from the standpoint of population growth and persistence. Rather, measures taken to improve

first year survival and female fecundity may be more important for conservation of short-lived, highly fecund fishes in estrogen impacted environments.

INTRODUCTION

Recent global circulation models suggest the possibility of reduced water flow in many western U.S.A. streams and rivers (Barnett et al. 2005). Coupled with increasing human populations it is expected that those streams and rivers will be dominated by processed wastewater (Strange et al. 1995, Dennehy et al. 1998, Brooks et al. 2006). Most wastewater treatment plants are not designed to remove pharmaceuticals and personal care products (PPCPs) and the impacts of these compounds on aquatic populations are largely unknown. Some of the most commonly found chemical constituents in wastewater are steroid and non-steroid estrogens (Kolpin et al. 2002). Further, estrogenic wastewater effluent has been shown to disrupt fish reproduction in the field (Vajda et al. 2008).

In vertebrates, the biochemical actions of estrogens are mediated predominantly through the estrogen receptors, but other modes of action are also possible (Tabb & Blumberg 2006). Most research on the effects of EE2 has focused on alteration of the vertebrate endocrine system with effects identified at the cellular to organ levels in a variety of wildlife species (Tyler et al. 1998). However, studies evaluating effects at higher levels of biological organization are largely lacking.

Although an increasing number of studies identify effects on survival and fecundity of individuals, few studies link those data to population dynamics and persistence (Mills & Chichester 2005). One way to estimate effects of endocrine disrupting chemicals (EDCs) on

populations is through models. Assessing effects of chemical pollutants on populations using a modeling framework is becoming more common (Relyea & Hoverman 2006, Newman & Clements 2008, Forbes et al. 2008, 2011, Newman 2010). Miller et al. (2007) linked androgen exposure to population effects by demonstrating that reduced fecundity was highly correlated to reduced vitellogenin, an egg yolk precursor protein. The decline in the protein was used in the model to adjust fecundity and ultimately predicted the effects of androgens on fathead minnow (*Pimephales promelas*) population dynamics. Miller et al. (2013) also demonstrated that pulp mill effluent disrupted the population dynamics of white suckers (*Catostomus commersonii*) using field collected data. An et al. (2009) linked the incidence and severity of intersexuality in male roach (*Rutilus rutilus*) to reduced fertility and subsequent effects on population growth rate (PGR) in a stage-structured model. The reduced fertility presumably resulted from exposure to estrogens in wastewater effluent. An et al. (2009) also concluded that while intersexuality reduced PGR compared to reference sites, PGR was still positive even under selective harvest of males. Finally, Raimondo et al. (2009) demonstrated that water borne exposure to the steroid estrogen 17 β -estradiol reduced PGR in the sheepshead minnow (*Cyprinodon variegatus*) using a stage-structured model.

Despite the increasing use of population models in ecotoxicology, few models incorporate variation in vital rates, despite their inherent contributions to population dynamics. Most published approaches rely on deterministic models and analytical solutions to examine effects of vital rates on PGR (Miller et al. 2007, 2013, An et al. 2009, Raimondo et al. 2009). However, disregarding variation in the vital rates may give potentially misleading estimates of PGR (Wisdom et al. 2000). For instance, estimates of PGR using mean survival and fecundity

may be positive; however, variation in one or both parameters could yield a distribution of PGR indicative of population decline.

A sophisticated analysis of the effects of environmental stressors on PGR requires understanding the causes of variation in the vital rates (Nicholson & Possingham 2007), particularly variation caused by exposure to contaminants (Forbes et al. 2011). One method of modeling variation in vital rates is a stochastic simulation method called life-stage simulation analysis (LSA) (Wisdom et al. 2000). In LSA the vital rates are sampled from probability distributions based on the mean and variance of the collected data. At each step in the simulation, PGR is calculated and stored. The resulting distribution is the stochastic PGR (λ_S). Additionally, by calculating the coefficient of determination (R^2) between λ_S and a vital rate in a linear model, one can estimate the sensitivity of PGR to changes in the vital rate.

We used LSA to estimate λ_S in fathead minnow populations exposed to varying concentrations of EE2. Stochastic PGR was then compared among the populations. Because EE2 reduces both fecundity and survival (see Chapter 3), we hypothesized that these individual effects would translate to reduced population growth. To test this hypothesis we developed stage-structured population models and parameterized those models with empirically derived vital rates presented in Chapter 3. In addition to testing the effects of EE2 on PGR, we used the LSA to estimate the sensitivity of PGR to stochastically varying vital rates. The results from our study demonstrate the utility of population models in ecotoxicology in linking individual effects to population-level outcomes. More importantly, our results suggest that extrapolation of individual effects to population consequences without a model is potentially misleading. This is because a lack of effect on individual vital rates can still result in population consequences using a simple model as demonstrated herein.

MATERIALS AND METHODS

Data Sources

Population data used to calculate the vital rates (fecundity and survival) were obtained from Chapter 3 and are presented in Table 4.1. Briefly, the data were gathered from a three month study of fathead minnow (*Pimephales promelas*) population dynamics conducted in outdoor mesocosms. Fish were exposed daily as static renewal to 0 (control), 3.2, 5.3, or 10.9 ng/L 17 α -ethinylestradiol (EE2) in a randomized and replicated design ($n = 7$) with the goal of assessing the short term effects of EE2 on the populations. Therefore, mean and standard deviations from seven mesocosms were estimated for fecundity and survival within each treatment. Overwinter survival was initially evaluated from literature sources (Danylchuk & Tonn 2003, Divino & Tonn 2007) that ranged from 0 to 53%. Average survival from these literature values was $23 \pm 9.5\%$ (SEM) but using 23% survival in the models gave very high PGR estimates. Therefore, we used an iterative process to adjust the overwinter survival to give a PGR of λ_s close to one for the control population (0 ng/L). Following the iterative process, overwinter survival was set to 8.65% and the variance was set at 0.0001 to reduce the influence that overwinter survival has in model because we had no empirical estimate. We assume that the EE2 treatment does not affect overwinter survival and use the same value for all treatments. We also assumed that fish smaller than 20 mm total length would not survive the winter (Toneys & Coble 1979). The proportion of fish larger than 20 mm for each mesocosm was calculated and averaged across treatments and included in the model. Egg to juvenile survival was calculated as the ratio of juveniles to eggs. Adult survival was the ratio of adults surviving the experiment to the number of adults in the founding population (Chapter 3). Therefore, excepting overwinter

survival, the shape parameters for each vital rate were empirically determined and used in the stochastic life-stage simulation analysis described below.

Life History

Our model assumes an after birth-pulse census since juveniles and adults were the only stages present during the census at time $t + 1$ in our mesocosm study (Noon et al. 2001; Figure 4.1) described in Chapter 3. Juveniles do not reproduce during their first season and transition to adults the following summer and reproduce (Figure 4.1). Adults that survive the winter also reproduce (Figure 4.1). Our assumption is that fecundity (m_1 and m_2), overwinter survival, and summer survival are the same across generations. Summer survival was different between the sexes (Table 4.1). The time step for the model was one year (Figure 4.1).

Population Model

We chose to model these populations using stage-structured matrices (Caswell, 2001) because of the ability to easily estimate the effects of EE2 on multiple vital rates simultaneously. While it is more common to ignore males in stage-structured models, we included both male and female fathead minnows because EE2 significantly reduced male survival (Chapter 3, Table 4.1). Including males allows us to estimate the effect of reduced male survival on λ_S .

Based on our empirical experiment (Figure 4.1) we constructed the following model:

$$\begin{bmatrix} p_j^{1-k} \cdot m_1 \cdot p_j^k & 0 & p_a^{1-k} \cdot m_2 \cdot p_j^k & 0 \\ p_j^{1-k} \cdot m_1 \cdot p_j^k & 0 & p_a^{1-k} \cdot m_2 \cdot p_j^k & 0 \\ p_j^{1-k} \cdot p_{af}^k & 0 & p_a^{1-k} \cdot p_{af}^k & 0 \\ 0 & p_j^{1-k} \cdot p_{am}^k & 0 & p_a^{1-k} \cdot p_{am}^k \end{bmatrix} \times \begin{bmatrix} N_{jf}^t \\ N_{jm}^t \\ N_{af}^t \\ N_{am}^t \end{bmatrix} = \begin{bmatrix} N_{jf}^{t+1} \\ N_{jm}^{t+1} \\ N_{af}^{t+1} \\ N_{am}^{t+1} \end{bmatrix}$$

where:

$$p_j^k = \text{egg to juvenile survival}$$

$$p_j^{1-k} = \text{survival of Age} - 0 > 20\text{mm} \times \text{overwinter survival}$$

$$p_a^k \text{ (f or m)} = \text{sex specific summer survival}$$

$$p_a^{1-k} = \text{overwinter survival}$$

$$m_1, m_2 = \text{fecundity}$$

$$N_{jf}, N_{jm} = \text{number of juvenile females and males, respectively}$$

$$N_{af}, N_{am} = \text{number of adult females and males, respectively}$$

We did not account for density dependence in our models because the mesocosms were likely not resource limited because we fed the fish daily (Chapter 3). A follow-up study was conducted in the same mesocosms where growth and survival were estimated in mesocosms stocked with varying densities of fish. Density did not affect the growth or survival of the fish until the population size was 5× our most dense mesocosm (three adult males, three adult females, and 196 age-0 fish) (data not shown).

Life-stage Simulation Analysis (LSA)

LSA allows examination of the relative effect of each stochastic vital rate on λ_s (Wisdom et al. 2000, Biek et al. 2002). As described in Wisdom et al. (2000), the steps in LSA are to: 1) randomly draw new values of each vital rate bounded by the shape parameters from specified

probability distributions; 2) construct a static matrix for the new set of vital rates and calculate λ ; 3) repeat steps 1 and 2 until a large number of matrix replicates have been obtained; 4) analyze the data across the matrix replicates and evaluate the influence of the stochastic vital rates on λ_s .

We performed 1,000 simulations (Wisdom et al. 2000) for each experimental treatment and compared the probability distributions of λ_s across EE2 treatments and calculated the proportion of simulations where λ_s was < 1 (indicating a declining population). We also evaluated the sensitivity of λ_s to the stochastic vital rates. Sensitivity was estimated as the coefficient of determination (R^2) from linear models of λ_s regressed against each stochastic vital rate (Wisdom et al. 2000). The coefficient of determination R^2 represents the proportion of variation in λ_s that is explained by the variation in each vital rate. The 10.9 ng/L EE2 population was included in these analyses for the purposes of comparing λ_s among treatments; however, male survival was 0. Some reproduction occurred in this treatment (Table 4.1) so we included in the model the chance, however slight, that male survival could creep above 0 during the course of the simulations. As such mean male survival and variance was adjusted to 1×10^{-10} in the 10.9 ng/L EE2 population. Our assumptions for this analysis include: 1) no density dependence and 2) closed populations. We also assume that the relationships (R^2) between λ_s and the vital rates can be estimated with linear models. Program R version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria) was used for this analysis.

