Effects of early life stage exposure of largemouth bass to atrazine or a model estrogen (17α-ethinylestradiol)

Jessica K Leet [Corresp., 1] , **Catherine A Richter** [1] , **Robert S Cornman** [2] , **Jason P Berninger** [1] , **Ramji K Bhandari** [3] , **Diane K Nicks** [1] , **James L Zajicek** [1] , **Vicki S Blazer** [4] , **Donald E Tillitt** [1]

[1] Columbia Environmental Research Center, United States Geological Survey, Columbia, Missouri, United States

[2] Fort Collins Science Center, United States Geological Survey, Fort Collins, Colorado, United States

[3] Department of Biology, University of North Carolina at Greensboro, Greensboro, NC, United States

[4] Leetown Science Center, United States Geological Survey, Kearneysville, WV, United States

Corresponding Author: Jessica K Leet
Email address: jleet@usgs.gov

Endocrine disrupting contaminants are of continuing concern for potentially contributing to reproductive dysfunction in largemouth and smallmouth bass in the Chesapeake Bay watershed (CBW) and elsewhere. Exposures to atrazine (ATR) have been hypothesized to have estrogenic effects on vertebrate endocrine systems. The incidence of intersex in male smallmouth bass from some regions of CBW has been correlated with ATR concentrations in water. Fish early life stages may be particularly vulnerable to ATR exposure in agricultural areas, as a spring influx of pesticides coincides with spawning and early development. Our objectives were to investigate the effects of early life stage exposure to ATR or the model estrogen 17α-ethinylestradiol (EE2) on sexual differentiation and gene expression in gonad tissue. We exposed newly hatched largemouth bass (LMB, *Micropterus salmoides*) from 7 to 80 days post-spawn to nominal concentrations of 1, 10, or 100 µg ATR/L or 1 or 10 ng EE2/L and monitored histological development and transcriptomic changes in gonad tissue. We observed a nearly 100% female sex ratio in LMB exposed to EE2 at 10 ng/L, presumably due to sex reversal of males. Many gonad genes were differentially expressed between sexes. Multidimensional scaling revealed clustering by gene expression of the 1 ng EE2/L and 100 µg ATR/L-treated male fish. Some pathways responsive to EE2 exposure were not sex-specific. We observed differential expression in male gonad in LMB exposed to EE2 at 1 ng/L of several genes involved in reproductive development and function, including *star*, *cyp11a2*, *ddx4* (previously *vasa*), *wnt5b*, *cyp1a*, and *samhd1*. Expression of *star*, *cyp11a2*, and *cyp1a* in males were also responsive to ATR exposure. Overall, our results confirm that early development is a sensitive window for estrogenic endocrine disruption in LMB and are consistent with the hypothesis that ATR exposure induces some estrogenic responses in the developing gonad. However, ATR-specific and EE2-specific responses were also observed.

Effects of early life stage exposure of largemouth bass to

atrazine or a model estrogen (17α-ethinylestradiol)

Jessica K. Leet[1*], Catherine A. Richter[1], Robert S. Cornman[2], Jason P. Berninger[1], Ramji K.

Bhandari[3], Diane K. Nicks[1†], James L. Zajicek[1], Vicki S. Blazer[4], and Donald E. Tillitt[1]

[1]Columbia Environmental Research Center, United States Geological Survey, Columbia,
Missouri, United States of America

[2]Fort Collins Science Center, United States Geological Survey, Fort Collins, Colorado, United
States of America

[3]Department of Biology, University of North Carolina at Greensboro, Greensboro, North
Carolina, United States of America

[4]Leetown Science Center, United States Geological Survey, Kearneysville, West Virginia,
United States of America

* Corresponding author:

Jessica K. Leet

Columbia Environmental Research Center, United States Geological Survey, Columbia,

Missouri, United States of America

E-mail: jleet@usgs.gov

† Deceased

Abstract

29 Endocrine disrupting contaminants are of continuing concern for potentially contributing to

30 reproductive dysfunction in largemouth and smallmouth bass in the Chesapeake Bay watershed