Estimates of the vital rates came from the population data collected in the mesocosms (Chapter 3). Our fecundity data were counts with high variance (Table 4.1) resulting in overdispersion that could not be corrected by log transformation. Therefore, the negative binomial distribution was used and shape parameters were estimated using the "fitdistr" function in Program R with starting values calculated according to Bolker (2009: 160). The beta

distribution was specified for the survival rates and shape parameters were calculated accordingly:

$$\alpha = (\mu^2 - \mu^3 - \mu\sigma^2)/\sigma^2 \text{ and } \beta = (\mu - 2\mu^2 + \mu^3 - \sigma^2 + \mu\sigma^2)/\sigma^2$$

where:

$$\mu = \text{mean and } \sigma^2 = \text{variance}$$

We restrict our sensitivity analysis to the LSA method (Wisdom et al. 2000) using the coefficient of determination (R^2) and did not calculate analytical sensitivity or elasticity. The primary reason to exclude analytical solutions is that many of the components of the population model are products of the vital rates. For instance, fecundity is a product of the number of eggs produced, egg survival, and age-0 overwinter survival and the analytical sensitivity of any particular value of this product would not be informative. Another reason to not use analytical solutions is that variation in vital rates may cause some values to be 0 and the sensitivity calculation cannot be performed.

Variance and Relationship among Vital Rates

The nature of our experiment was such that, at census time, all individuals in each mesocosm were counted and means and variances were estimated for each treatment. Therefore, the total variance represented in these estimates can be considered process variance because there was no variance attributable to sampling. However, both process variance and sampling variance are present in the fecundity data. We counted eggs every 7-d, but fathead minnows hatch every 4- or 5-d, so some eggs may not be accounted for in our estimates. Therefore, survival from eggs to juveniles and fecundity contain both process and sampling variance because the probability of egg to juvenile survival was estimated as the ratio of eggs to age-0 fish

at the census. To estimate the influence of an unknown quantity of sampling variance on the LSA and λ_S we performed simulations where the variance in fecundity was reduced by 10%, 25%, and 50%. While it is unlikely that 50% of the variance in the fecundity data is sampling variance we included the 50% reduction for comparative purposes.

Correlation among vital rates can obscure interpretation of sensitivity analyses. Measurements of, for example, fecundity and female survival may not be independent. For instance, if all females contribute equally to egg production then, as females die, fecundity should decrease. We assessed correlation among vital rates with Pearson correlation coefficients using Proc CORR in SAS version 9.3 (©2012, SAS Institute Inc. Cary, NC). We included a Bonferroni adjustment because 10 pairwise comparisons were made. This is resulted in $\alpha = 0.005$ for the correlation analysis.

RESULTS

Because overwinter survival was unknown, we adjusted it until the λ_S in the control population was near stability (average = 1.05, 0.29 - 2.91, 95% C.I.). We compared the control population λ_S to the other EE2 treatments and EE2 reduced λ_S in a concentration dependent manner (Figure 4.2). Stochastic λ declined to a mean of 0.36 (0.05 - 4.42, 95% C.I.), 0.42 (0.08 - 1.75, 95% C.I.), and 0.04 (0.03 - 0.09, 95% C.I.) in the 3.2 ng/L, 5.3 ng/L, and 10.9 ng/L EE2 exposed populations, respectively (Table 4.2). The proportion of simulations where $\lambda_S < 1$ was 43% in the control population and 76%, 86%, and 100% in the 3.2 ng/L, 5.3 ng/L, and 10.9 ng/L EE2 populations (Table 4.2).

Results from the LSA indicate that variation in survival from eggs to juveniles is most important for explaining the variation in λ_S with R^2 ranging from 0.42 - 0.71 (Figure 4.3).

Interestingly, as the concentration of EE2 increases the R^2 for egg production increases from 0.18 to 0.33 in EE2 exposed groups (Figure 4.3). Conversely, the R^2 for egg to juvenile survival decreases from 0.71 - 0.42 as EE2 increases (Figure 4.3). Adult male or female survival has little correlation with λ_S since $R^2 \leq 0.004$, regardless of treatment (Figure 4.3). The variance in egg production and egg to juvenile survival combined account for 92%, 74%, and 75% of the variance in λ_S in the control, 3.2 ng/L, and 5.3 ng/L EE2 exposed populations, respectively. There is some indication of increasing variance in our regressions of λ_S versus egg production and egg to juvenile survival (Figure 4.3).

We assessed correlation among the vital rates. After the Bonferroni adjustment Pearson correlation coefficients (r) were not significantly different than zero (Table 4.3). We evaluated the influence of sampling variance in our fecundity estimates on the R^2 for each vital rate in each treatment. Reducing the total variance in fecundity by 10, 25, or 50% increased egg to juvenile survival R^2 and reduced fecundity R^2 relative to the R^2 derived from models with no reduction in variance (Table 4.4). This pattern was evident regardless of treatment and the magnitude of the difference was most evident at a 50% reduction in total variance (Table 4.4).

DISCUSSION

We evaluated the effects of a steroid estrogen on PGR using a stochastic stage-structured population model. We found a significant decline in λ_S (mean = 0.36) at 3.2 ng/L, suggesting that environmentally relevant concentrations of EE2 (Kostich et al. 2013) potentially have serious consequences for exposed populations. A key conclusion from our work is that population consequences are evident despite statistically insignificant effects of EE2 on single vital rates (Chapter 3). The results from the sensitivity analysis indicate that PGR is most sensitive to

survival from the egg to juvenile and secondarily to fecundity. Interestingly, male survival was largely inconsequential from the standpoint of PGR. This is despite a significant reduction in male survival observed in the empirically derived data (Chapter 3). Within the confines of our experiment, these results indicate that population sustainability depends on protection of survival from the egg to the juvenile and fecundity.

A concentration response relationship between EE2 and λ_S was identified in our analyses. The lowest ecologically relevant concentration of EE2 used in our study (3.2 ng/L) resulted in an estimated λ_S that would result in population decline, a finding that is consistent with Grist et al. (2003). Interestingly, the effect of EE2 on the population was not readily apparent by examining our empirical data (Chapter 3). That is, using standard statistical analyses we did not find a significant effect on egg, embryo, or juvenile fish production until our highest EE2 concentration (10.9 ng/L). However, our modeling suggests that λ_S was < 1 (declining population) 76% of the time at our lowest EE2 concentration. The population model suggests that conclusions based solely on empirical population-level endpoints, like fecundity and survival, may not tell the whole story. A population model that integrates the effects of EE2 on the vital rates in the form of λ_S tells a more complete story. Until ecologically relevant, but perhaps statistically insignificant, endpoints are linked to population dynamics with a model, it is difficult to gain much in the way of ecological inference (Forbes et al. 2008, 2011).

The LSA indicated that survival from eggs to juveniles and fecundity are most important for population growth, which is consistent with predictions of ecological theory (Pianka 1970, Southwood et al. 1974). Short-lived species such as the fathead minnow (lifespan, 1-3 y) depend on successful reproduction in the first year of sexual maturity and egg to juvenile survival is critical for population persistence (e.g. Held & Peterka 1974, Shaw et al. 1995, Duffy 1998). Our

models included both sexes because EE2 reduced male survival to 17% and 14% in the 3.2 ng/L and 5.3 ng/L EE2 populations (Chapter 3). However, our sensitivity analyses suggest that male (and female) survival is largely unimportant for maintaining population growth. This is also consistent with life history theory that suggests that adult survival is less important in short-lived species (Pianka 1970, Southwood et al. 1974). A great deal of research has documented the effects of EE2 on the physiology and endocrinology of adult male fish. While interesting from the standpoint of identifying mechanisms, and effects on individual males, the results appear to have little importance from an ecological perspective. We are not suggesting that effects on individuals are not worth reporting; rather, effects at multiple levels of biological organization are needed to assess the ecological impact of chemical pollution.

The effect of reducing total variance in fecundity had little effect on sensitivity of the vital rates until 50% reduction. Since egg counts were collected using digital images we do not believe that missed eggs were responsible for 50% of the total variance in the fecundity estimate. Even if sampling variance was up to 25% of the total variance, effects on the R^2 were negligible. This suggests that sampling variance in our fecundity estimates was negligible and was likely not biasing our sensitivity analysis. We did not incorporate dependence among our vital rates into our models because there was no detectable correlation among our measured vital rates.

We modeled PGR using estimates derived from experiments using EE2, but in wastewater dominated systems wild fish populations are exposed to a suite of steroid and non-steroid estrogens (Vajda et al. 2008). Our assumption is that EE2 has similar effects on fecundity and survival as estrogens, which has been demonstrated previously (Thorpe et al. 2007). Average EE2 concentrations used in our studies range from 3.2 - 10.9 ng/L and were collected 30 min after the spike (Chapters 1 & 3). However, we administered EE2 to the mesocosms as a static

renewal so the concentrations in the mesocosms were not constant and actually declined over a 24-h period (Chapters 1 & 3). This suggests that while significant effects on λ_S were found at 3.2 ng/L the average daily exposure was much lower at 1.06 ng/L (Chapters 1 & 3) and these concentrations are ecologically realistic (Kostich et al. 2013).

Anderson et al. (2012) argued that only wastewater dominated streams or rivers in the USA are at risk from pollution by estrogens. Other investigators predict that concentrations of estrogens will double by 2050 due mainly to increasing human populations and reduced river flows resulting from climate change (Green et al. 2013). Presently, many waterways in the western and southwestern USA are wastewater dominated (Strange et al. 1995, Dennehy et al. 1998, Brooks et al. 2006) indicating a current shortage of river water to dilute wastewater effluent. For example, in 285 of 582 permitted wastewater discharges in Texas, Oklahoma, New Mexico, Arkansas, and Louisiana sewage effluent comprises over 90% of the stream flow (Brooks et al. 2006). Maximum EE2 concentrations measured using state-of-the-art methods in surface waters range from non-detectable to 11.6 ng/L (Kostich et al. 2013). Given our results and research (Grist et al. 2003, Raimondo et al. 2009, An et al. 2009, Miller et al. 2013, Chapter 3) the ecological effects of EE2 (and other estrogens or anti-androgens) may be more common than previously thought (Anderson et al. 2013). Until very recently the application of methods, namely population models, capable of detecting ecological effects have not been sufficiently used. The perspective of Sumpter & Jobling (2013: 251) suggests that little additional information, of any real importance, can be gleaned from additional studies on the effects of estrogens in the environment. It is our view that we are just beginning to learn the long-term ecological consequences of estrogens and estrogenic chemicals.

In our study we integrated empirically derived reproductive and survival data on the effects of EE2 on fathead minnows into stage-structured population models and estimated effects on λ_S and the sensitivity of the vital rates. A key conclusion is that population effects of estrogens may still be evident even though standard statistical tests are not capable of resolving treatment effects on population-level endpoints. We also clearly demonstrate that fecundity and survival from eggs to juveniles are most important for sustaining populations of fathead minnows, and probably other species with similar life history. We recommend the inclusion of stochastic population models with sensitivity analysis as another tool for risk assessment aimed at estimating the effects of chemical stressors at multiple levels of biological organization.

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Table 4.1. Vital Rates (mean $\pm$ SD) Used in Models of Fathead Minnow (*Pimephales promelas*) Populations Exposed to 17 α -Ethinylestradiol (EE2).

Vital Rates	Control	3.2ng/L EE2	5.3ng/L EE2	10.9ng/L EE2
<i>Fecundity</i>	457 $\pm$ 124	304 $\pm$ 193	258 $\pm$ 133	25 $\pm$ 32
<i>Survival</i>				
Egg to Juvenile	0.06 $\pm$ 0.03	0.06 $\pm$ 0.08	0.05 $\pm$ 0.03	0.003 $\pm$ 0.007
Adult Female	0.66 $\pm$ 0.19	0.71 $\pm$ 0.19	0.49 $\pm$ 0.28	0.46 $\pm$ 0.22
Adult Male	0.66 $\pm$ 0.22	0.17 $\pm$ 0.24	0.14 $\pm$ 0.15	0
Survival of Age-0 >20mm	0.98 $\pm$ 0.04	0.94 $\pm$ 0.10	0.89 $\pm$ 0.20	0.14 $\pm$ 0.38
Overwinter ¹	0.086 $\pm$ 0.0001	0.086 $\pm$ 0.0001	0.086 $\pm$ 0.0001	0.086 $\pm$ 0.0001

¹Overwinter survival was adjusted to give a λ_s of approximately one in the control stochastic model and is considered to be constant among treatments.

Table 4.2. Population Parameters Extracted from Models of Fathead Minnows (*Pimephales promelas*) Exposed to 17 α -Ethinylestradiol (EE2).

Estimated Population Parameters	Control	3.2 ng/L EE2	5.3 ng/L EE2	10.9 ng/L EE2
<i>Life-Stage Simulation Analysis</i>				
λ_S^1	1.05 (0.29-2.91)	0.36 (0.05-4.42)	0.42 (0.08-1.75)	0.04 (0.03-0.09)
% of iterations Where $\lambda_S < 1$	43%	76%	86%	100%
R^2 Fecundity ²	0.21	0.18	0.33	NA ³
R^2 Egg to Juvenile Survival	0.71	0.56	0.42	NA
R^2 Adult Female Survival	0.004	0.002	0.003	NA
R^2 Adult Male Survival	0.0003	0.0004	0.0006	NA
R^2 Survival of Age-0 >20mm	0.0003	0.006	0.07	NA
R^2 Overwinter Survival	0.0003	0.0003	0.00003	NA

¹ λ_S = Geometric mean stochastic population growth rate with 95% confidence intervals in ()

² R^2 = Proportion of variation in λ_S explained by the variation in the vital rate.

³NA = Not Applicable (because no males survived)

Table 4.3. Pearson Correlation Coefficients (*r*) and *p*-values Among Vital Rates in the Control, 3.2 ng/L, 5.3 ng/L, and 10.9 ng/L EE2 Exposed Populations.

Control Population Parameters	Fecundity	Male Survival	Female Survival	Survival >20mm
Male Survival	$r = 0.07356$ $p = 0.8755$			
Female Survival	0.09825 0.8340	-0.2475 0.5926		
Survival >20mm	0.6036 0.1512	0.2623 0.5698	-0.0812 0.8626	
Egg to Juvenile Survival	-0.5327 0.2183	-0.1792 0.7006	-0.3142 0.4925	-0.2513 0.5868
3.2 ng/L Population Parameters	Fecundity	Male Survival	Female Survival	Survival >20mm
Male Survival	0.2092 0.6526			
Female Survival	-0.1850 0.6912	-0.4819 0.2735		
Survival >20mm	0.5637 0.1875	-0.4301 0.3355	0.0432 0.9267	
Egg to Juvenile Survival	-0.2940 0.5222	0.4698 0.2875	-0.5972 0.1568	-0.2995 0.5141
5.3 ng/L Population Parameters	Fecundity	Male Survival	Female Survival	Survival >20mm
Male Survival	-0.0058 0.9901			
Female Survival	0.6575 0.1085	0.2930 0.5236		
Survival >20mm	-0.1335 0.7754	0.7963 0.0321	0.2101 0.6511	
Egg to Juvenile Survival	0.0376 0.9363	0.6297 0.1297	0.1769 0.7043	0.3838 0.3953

Table 4.4. Effect of Reducing Variance in Fecundity on the Proportion of Variance in λ_S explained by the Variance in Each Vital Rate (R^2).

Estimated Parameters	Percent Reduction in Variance								
	10%			25%			50%		
	Control	3.2 ng/L	5.3 ng/L	Control	3.2 ng/L	5.3 ng/L	Control	3.2 ng/L	5.3 ng/L
R^2 Fecundity	0.18 (0.03) ¹	0.17 (0.01)	0.32 (0.01)	0.16 (0.05)	0.14 (0.04)	0.31 (0.02)	0.11 (0.10)	0.10 (0.08)	0.22 (0.11)
R^2 Egg to Juvenile Survival	0.74 (-0.03)	0.63 (-0.07)	0.44 (-0.02)	0.76 (-0.02)	0.70 (-0.14)	0.44 (-0.02)	0.82 (-0.11)	0.76 (-0.20)	0.54 (-0.12)
R^2 Adult Female Survival	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.0001	0.004
R^2 Adult Male Survival	0.0001	0.00009	0.0009	0.00007	0.0001	0.0006	0.0002	0.00002	0.0002
R^2 Survival of Age-0 >20mm	0.001	0.005	0.07	0.001	0.005	0.08	0.002	0.004	0.09
R^2 Overwinter	0.0008	0.002	0.00004	0.0002	0.0004	0.00002	0.00009	0.002	0.0001

¹Data in () are $\Delta R^2 = R^2 Var_{\text{total}} - R^2 Var_{\text{reduced 10, 25, or 50\%}}$ where Var = estimated variance. Estimates or Var_{total} are in Table 4.2. ΔR^2 was calculated only for fecundity and egg to juvenile survival because of negligible R^2 for the other vital rates.

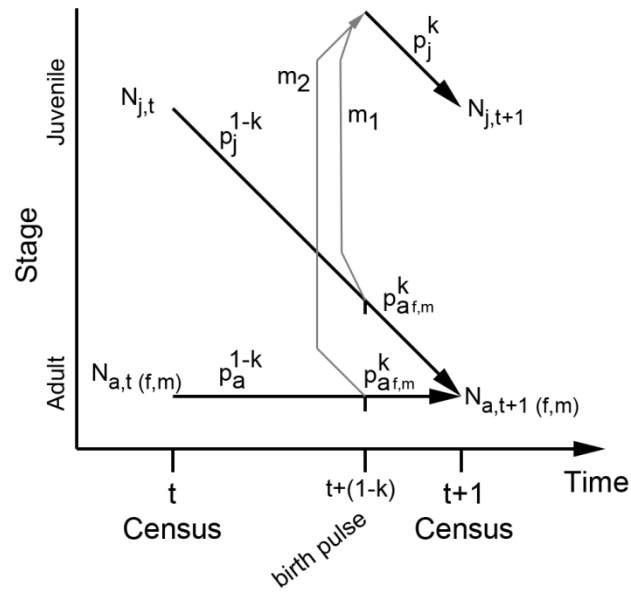


Figure 4.1. Life history of the fathead minnow (*Pimephales promelas*) pertaining to stage-structured matrix models of populations exposed to 17α -ethinylestradiol. N_a = number of adults. N_j = number of juveniles. p_j = egg to juvenile survival. p_a = adult survival. m_1, m_2 = fecundity. Adult survivals are sex specific (f, m).

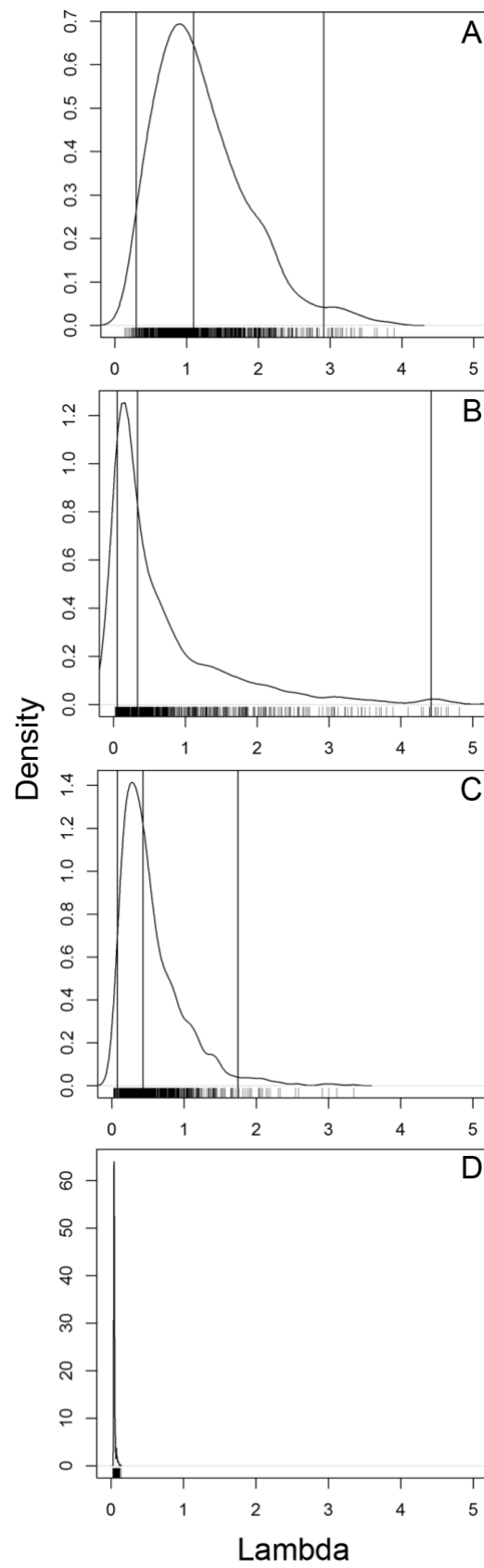


Figure 4.2. Probability densities of stochastic population growth rate (λ_s) from control fathead minnow (*Pimephales promelas*) populations (A) or populations exposed to 3.2 ng/L (B), 5.3 ng/L (C), and 10.9 ng/L 17 α -ethinylestradiol. Mean λ_s was 1.05 (0.29 - 2.91 95% CI) in (A), 0.36 (0.05 - 4.42) in (B), 0.42 (0.08 - 1.75) in (C), and 0.04 (0.03 - 0.09) in (D). The densities were plotted from results of 1000 simulations where vital rates were drawn randomly from probability distributions after that asymptotic λ was calculated as the dominant Eigen-value for each matrix. Individual estimates of λ are shown in the "rug" below the x-axis. The shape parameters for each vital rate were calculated from the mean and standard deviation in Table 4.1. Vertical lines on plots A - C represent the 2.5%, 50% (median), and 97.5% quartiles.