31 (CBW) and elsewhere. Exposures to atrazine (ATR) have been hypothesized to have estrogenic

32 effects on vertebrate endocrine systems. The incidence of intersex in male smallmouth bass from

33 some regions of CBW has been correlated with ATR concentrations in water. Fish early life

34 stages may be particularly vulnerable to ATR exposure in agricultural areas, as a spring influx of

35 pesticides coincides with spawning and early development. Our objectives were to investigate

36 the effects of early life stage exposure to ATR or the model estrogen 17α-ethinylestradiol (EE2)

37 on sexual differentiation and gene expression in gonad tissue. We exposed newly hatched

38 largemouth bass (LMB, *Micropterus salmoides*) from 7 to 80 days post-spawn to nominal

39 concentrations of 1, 10, or 100 µg ATR/L or 1 or 10 ng EE2/L and monitored histological

40 development and transcriptomic changes in gonad tissue. We observed a nearly 100% female sex

41 ratio in LMB exposed to EE2 at 10 ng/L, presumably due to sex reversal of males. Many gonad

42 genes were differentially expressed between sexes. Multidimensional scaling revealed clustering

43 by gene expression of the 1 ng EE2/L and 100 µg ATR/L-treated male fish. Some pathways

44 responsive to EE2 exposure were not sex-specific. We observed differential expression in male

45 gonad in LMB exposed to EE2 at 1 ng/L of several genes involved in reproductive development

46 and function, including *star*, *cyp11a2*, *ddx4* (previously *vasa*), *wnt5b*, *cyp1a*, and *samhd1*.

47 Expression of *star*, *cyp11a2*, and *cyp1a* in males were also responsive to ATR exposure. Overall,

48 our results confirm that early development is a sensitive window for estrogenic endocrine

49 disruption in LMB and are consistent with the hypothesis that ATR exposure induces some

50 estrogenic responses in the developing gonad. However, ATR-specific and EE2-specific

51 responses were also observed.

52

Introduction

54   Early life stages in fish and other vertebrates tend to be sensitive to effects of endocrine

55 disrupting chemicals (EDCs) (van Aerle et al. 2002), and EDCs are considered a global concern.

56 Effects during gonad development are likely to be permanent, organizational events with

57 potential for complete sex reversal, in contrast to the generally reversible, activational effects

58 that occur during adulthood. Fish early life stages are of particular concern in agricultural areas,

59 as a spring influx of pesticides coincides with spawning and early development (Gall et al.

60 2011).

61   Evidence of endocrine disruption in wild fish has been observed in many areas. A high

62 prevalence of testicular oocytes, an intersex condition, has been observed in smallmouth bass

63 within the Potomac (Blazer et al. 2007; Blazer et al. 2010; Iwanowicz et al. 2009) and

64 Susquehanna (Blazer et al. 2014) river basins of the Chesapeake Bay watershed in the eastern

65 United States. Throughout North America, intersex gonads have been found in black bass

66 species including smallmouth bass (*Micropterus dolomieu*) and largemouth bass (*Micropterus*

67 *salmoides)* (Abdel-Moneim et al. 2015; Blazer et al. 2018; Grieshaber et al. 2018; Hinck et al.

68 2009; Iwanowicz et al. 2016; Kellock et al. 2014; Yonkos et al. 2014). The individual chemicals,

69 complex mixtures of chemical compounds and/or other environmental stressors contributing to

70 the development of intersex in specific areas are not yet fully understood. A significant positive

71 correlation was observed between the incidence of intersex and the concentration of Atrazine

72 (ATR) in the water during monitoring of the Potomac river basin at six sites in Maryland,

73 Virginia, and West Virginia during the spring (Kolpin et al. 2013). This observation highlighted

74 ATR as a contaminant of interest for further investigation into its potential role as an estrogenic

75 endocrine disrupting contaminant (EDC) in the Chesapeake Bay Watershed.