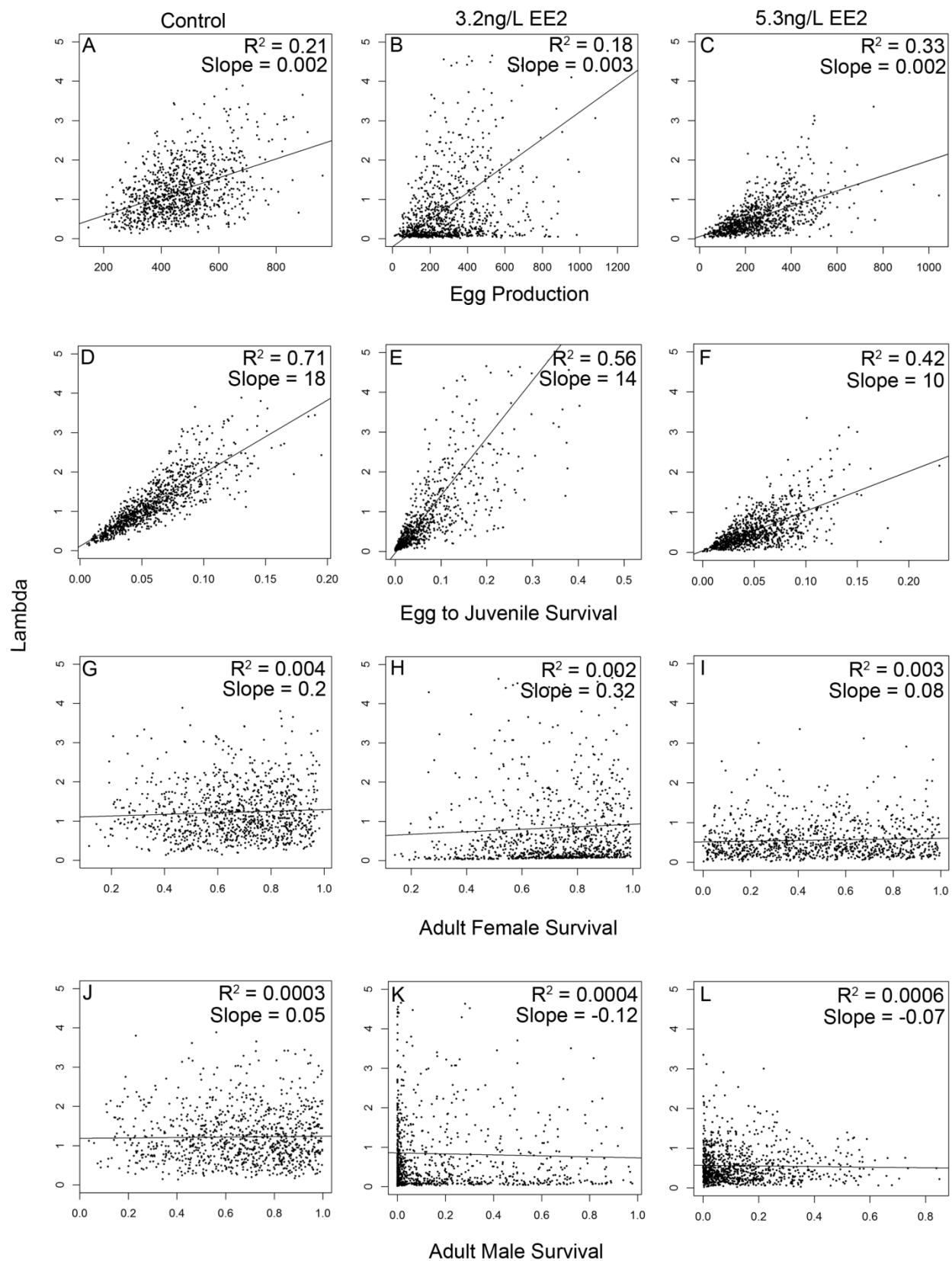


Figure 4.3. Effect of stochastic vital rates on population growth rate (λ) in fathead minnow (*Pimephales promelas*) populations exposed to 17 α -ethinylestradiol (EE2). Control populations (0 ng/L EE2) are represented in (A, D, G, and J), populations exposed to 3.2 ng/L EE2 or 5.3 ng/L are represented in (B, E, H, and K) and (C, F, I, and L), respectively. R^2 is proportion of variation in λ explained by the variation in each vital rate; egg production (A, B, and C), egg survival (D, E, and F), embryo survival (G, H, and I) or overwinter survival (J, K, and L). Data for the regressions were acquired from 1000 random draws from probability distributions for each vital rate and related to asymptotic λ , the dominant Eigen value from stage-structured matrices calculated at each iteration.

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APPENDIX I: EXPERIMENTAL FACILITIES AND STRATEGIES FOR DATA ANALYSIS -
RELEVANT EXCERPTS FROM THE RESEARCH PROPOSAL

INTRODUCTION

Experiments will be conducted at the Foothills Fisheries Laboratory (FFL) on the Foothills Campus of Colorado State University. A new constructed outdoor mesocosm facility (26 total) will serve as the experimental system (Figure AI.1). **Author's note:** two additional mesocosms were added in 2011 to increase statistical power. The polyethylene mesocosms (Rubbermaid Corporation, Atlanta, GA) measure 1.7 m in diameter by 0.635 m deep and can be filled to an average of 1056 L (range 1009-1105 L) of water. The water is sourced from College Lake and Horsetooth Reservoir and delivered by underground pipe to the FFL. Upon entry to the FFL, the lake water is filtered and sterilized by ultra-violet light. The mesocosms are gravity fed by polyvinyl chloride (PVC) pipe from an approximately 3000 L head tank. Flow rate is maintained 1 - 2 L/min with valves at each mesocosm. Ambient water temperature ranges from 17 to 27 °C during the summer months. Mesocosms will be aerated continually. The effluent is treated with activated charcoal, then released to a holding pond, and ultimately treated by municipal wastewater facilities before entering the Poudre River, Colorado.

The USEPA Region 8 Laboratory will be used for assessing fish molecular biomarkers indicative of estrogen exposures. Histology will be performed by the Central Histology Facility, Sacramento, CA. Scoring of tissue abnormalities evident in histological sections will be performed microscopically at Colorado State University, USEPA Region 8 lab, or the University of Colorado, Denver, CO. Ethinylestradiol concentrations will be measured by the University of Nebraska Water Sciences Laboratory, Lincoln, NE, the USEPA Region 8 Laboratory, or other collaborating or contract laboratory. **Author's note:** we developed a method for quantifying EE2 in the water, the results of which were described in Chapter 1.

Data Analysis

Data will be tested to assess if assumptions for parametric tests are met. Biological endpoints comprised of continuous data will be tested for differences by ANOVA (or Kruskal Wallis) and for trends by linear regression. In the case of a significant main effect for ANOVA differences comparing control to treatment will assessed by Dunnett's test, other multiple comparisons will tested by REGWQ to control for maximum experiment-wise error rate. Should unplanned comparisons be tested, Scheffe will be used for multiple comparisons. For categorical data Chi-squared or logistic regression will be used. SAS statistical software will be used for these analyses (SAS Institute, Cary, North Carolina). Statistical significance will be set at $p < 0.05$. **Author's note:** generalized linear mixed models were chosen over ANOVA because of our interested in incorporating random effects and the ability to specify distributions in the analyses.

Power Analysis

Based on the mean population numbers of the Age-0 fish (Table AI.1) I conducted a power analysis for an ANOVA model with three different standard deviations. The analyses were based on the average of the standard deviations (52), the minimum (34), and the maximum (70) standard deviation (Table AI.1) with the goal of calculating the sample size per treatment (Figure AI.2). I believe that our proposed daily (as opposed to every other day) dosing schedule and increased sample size ($n = 6$) will result in larger differences among the mean population numbers than I observed in a pilot study (Appendix II). **Author's note:** we decided on a $n = 7$ and to drop the 2.5 ng/L treatment for the definitive study. Assuming the standard deviations will remain roughly the same, this should improve my ability to detect statistical significance among population means. The null hypothesis for all analyses is that all means are equal.

Table AI.1: Descriptive Statistics of Age-0 Fish Counts from the Preliminary Population Study
in Appendix II.

Treatment (EE2 ng/L)	0	2.5	5	10	20
Mean	190.6	197.8	196.8	147.4	153.8
Standard Deviation	52.20919	51.7465	70.17621	34.19503	51.06564
Standard Error	23.34866	23.14174	31.38375	15.29248	22.83725



Figure AI.1. Mesocosm array at the Foothills Fisheries Lab, Colorado State University.

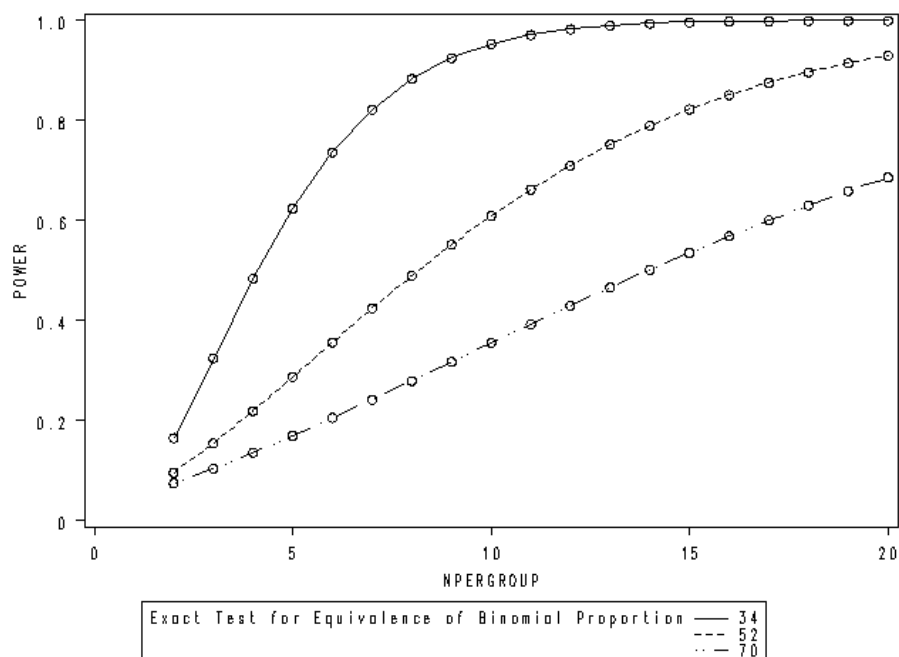


Figure AI.2. Power analysis using data from a preliminary experiment described in Appendix II. Standard deviation = 34 (uppermost line), 52 (middle), and 70 (bottom line).