76   Atrazine has been implicated in both reproductive dysfunction in adults and alterations

77 during early development in fish, as well as in other vertebrates (Wirbisky & Freeman 2015).

78 Adult exposures to atrazine impaired reproduction in medaka (*Oryzias latipes*) (Papoulias et al.

79 2014) and fathead minnow (*Pimephales promelas*) (Tillitt et al. 2010). However, the molecular

80 mechanism of endocrine disruption by atrazine remains unclear and appears distinct from the

81 mechanism of estrogenic EDCs (Richter et al. 2016). Atrazine does not directly activate the

82 estrogen receptor, but has been hypothesized to induce expression of *cytochrome P450, family*

83 *19, subfamily A, polypeptide 1a* (*cyp19a1a*, previously *aromatase*) through inhibition of

84 phosphodiesterase; activation of nuclear receptor subfamily 5, group A, member 1a (Nr5a1a); or

85 alterations in miRNA expression (Sanderson et al. 2001; Roberge et al. 2004; Suzawa &

86 Ingraham 2008; Wang et al. 2019). Early life stage exposures to atrazine have been reported to

87 alter sex ratios (Suzawa & Ingraham 2008; Wang et al. 2019).

88   Exposure to estrogenic compounds, including the pharmaceutical estrogen 17α-

89 ethinylestradiol (EE2), has been shown to induce intersex in certain fish species (Abdelmoneim

90 et al. 2019). There have been many studies evaluating the effects of EE2 on various fish species

91 at different life stages, especially the model species zebrafish (*Danio rerio*), medaka, and fathead

92 minnow. However, the exact mechanism causing development of intersex gonads has not been

93 identified and there is a particular data gap in this area for black bass. This early life stage

94 evaluation has not been done in a laboratory setting in black bass species, which are

95 economically important sport fish. Identifying estrogen-specific responses in early life stage

96   largemouth bass can provide a baseline to build on when identifying mechanisms of action, as

97   estrogens have been shown to have additive effects (Brian et al. 2005).

98   To evaluate the role of atrazine as an EDC and the role of estrogenic EDCs in intersex,

99   largemouth bass fry were exposed to a range of concentrations of ATR or EE2 during sexual

100   differentiation. To our knowledge this study is the first to measure effects of laboratory ATR and

101   EE2 exposure in early life stage LMB. The objectives of this study were to: monitor the growth

102   of LMB and development of the gonads through histological examinations; identify differences

103   in gene regulation associated with ATR and EE2 exposure; and develop testable hypotheses for

104   potential biochemical pathways and cellular mechanisms leading to altered gonad development,

105   the condition of intersex, and ultimately impairment of reproductive function in largemouth bass.

106   The focus of this manuscript is on potential molecular initiating pathways that could lead to

107   altered gonad development and function.

108

109   Materials and methods

110   Experimental design

111   Largemouth bass fry were exposed to solvent control (0.0001% ethanol), 17α-

112   ethinylestradiol (EE2, 1 or 10 ng/L as a model estrogen control), or atrazine (ATR, 1, 10, or 100

113   μg/L) starting at 7 days post spawn (dps). Exposures continued to 80 dps, during early gonadal

114   and sexual differentiation. Each treatment was conducted in quadruplicate (n=4), for a total of 24

115   exposure tanks (6 treatments x 4 replicates). Fry/juveniles were sampled at 17, 33, and 80 dps.

116   Each tank began with approximately 500 viable fry at 7 dps. At the first two time points, 20 fish

117   per tank were collected for histological examination of gonadal development. At 80 dps 140 fish

118   per treatment (35 fish per tank) were sampled to assess growth, sex identification. Of those fish

119   sampled at 80 dps, gonads of 20 fish per treatment (5 fish per tank) were removed for

120   histological sex identification and gene expression analysis. Four treatments were chosen for

121   gene expression analysis: solvent control, low EE2 (1 ng/L), and low and high ATR (1 and 100

122   µg/L). Four fish were randomly selected per treatment per sex (4 fish x 4 treatments x 2 sexes)

123   for a total of 32 samples analyzed by RNAseq.

124   All exposure, sample processing, and data analysis was conducted at the U.S. Geological

125   Survey Columbia Environmental Research Center (CERC, Columbia MO, USA) unless

126   otherwise stated. This study was in compliance with all applicable sections of the Final Rules of

127   the Animal Welfare Act regulations (9 CFR) and with all CERC Institutional Animal Care and

128   Use Committee guidelines for the humane treatment of the test organisms during culture and

129   experimentation.

130

131   **Spawning, egg collection, feeding, and animal care**

132   The original broodstock of virgin largemouth bass were obtained from the U.S. Fish and

133   Wildlife Service's Genoa National Fish Hatchery, Genoa, WI. Eggs for the current study were

134   collected from second generation broodstock reared at CERC. Approximately 12-18 sexually

135   mature largemouth bass (6-9 from each sex) were spawned in a 0.2 acre pond containing 45 cm x

136   80 cm spawning mats placed in shallow near-shore water. Mats with freshly deposited eggs were

137   collected daily, brought into the laboratory and treated with sodium sulfite (0.015% solution) for

138   3-5 minutes to release the eggs from the mats. The eggs were then placed in modified

139   MacDonald egg incubation tubes and rolled at a temperature no more than 1 °C from the pond

140   temperature. Eggs were collected until the total was greater than 24,000 eggs to allow for 1000

141   embryos per tank (total of 24 tanks). Eggs were rolled with a stream of fresh well water in the

142   egg incubation rack until hatch. Hatched fry were collected in mixed cultures and maintained at

143   temperature (22-24°C) until placed in the exposure tanks. Upon placement in the exposure tanks,

144   the temperature was acclimated to 22°C and maintained there for the remainder of the study

145   period under a 16:8 light:dark photoperiod.

146   Hatched fry were fed a combination of three different food sources over the course of the

147   study. Live freshwater rotifers (*Brachionus calyciflorus*) were fed in conjunction with live,

148   newly hatched brine shrimp (*Artemia* sp.) nauplii to satiation three times a day during the first

149   week after initiation of exogenous feeding. After the first week, fish were transitioned off rotifers

150   and offered a combination of live, newly hatched *Artemia* nauplii three times a day and a dry

151   manufactured diet (Otohime, Reed Mariculture) to satiation twice a day. The manufactured diet

152   was administered to the fish via an automatic feeder to each tank. The manufactured diet was

153   adjusted throughout the study based on growth rates and gape width. Tanks were siphoned clean

154   once every other day to remove uneaten food and waste. Additionally, fish were prophylactically

155   treated in-tank twice a week with 20 parts per million (ppm) Chloramine T for the first four

156   weeks followed by 10 ppm twice a week for the remainder of the study to prevent bacterial

157   outbreaks from occurring.

158   Well water was used in this study and conditions were maintained within criteria set forth

159   by the American Standards and Testing Materials (American Society for Testing and Materials

160   (ASTM) 2004) for toxicity testing with aquatic organisms. General water quality was expected to

161   be 275 mg/L hardness, 245 mg/L alkalinity, 8.3 pH, 0.0200 mg/L ammonia. Alkalinity, hardness,

162   pH, gas saturation, and ammonia were monitored weekly. Dissolved oxygen and temperature

163   were measured every other day. Tanks were rectangular glass aquaria with 64 L capacity, filled

164   to 48 L. Each tank had an overflow outlet into the waterbath and an airstone. The sides of each

165 tank were covered with contact paper to prevent interactions of fish tank to tank. Volume of

166 water flowing into the tanks was checked weekly.