APPENDIX II: RESULTS FROM A PRELIMINARY STUDY ON THE EFFECTS OF 17 α -
ETHINYLESTRADIOL ON POPULATIONS OF FATHEAD MINNOWS (*PIMEPHALES*
PROMELAS) IN OUTDOOR AQUATIC MESOCOSMS

INTRODUCTION

I conducted a pilot study in the spring of 2010 that provides the framework for the proposed research. Specifically our goals were to: 1) construct and plumb an array of 26 mesocosms; 2) evaluate the suitability of the mesocosms for supporting fish populations for several months; 3) test an 17 α -ethinylestradiol (EE2) spiking method; 4) estimate the half-life of EE2 in the water column, and; 5) gather initial biomarker, reproduction, and population data in response to EE2 exposures. Details of the mesocosms are found in Appendix I above.

MATERIALS AND METHODS

Mesocosms (Appendix I, Figure AI.1) were assigned using a stratified random process to ensure treatments were equally represented in each row of the mesocosm array. A total of 25 mesocosms were used with five treatments at 0, 2.5, 5.0, 10.0, 20.0 ng/L nominal EE2 concentrations and five mesocosms per treatment. Mesocosms were conditioned with lake water for 13-d prior to the addition of fish and were allowed to colonize naturally with invertebrates and algae throughout the experiment. Dissolved oxygen, pH, conductivity, was measured weekly with a YSI meter (YSI, Inc. Yellow Springs, Ohio) and water temperatures were collected every other day.

Fish

Five month old virgin fathead minnows (*Pimephales promelas*) were obtained from Aquatic Biosystems (Fort Collins, CO). Fish were sexed using external characteristics; nuptial tubercles on male fish and presence of an ovipositor on female fish. Males and females of the founding population were anesthetized in 50 mg/L tricaine methanesulfonate (MS-222). Total

length (mm) and mass (g) were collected and fish were uniquely identified as male or female with elastomer tags (Northwest Marine Technology, Shaw Island, WA) injected subcutaneously behind the left eye and left of the dorsal fin. Males and females were allocated to experimental tanks ensuring an even size distribution. The founding populations consisted of 10 breeding pairs. Breeding habitat was 7.5 cm diameter PVC pipe cut lengthwise and then in half at 15 cm increments forming "half-pipes" placed upside down on the bottom of the mesocosm. Fathead minnows spawn on the underside of submerged structures. Ten habitats were added to each mesocosm. For adult fish, brine shrimp flake supplemented the natural food in each mesocosm and newly hatched brine shrimp nauplii supplemented food for larval fish. Fish were treated in accordance with Institutional Animal Care and Use Committee guidelines under protocol number 10-1685A.

Chemicals, Mesocosm Dosing, and Water Quality

I purchased 17 α -ethinylestradiol (EE2) (purity > 98%) from Sigma Chemical, St. Louis, Missouri. A concentrated stock solution of EE2 was made in high pressure liquid chromatography grade methanol and stored at -20 °C in an acid rinsed amber glass bottle. Serial dilutions of the stock solution were made just prior to spiking in methanol rinsed glass vials so that nominal concentrations were 0, 2.5, 5, 10, and 20 ng/L. The EE2 additions began the day after fish were introduced to the tanks. The control (0 ng/L) treatment received an equivalent volume of methanol (1 ml) served as the vehicle control. This is approximately 1 ppm of methanol in the mesocosm and no water controls were used. I spiked mesocosms every other day between 17:00-18:00 h. On the spiking days, water to the mesocosms was turned off and the appropriate amount of EE2 was added by glass serological pipet to the plume of air bubbles to

ensure mixing. Water remained off overnight and was turned on the following day between 08:00-09:00 h for a 15-h total exposure period. With a 1 L/min flow rate and average water volume of 1056 L, turnover was approximately 18-h. Following approximately 95% water turnover, fish were unexposed for approximately 15-h before the next EE2 spike.

EE2 analysis

Biweekly water samples were collected during the breeding season for analysis of EE2 concentrations according to the following protocol. Thirty minutes after spiking the mesocosms, 250 ml amber glass bottles were rinsed in the respective mesocosm water. Similarly a methanol rinsed 1 L beaker was also rinsed in the respective mesocosm. Then four 250 ml water samples were collected from four different areas around the mesocosm and pooled in the 1 L beaker. From the pooled 1 L sample, a 250 ml subsample was collected in the amber glass bottle and frozen at -20 °C until analysis by gas chromatography mass spectrometry (University of Nebraska, Water Sciences Laboratory, Lincoln, NE). **Author's note:** we developed our own EE2 method that is presented in Chapter 1. On one occasion, water samples were collected 30 min, 4-h, and 12-h after spiking to estimate the half-life of EE2 in the water column.

Cage Study

To investigate the short-term effects of EE2, I conducted a 14-d exposure of adult male fathead minnows in PVC cages in the mesocosms. The cylindrical cages measured 30.5 cm in length × 15 cm in diameter. Eight adult males from the same allotment of fish stocked in the mesocosms were randomly allocated to each of 25 cages ensuring an equal size distribution among the cages. Plastic netting (3 mm opening) was fastened to the ends of the cages with cable

ties and the cages were submerged to the bottom of the mesocosms. Following the 14-d exposure the fish were sampled according to the procedures described in the following section.

Fish Sampling

Egg production was estimated on a weekly basis because FHM eggs hatch every 5-d. Digital photographs (Sony a700 digital SLR, Sony Corporation, Japan) of each breeding habitat were captured and eyed-eggs were enumerated using ImageJ software (NIH, Bethesda, MD). At the end of the 105-d experiment, fish were initially netted out of the mesocosms, separated from algae and other debris, and allocated to buckets filled with water from the mesocosm. Next, water was pumped from the mesocosms and the remaining fish in the bottom of the mesocosms were hand netted and transferred to buckets. All fish were euthanized in 250 mg/L MS-222 within 1-h of netting from the mesocosms. Wet weight and total length were collected from the adults. Blood plasma was collected following centrifugation and stored on dry ice until transport to a -60 °C freezer. Livers were removed aseptically, transferred to 1.5 ml cryo-vial, snap-frozen in liquid N₂, transferred to dry ice, and transported to -60 °C freezer. The carcass was placed in 15 ml vials, fixed in Davidson's for approximately 48-h, and then transferred to 10% buffered formalin. Secondary sex characteristics of adult fish were not collected because the fish were no longer in breeding condition. Young of the year were arbitrarily divided into three groups and processed accordingly: 1) 70% ethanol; 2) Davidson's fixative; 3) liquid N₂. Young of the year in Davidson's fixative were transferred to 10% buffered formalin after approximately 48-h.

Endpoints

Egg-production, survivorship, change in length, VTG mRNA, plasma VTG, and gonad histopathology will be evaluated in the adult fish. Only adult survivorship, change in length, egg production, and population estimates are presented here because the other analyses are in process. Length-frequency diagrams, size of ovipositors, histopathology, and whole-fish VTG mRNA will be estimated for the age-0 fish.

RESULTS AND DISCUSSION

Exposure of fathead minnows to 0, 2.5, 5.0, 10.0, and 20.0 ng/L of EE2 resulted in a dose dependent decline the total length (Figure AII.1) and reduced survivorship (Figure AII.2) of adult males after the 105-d experiment. Eyed egg production did not follow a dose response, with production being highest in the 2.5 and 5.0 ng/L groups, lowest at 10 ng/L and intermediate at 0 and 20.0 ng/L EE2 (Figure AII.3). The reasons for this are unknown but may be due to the variable effect of EE2 on male ability to fertilize eggs and guard the nests. Mesocosms exposed to 10 and 20 ng/L produced approximately 200 fewer offspring than the other treatments (Figure AII.4) although this decline is not significant ($F_{4,20} = 1.07$, $p = 0.3955$). Considering that the 0 ng/L and 20 ng/L treatments produced similar numbers of eggs but approximately 200 fewer survived to the end of the experiment in the 20 ng/L treatment indicates reduced hatch success or survival of the age-0 shortly after hatching. Regardless, greater reproductive effort was required from the 20 ng/L treatment per juvenile produced. This may be one explanation for the increased mortality in the adult male fish in the 20 ng/L treatment. Ovipositors and spinal deformities were observed in the Age-0 and analysis of those endpoints is in process. Relationships to treatments are currently being assessed with the other biological endpoints.

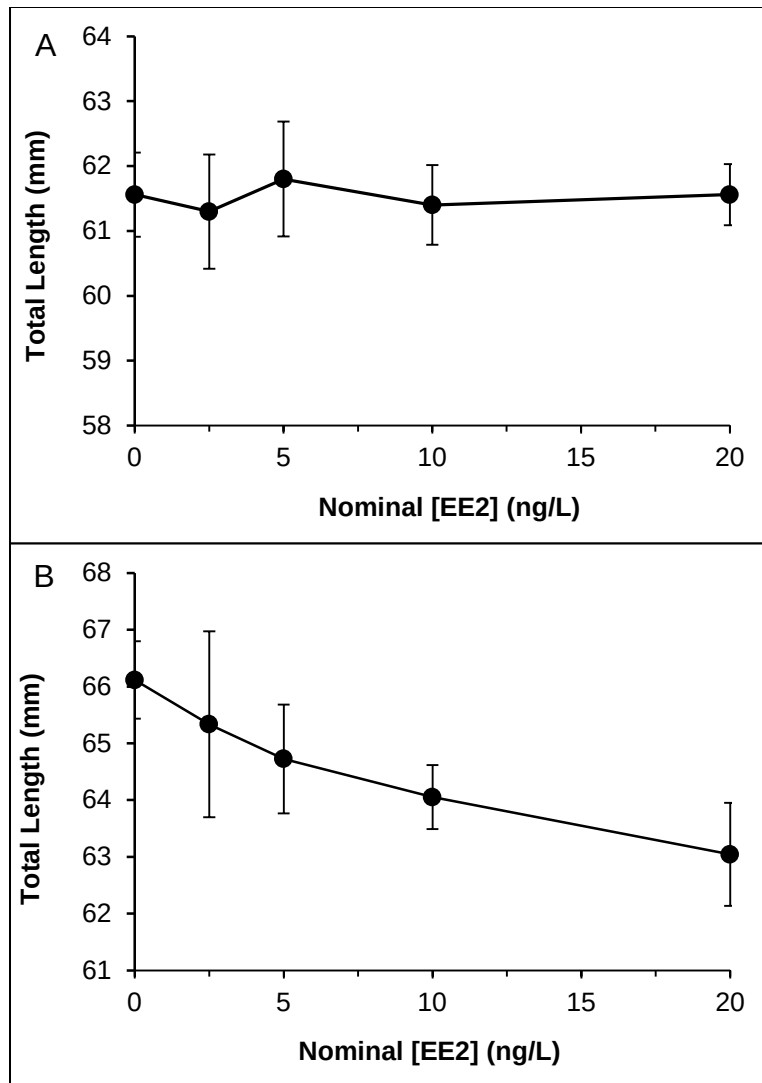


Figure AII.1. Initial total length (A) and effect of 17alpha-ethinylestradiol on length (B) of adult fathead minnow (*Pimephales promelas*). Y-axis origin is 58 mm (A) or 61 mm (B). Data are mean $\pm$ SEM.