167

168 **Exposure and chemical analysis**

169   Exposures were conducted in proportional, flow-through diluters with approximately four

170 tank turnovers per day. Exposure chemical concentrations were confirmed twice weekly for the

171 first two weeks, then weekly for the remainder of the exposures. Atrazine (98% purity, Fluka,

172 College Park, Georgia, USA) was prepared in stock solutions of 25 mg/L in CERC well water

173 and stored in amber bottles at 4°C prior to use. 17α-Ethinylestradiol (Sigma-Aldrich, St. Louis,

174 Missouri, USA) was prepared in a stock solution of 10 µg/mL in ethanol. Working solutions in

175 CERC well water of ATR were prepared for final exposure concentrations of 0, 1, or 100 ug/L

176 and of EE2 for final exposure concentrations of 1 or 10 ng/L and solvent concentration of

177 0.0001% ethanol. Diluters were intermittent-flow exposure systems with Hamilton syringes

178 delivering the test chemicals to replicate exposure chambers. System cycling occurred at a rate of

179 6 cycles per hour. The system was equilibrated with the test chemicals for 5 days prior to

180 stocking the fish. At the commencement of the exposure, newly hatched fry (7 dps) were placed

181 in floating baskets within randomly assigned aquaria for each treatment/replicate combination.

182 Atrazine in the tank water was quantified using enzyme-linked immunosorbant assay (ELISA)

183 kits (Abraxis, Warminister, Pennsylvania, USA) in accordance with manufacturer's protocols.

184 Confirmatory analysis was performed on selected water samples by gas chromatography

185 (Jiménez et al. 1997). Briefly, water samples were extracted using methylene chloride; the

186 extract dried with sodium sulfate and filtered through glass fibers; volume reduced to 0.1 mL in

187 methyl tertiary butyl ether; and triphenylphosphate (Chem Service Inc., West Chester,

188   Pennsylvania, USA, 500 µg/mL in MtBE) was added as an instrumental internal standard. The

189   extracts were analyzed by gas chromatographic nitrogen/phosphorus detector (GC/NPD) and

190   quantified by Perkin-Elmers TotalChrom™ workstation chromatography data software. Quality

191   control samples were analyzed with each sample set and included: ATR-spiked water, matrix

192   (well) water blank, and a procedural blank. Concentrations of EE2 in exposure tank water were

193   monitored using an ELISA kit by Ecologiena, (Tokiwa Chemical Industries Co. Ltd, Japan)

194   according to manufacturer's instructions. Water samples were brought to pH 7.0 prior to filtering

195   through a glass fiber filter. Samples were then extracted and concentrated using C18-solid phase

196   extraction. Extracted samples were added to a 96-well microtiter plate that was coated with

197   polyclonal rabbit anti-EE2 antibodies. Following incubation, a tracer conjugated with

198   horseradish peroxidase was applied. After tracer incubation the plate underwent washing and the

199   addition of a color substrate (3,3',5,5'-tetramethylbenzidine, TMB). After color development a

200   stop solution (sulfuric acid) was added before reading the absorbance at 450 nm. The EE2

201   concentrations were determined by quantifying the absorbance values in relation to the measured

202   values of EE2 calibration standards that had be assayed in the same manner.

203

204   **Fish collection**

205   Fish were euthanized with an overdose (300 mg/L) of MS-222 (Ethyl 3-aminobenzoate

206   methanesulfonate, Sigma-Aldrich), blotted dry, then weight and total length were measured.

207   Midsections were sampled from 30 fish per treatment by removing the head just posterior to the

208   opercular flap and tail was removed just posterior to the anal pore and were preserved (see

209   below) for histological analysis.

210   Fish sampled for gene expression analysis had an incision made from anal pore to

211   opercular flap, and a panel of muscle tissue removed to obtain access to the juveniles' gonads.

212   RNAlater (Sigma-Aldrich) was used to immediately preserve the gonads still in the body cavity,

213   and to increase visualization and integrity of the gonad tissue. The gonad was then carefully

214   removed and divided. One lobe was preserved (see below) for histological analysis and sex

215   identification. The second lobe was stored in RNAlater at 4°C overnight, then transferred to -

216   20°C until RNA was extracted.