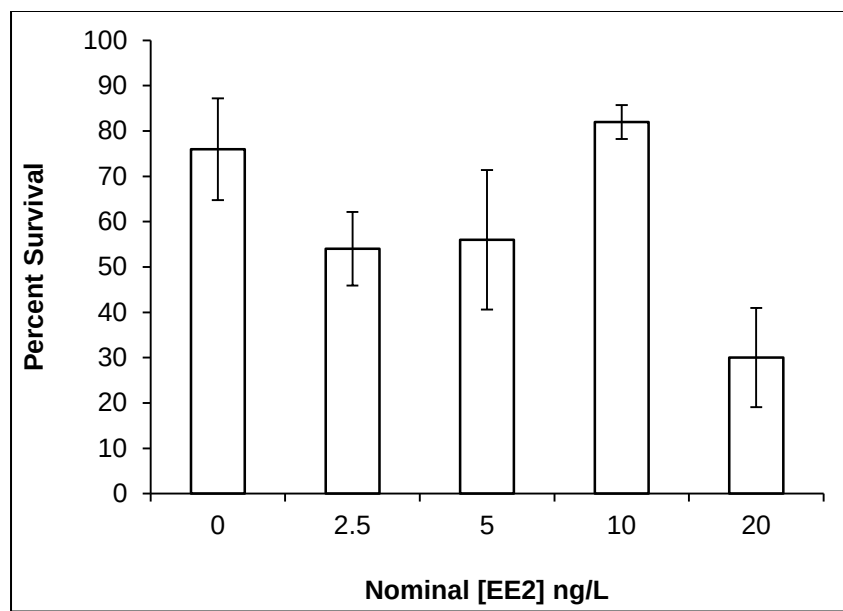


Figure AII.2. Adult male fathead minnow (*Pimephales promelas*) survival after 105-d of exposure to varying concentrations of 17alpha-ethinylestradiol. Data are mean percent $\pm$ SEM.

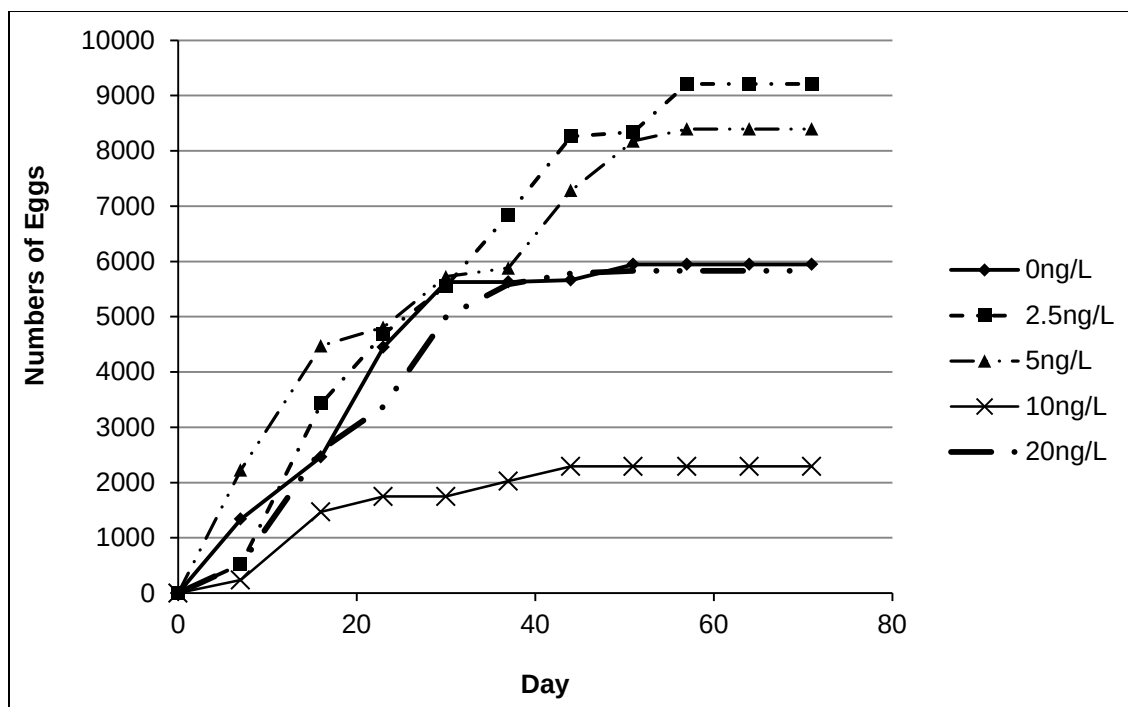


Figure AII.3. Effect of 17 α -ethinylestradiol on eyed-egg production through time. Data are sum totals of eggs from all treatments.

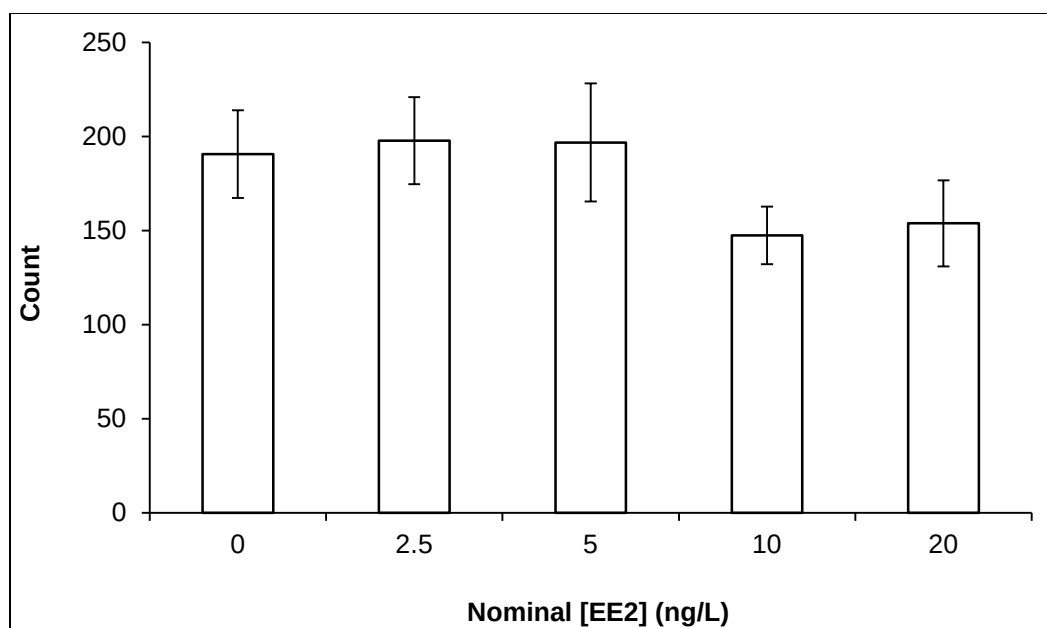


Figure AII.4. Effect of 17 α -ethinylestradiol (EE2) on numbers of age-0 fathead minnows (*Pimephales promelas*). Data are mean $\pm$ SEM.

APPENDIX III: ^1H -NUCLEAR MAGNETIC RESONANCE DIFFERENCE SPECTRA AND
METABOLIC NETWORK MODELING OF MALE FATHEAD MINNOWS (*PIMEPHALES*
PROMELAS) EXPOSED TO 17α -ETHINYLESTRADIOL

MATERIALS AND METHODS

Metabolite Profiles

We used ^1H -nuclear magnetic resonance to generate hepatic metabolite profiles of fathead minnows exposed to EE2. To identify metabolites that were significantly different between treatments we generated ‘*t*-test filtered difference spectra’ (see Chapter 4 Materials and Methods for details). This allowed us to assess if differences within a particular metabolite were significantly different between exposed and control fish. If the *t*-test for a particular bin comparison was not significant ($p \geq 0.05$), we set the average difference to zero, otherwise we reported the average difference. Differences that were > 0 indicate metabolites that were greater in the exposed males relative to the controls, and peaks with magnitudes of < 0 represented metabolites that were lower in the exposed males (Figure AIII.1). For those bins that were found to be significantly different based on the *t*-test, we identified metabolite peaks using Chenomx NMR Suite 7.0 (Chenomx, Inc. Edmonton, Canada) and previously published spectra standards (Ekman et al. 2008, Wishart et al. 2009, Teng et al. 2009).

Metabolic Network Modeling

We used metabolic network modeling (MNM) to characterize responses. Numerous applications are available for MMN and we chose Interactive Pathway Analysis (IPA) version 16542223 (©2013, Ingenuity Systems, Inc., Redwood City, CA). IPA assembles metabolic pathways drawing from an expertly curated database that is regularly reviewed and updated by subject matter experts. Output from the analysis includes a metabolic network with: 1) predicted biological functions based on published literature, 2) the ability to predict the downstream effects

of metabolites that are up or down-regulated by the experimental treatment, and 3) links to the primary literature used to build the network. The MNM does not allow strong inference regarding the molecular pathway but assembles likely treatment relevant pathways for purposes of generating hypotheses for further study. For our analysis we uploaded the metabolite profiles, one dataset for the 7-d exposure and one dataset for the 102-d exposure into the IPA graphic user interface. We divided the data in this way because the metabolite profiles were different across time but not different in relation to the EE2 concentrations within an exposure period. In other words a dose response was not evident within a timepoint. We then ran the "Core Analysis" using default parameters except that we directed IPA to build pathways based on knowledge of hepatic physiology by selecting "Liver" from the "Organ Systems" box under the Tissues and Cell Lines tab. We also deselected "Mutations" to prevent IPA from looking for pathways based on experimental data from genetically modified animals. The resulting networks do not contain all metabolites that were measured. The IPA uses the metabolite profile as a basis for which to build a network considering the data on known cellular processes in the literature and their relation to the metabolites in the profile.

RESULTS AND DISCUSSION

Effect of EE2 on the hepatic metabolite profile

EE2 induced or suppressed hepatic metabolites at 7-d compared to 102-d, but no obvious patterns emerged in terms of a dose response at either timepoint (Figure AIII.1). The energy producing intermediates AMP/ADP/ATP were down-regulated at 7-d EE2 exposure, but up-regulated after 102-d relative to control (0 ng/L) fish. Likewise the amino acids glutamine and

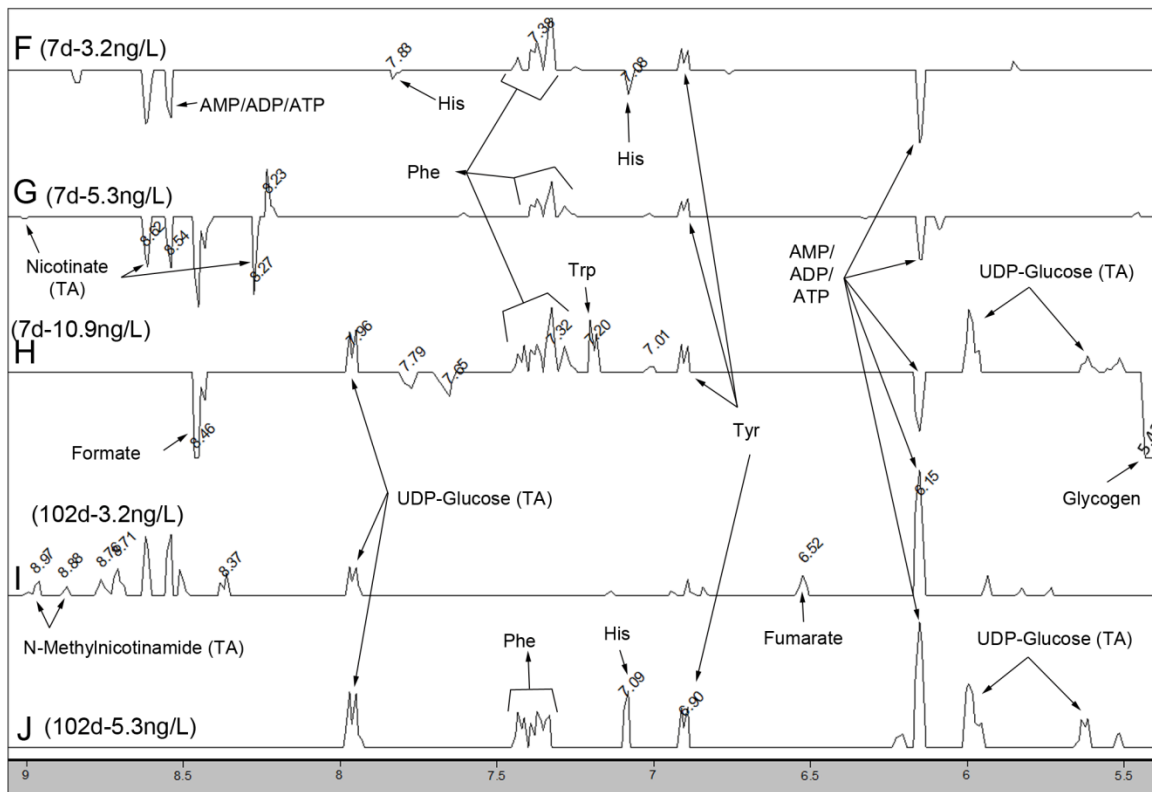
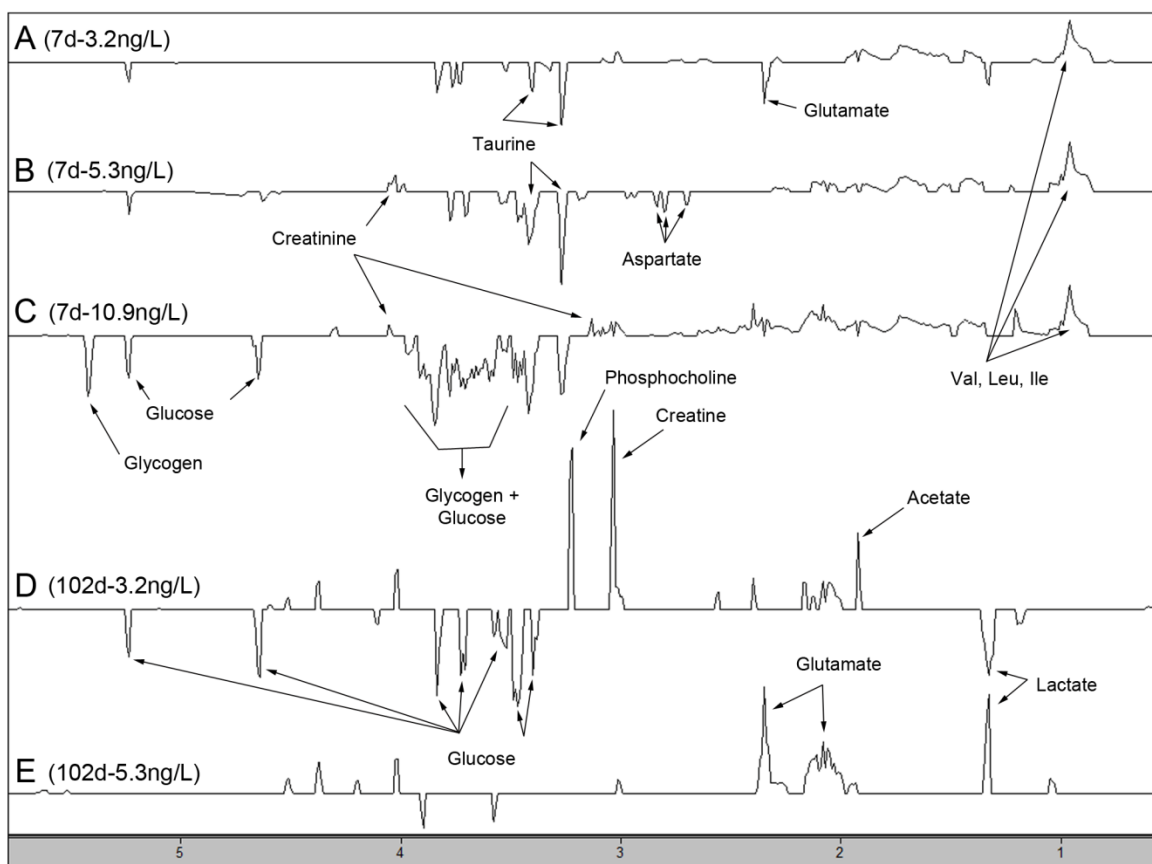
histidine were down-regulated at 7-d but up-regulated after 102-d exposure. The amino acid taurine was down-regulated at 7-d but was not different than controls at 102-d. In the liver, taurine is conjugated to cholic acid forming taurocholate, a bile acid (Mathews et al. 2000). The amino acids isoleucine, leucine, phenylalanine, tryptophan, tyrosine, and valine were all up-regulated at 7-d but were not different than control after 102-d EE2 exposure. Notably phosphocholine, an intermediate in the biosynthesis of phosphatidylcholine a component of cell membranes was up-regulated at 102-d. The high energy intermediate creatine (with associated phosphate) facilitates phosphorylation of ADP to maintain ATP at high levels was up-regulated at 102-d (Mathews et al. 2000). Nicotinate (vitamin B3/niacin) was down-regulated at 7-d and N-methylnicotinamide was up-regulated at 102-d; both are involved in the production of the coenzyme NADP that contains high energy phosphate groups for use in many metabolic pathways such as fatty acid biosynthesis and the Citric Acid cycle. The only metabolite that showed a concentration response was lactate, being down-regulated at 3.2 ng/L and up-regulated at 5.3 ng/L in the 102-d exposure. Lactate is a the main substrate in gluconeogenesis. The only metabolites that were not affected temporally were glucose and glycogen. Both were down-regulated relative to control regardless of timepoint. However, UDP-glucose was up-regulated at both 7-d and 102-d. UDP-glucose donates the glucosyl residue to glycogen in the glycogen biosynthesis pathway. Acetate was up-regulated at 102-d and is a substrate in acetyl-CoA biosynthesis an intermediate in the Citric Acid cycle as well as an intermediate in fat, carbohydrate, and protein metabolism.

The networks built by IPA were quite different between the 7-d and 102-d timepoints (Figures AIII.2 and AIII.3). The top 10 biological functions associated with the network assembled from 7-d EE2 exposure were: 1) carbohydrate metabolism, 2) small molecule

biochemistry, 3) molecular transport, 4), lipid metabolism 5) metabolic disease, 6) endocrine system disorders, 7) gastrointestinal disease, 8) hepatic system disease, 9) liver steatosis, and 10) connective tissue development. The top 10 biological functions associated with the network assembled from the 102-d exposure were: 1) lipid metabolism, 2) molecular transport, 3) small molecule biochemistry, 4) carbohydrate metabolism, 5) cellular compromise, 6) energy production, 7), endocrine system disorders, 8) metabolic disease, 9) cell morphology, and 10) cellular function and maintenance.

The network built from the 7-d exposure included several molecules from the metabolite profile including glucose, glycogen, and the amino acids tryptophan, and taurine (Figure AIII.1A-C, F-H). Both glucose and taurine were durably down-regulated across EE2 concentrations at the 7-d timepoint (Chapter 2, Table 2.2). So we perturbed the model to evaluate the downstream effects of depressed glucose and taurine levels on the network. This led to a cascade of predicted responses in a model hepatocyte (Figure AIII.2). Plasma membrane receptors and growth factors were up- and down-regulated, respectively. The majority of predicted metabolic effects appear to be controlled by peroxisome proliferator-activated receptor α (PPARA) that regulates lipid metabolism in the liver (Figure AIII.2). Taurine was down-regulated in our experiment which IPA predicted to reduce PPARA activity that resulted in predicted changes to enzymes in the glycolytic, gluconeogenic, and fatty acid synthesis pathways. On the glycolysis side, glucokinase was up-regulated but pyruvate kinase was down-regulated. The gluconeogenic enzyme phosphoenolpyruvate carboxy kinase was down-regulated as was glucose-6-phosphate dehydrogenase in the pentose phosphate pathway. Fatty acid synthesis was also predicted to be depressed as indicated by down-regulated carnitine palmitoyltransferase, fatty acid synthase, and fatty acid elongase. The 102-d model contained

phosphocholine and glucose from the metabolite profiles (Figure AIII.1D). The downstream effects predicted from this perturbation were only that insulin-like-growth-factor-2 was depressed (Figure AIII.3). All of the predicted changes are relative to the control (0 ng/L) fish.



Chemical Shift (ppm)

Figure AIII.1. Treatment averages of difference spectra of metabolites from fish exposed to 17 α -ethinylestradiol (EE2) for either 7-d (A-C, F-H) or 102-d (D-E, I-J). A-E are compounds read during the chemical shift from 1-5; whereas, F-J are read during the chemical shift from 5.5 to 9 so that F-J is a continuation of the same run as A-E. Fish exposed to 3.2 ng/L are represented in A, D, F, and I. Fish exposed to 5.3 ng/L are represented in (B, E, G, and J). Fish exposed to 10.9 ng/L for 7-d are represented in C and H. Peaks indicate metabolites up-regulated relative to control (0 ng/L) and troughs are metabolites down-regulated relative to control. The relative height of the peak or depth of the trough indicates the magnitude of difference from control.