217

218   **Histological analysis**

219   Two different histological samples were taken at 80 dps: 1) whole, intact midsections or

220   2) individual gonad lobes were extracted. Samples were preserved in Z-fix (Anatech Ltd, Battle

221   Creek, Michigan, USA) or PAXgene tissue fix (PreAnalytix, Hombrechtikon, Switzerland) for

222   12-16 hours then moved to 70% ethanol and processed within 3 weeks. Midsections were cut in

223   the center and positioned in the paraffin block so both anterior and posterior directions of the

224   midsection could be visualized. Samples were processed by dehydration and embedded into

225   paraffin (Luna 1968). The midsections were sectioned in 5 μm transverse cuts until the gonads

226   were clearly visible. Kidney and swim bladder were used to orient the visualization of the gonad,

227   as the gonads are on the ventral side of the swim bladder in the section of the fish where the hind

228   kidney is clearly visible. Slides were stained with hematoxylin and eosin (Luna 1968). In those

229   fish where gonads were extracted during sampling, RNAlater (ThermoFisher, Waltham,

230   Massachusetts, USA) was added to the open cavity of the fish to give the gonads integrity to be

231   more easily removed. Then gonads were removed with forceps, one lobe was taken for histology

232   and the other was preserved in RNAlater for RNAseq (5 fish per treatment). Individual lobe

233   samples collected for histological analysis were stored in Paxgene for 12-16 h and then

234   transferred to 70% ethanol and processed within 3 weeks. For each individual gonad lobe

235   approximately 20-40 sagittal 5 μm thick sections were taken.

236   The characteristics used to identify female fish were: gonadal tissue had the presence of

237   primary oocytes (POs; round cells with hematoxylin staining around the nucleus that was lighter

238   or stained with eosin, and larger than 25 μm in diameter), ovarian tissue was relatively larger

239   than testes (larger than 100 μm in diameter) and round in shape, typically with an ovarian cavity

240   present. Fry were identified as female if POs were present in any of the sections. A presumptive

241   male identification was made if only primordial germ cells (PGCs) and no POs were present in

242   the gonad sections. It is assumed these undifferentiated gonads were male because this concurs

243   with expected development of male LMB (Johnston 1951). Due to the lack of testis

244   differentiation at this developmental time point, there is a possibility that these undifferentiated

245   gonads could be ovaries with considerably delayed development. However, due to the likelihood

246   that these were males, those identified as presumptive males will hereafter be referred to as

247   males. In females the extreme anterior and posterior ends of ovaries appeared similar to

248   undifferentiated gonadal tissue, except with no PGCs present. When undifferentiated tissue with

249   no PGCs was sectioned further POs were consistently found, leading to a female identification.

250   Conversely, when undifferentiated gonadal tissue with any PGCs was sectioned through the

251   entire gonad no POs were revealed, leading to a male identification. If only connective tissue

252   was present and no gonadal tissue could be identified, sex identification was deemed

253   inconclusive for that fish.

254

255   **RNA extraction, library preparation, and sequencing**

256   Total RNA was extracted from an individual gonad lobe from each of the selected fish,

257   corresponding to a gonad lobe that was processed for histological sex identification. The

258   extraction was performed using an RNeasy Mini Kit (74104, Qiagen, Hilden, Germany). Briefly,

259   tissue was removed from RNAlater and homogenized in 150 µl of lysis buffer using a micro-tube

260   homogenizer and pestle (Fisher). The pestle was rinsed with an additional 200 µl of buffer

261   following homogenization (350 µl total) to assure no sample was lost. Extraction was performed

262   according to manufacturer's instruction, with DNase treatment (Qiagen). RNA was eluted off the

263   extraction column in two 14 µl elutions (28 µl final volume). Capillary electrophoresis on a

264   QIAXEL instrument (Qiagen) was used to characterize the concentration and quality of the RNA

265   following manufacturer's instructions. Four samples per treatment were chosen for the RNAseq

266   analysis. These samples had RNA integrity scores (RIS) with an average of 7.73 +/- 0.97.