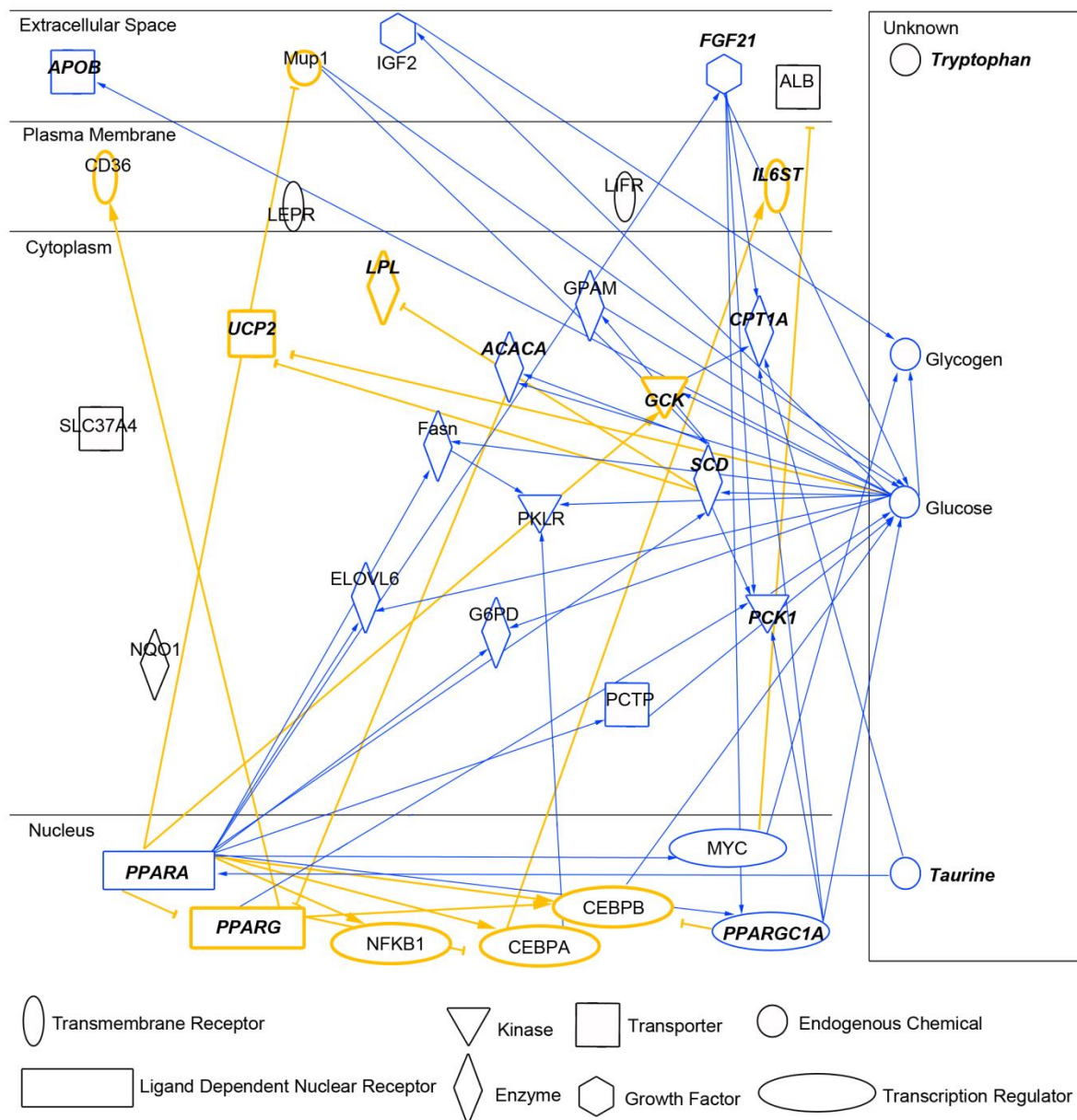


Figure AIII.2. Hepatic metabolic network of fathead minnows (*Pimephales promelas*) exposed from 3.2 - 10.9 ng/L 17 α -ethinylestradiol for 7-d. The model was built using Interactive Pathway Analysis software where metabolite profiles were uploaded and a computer algorithm builds the model. Glucose and taurine were down-regulated in our experiment and the arrows indicated predicted downstream effects resulting from that change. Molecules and arrows in blue indicate predicted inhibition and molecules and arrows in orange indicate predicted activation. Bold

italics are molecules associated with hepatic steatosis in mammals. Molecules are organized based on location in the hepatocyte or extracellular space and are separated by black horizontal lines.

Abbreviations:

ACACA - acetyl-CoA carboxylase α
ALB - albumin;
APOB - apolipoprotein B;
CD36 - CD36 molecule (thrombospondin receptor);
CEBPA - CCAAT/enhancer binding protein (C/EBP) α ;
CEBPB - CCAAT/enhancer binding protein (C/EBP) β ;
CPT1A - carnitine palmitoyltransferase 1A (liver);
ELOVL6 - fatty acid elongase 6;
Fasn - fatty acid synthase;
FGF21 - fibroblast growth factor 21;
G6PD - glucose-6-phosphate dehydrogenase;
GCK - glucokinase;
GPAM - glycerol-3-phosphate acyltransferase (mitochondrial);
IGF2 - insulin like growth factor 2;
IL6ST - interleukin 6 signal transducer;
LEPR - leptin receptor;
LIFR - leukemia inhibitory factor receptor α ;
LPL - lipoprotein lipase;
Mup1 - major urinary protein 1;
MYC - v-myc myelocystomatosis viral oncogene homolog;
NFKB1 - nuclear factor of kappa light polypeptide gene enhancer;
NQO1 - NAD(P)H dehydrogenase, quinone 1;
PCK1 - phosphoenolpyruvate carboxykinase 1;
PCTP - phosphatidylcholine transfer protein;
PKLR - pyruvate kinase (liver and red blood cell);
PPARA - peroxisome proliferator-activated receptor α ;
PPARG - peroxisome proliferator-activated receptor γ ;
PPARGC1A - peroxisome proliferator-activated receptor γ , coactivator 1 α ;
SCD - stearoyl-CoA desaturase;
SLC 37A4 - solute carrier family 37 (G6P transporter) member 4;
UCP2 - uncoupling protein 2

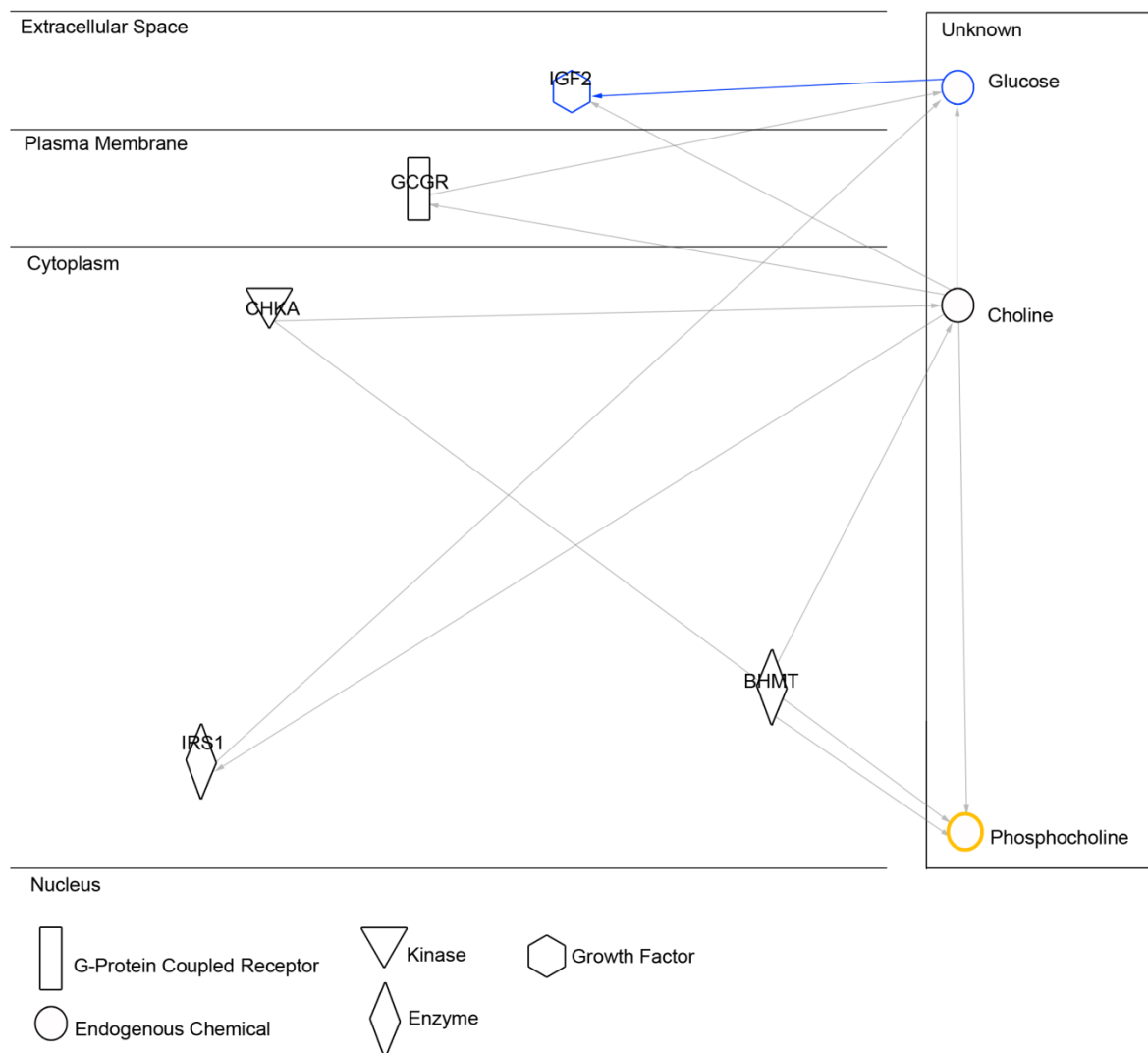


Figure AIII.3. Hepatic metabolic network of fathead minnows (*Pimephales promelas*) exposed from 3.2 - 10.9 ng/L 17 α -ethinylestradiol for 102-d. The model was built using Interactive Pathway Analysis software where metabolite profiles were uploaded and a computer algorithm builds the model. Glucose was down-regulated and phosphocholine was up-regulated in our experiment. Molecules and arrows in blue indicate predicted inhibition. Grey arrows indicate all known molecular interactions. Molecules are organized based on location in the hepatocyte or extracellular space and are separated by black horizontal lines.

Abbreviations:
BHMT - betaine-homocysteine S-methyl transferase;

CHKA - choline kinase α ;

GCCR - Glucagon Receptor; G6PD - glucose-6-phosphate dehydrogenase;

IGF2 - insulin like growth factor 2;

IRS1 - insulin receptor substrate 1

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