267   Concentrations of RNA were normalized for all samples. Library preparation and sequencing

268   was performed by the DNA Core facility at the University of Missouri (Columbia, Missouri,

269   USA). Libraries were prepared by Ultra Low RNA Library Preparation kit (Clontech) following

270   manufacturer's instructions. Transcripts were sequenced in four lanes of a 2x75 NextSeq

271   (Illumina) paired-end read.

272

273   **Assembly of gonad transcriptome**

274   Initial read processing and assembly into transcript contigs was performed by the

275   sequencing core. The reads were first trimmed of low-quality base calls and adapter sequence, if

276   present, by the Trimmomatic command (Bolger et al. 2014) in Trinity (Trinity-v2.3.2) with

277   default settings except the 5 bases were not removed from each end. Strand specific paired end

278   settings were used assuming the first read was on the sense (forward) strand and the second read

279   was on the antisense (reverse) strand. All settings were default including the k-mer size of 25.

280   The initial assembly included 743,843 contigs, which was reduced in number to 103,580 by

281   requiring at least 10 counts summed across all samples and imposing a threshold contig length >

282   200 nt.

283   Prior to differential expression analysis, contigs were clustered at 95% with cd-hit-est (Li

284   & Godzik 2006) to further reduce redundancy in the reference assembly, to 59,261 contigs (N50

285   of 3,545 nt). Assembled and clustered RNA contigs will be referred to as transcripts hereafter.

286   Transcripts were annotated using a BlastX search against all *Danio rerio* peptides (version

287   GRCz10, NCBI accession number GCF_000002035). A reciprocal search identified 11,916 one-

288   to-one matches at a minimum bit score of 100, for which functional annotations were used in

289   enrichment analysis (see below). Annotated transcripts will be referred to as gene transcripts or

290   expressed genes. The average number of mapped reads was 47.4 +/-7.4 million reads/sample.

291

292   **Analysis of differential transcript expression**

293   For quantification of transcripts, raw reads were reprocessed using a different workflow

294   than that used for assembly. Reads were trimmed with CLC Genomics Workbench v 9.5

295   (Qiagen) at an error probability of 0.05, allowing a maximum of 2 ambiguous bases and

296   requiring a minimum trimmed length of 50 bp. Sequencing adapters were removed using default

297   scoring parameters. Abundance was estimated with kallisto (Bray et al. 2016) using a k-mer size

298   of 31 and with sequence composition bias correction. Note that counts estimated by kallisto are

299   probabilistically adjusted to account for sequence shared among multiple transcripts (as in close

300   paralogs or alternative isoforms), resulting in fractional count values. Counts were rounded to

301   integer values for analysis with edgeR (Robinson et al. 2009).

302   The multidimensional scaling function of edgeR was used to check for consistency of

303   response by treatment. Two samples (2-2 and 5-5) were strong outliers by this approach and

304   therefore removed from all further analyses. For each contrast tested, low-abundance transcripts

305   were filtered by requiring at least 2 cpm in at least 3 samples, unless the sample size had been

306   reduced due to the exclusions noted above, in which case at least 2 samples with $\geq$ 2 cpm were

307   required. The p-value for each transcript was calculated using the "exactTest" function in edgeR,

308   based on the normalized and expression-filtered data for each pairwise contrast and adjusted

309   using the Benjamini-Hochberg correction (Benjamini & Hochberg 1995) to account for false

310   discovery and an adjusted value of 0.05 was considered the threshold of significant differential

311   expression. No minimum fold-change threshold was used. Genes with significantly male-biased

312   and female-biased expression were identified by comparing male and female expression in the

313   controls using the same thresholds.

314

315   **Functional enrichment analysis**

316   Three differentially expressed (DE) gene sets of interest were further analyzed in this

317   study: 1) female-biased DE genes (female expression significantly greater than male) that were

318   also differentially expressed in response to 100 µg ATR/L and 1 ng 17α-ethinylestradiol/L

319   treatments in males; 2) DE genes in common among both 1 and 100 µg/L ATR treatments in

320   males; and 3) DE genes in common among both 1 and 100 µg/L ATR treatments in females. For

321   each comparison (gene sets 1, 2, and 3; see section 'GO analysis'), enrichment of ontologies and

322   pathway associations among DE genes was evaluated with go-seq (Young et al. 2010), using

323   only the annotations associated with transcripts with one-to-one matches to *D. rerio*. Go-Slim

324   gene ontology annotations for the parent Ensembl (Hubbard et al. 2002) gene ID of each

325   matched *D. rerio* protein were downloaded from BioMart (Smedley et al. 2009) on 01/10/2017.

326   NCBI gene IDs corresponding to each Ensembl gene were downloaded from the public ftp site

327   on 01/18/2017.

328

329   **Statistical Analysis**

330   Statistical analysis of physiological endpoints was performed using JMP 13.1.0 (SAS,

331   Cary NC) with significance set at 5% ($p < 0.05$) for all comparisons. Survival, weight, and length

332   were analyzed using analysis of variance (ANOVA) followed by Dunnett's post hoc test to

333   identify treatments that differed significantly from the solvent control. Sex ratios were compared

334   across treatments using a chi-square test.

335

336   # Results

337   **Exposure, mortality, growth, and sex identification**

338   *Chemical Exposure*

339   Concentrations of ATR during the course of the exposure averaged 0.91 (± 0.06), 9.87 (±

340   0.68), and 105.09 (± 8.57) µg ATR/L corresponding to nominal concentrations of 1, 10, and 100

341   µg ATR/L, respectively. Concentrations of EE2 averaged 0.84 (± 0.04) and 7.34 (± 0.46) ng

342   EE2/L for corresponding nominal concentrations of 1 and 10 ng EE2/L, respectively. The

343   concentrations in all treatments remained stable throughout the study period (Fig 1).

344   Mortality over the 73 d study ranged from 52.9% to 58.4% among tanks in all treatment

345   groups, and there were no significant differences between treatments (Table 1 and S1). High

346   mortality is expected during early development. We also observed cannibalism among the test

347 organisms that limited survival, consistent with the ecological role of LMB as voracious

348 piscivores from early in development.

349

350 *Gonad development and sex identification*

351   Developing gonads in midsections at 17 dps were small and had connective tissue and

352 primordial germ cells (PGCs) (Fig 2, A-B). At 33 dps the gonads were slightly larger and PCGs

353 were observed (Fig 2, C-D). At 80 dps testis tissue remained mainly undifferentiated with the

354 presence of clear PCGs, but no testis-specific characteristics (Fig 2, E-F). Ovarian tissue began

355 to differentiate by 80 dps, exhibiting ovarian cavities and primary oocytes in addition to PCGs,

356 and ovaries were much larger than testes in cross-section at this timepoint (Fig 2, G-H).

357   Equal effort was made for sex identification across tanks; the reported n of each sex for

358 each treatment reflects confident sex identification (Table 1 and S1). To verify consistency in the

359 sex identification protocol, 70% of samples had one or two additional blind identifications

360 performed, of which 100% concurred with the original sex identification. The only significant

361 difference in sex ratio from the expected 1:1 female:male was the 10 ng EE2/L treatment, where

362 all fish in each replicate were identified as female, except a single individual from one replicate

363 identified as a male.

364

365 *Juvenile growth*

366   There were no significant differences in length or weight between treatment groups

367 relative to control when averaged across both sexes. When sex-specific responses were analyzed,

368 female length was significantly greater in the both the 1 and 10 ng EE2/L treatments compared to

369 control (Table 1 and S1). Female weight was significantly increased in the 1 ng EE2/L treatment

370   group, but not the 10 ng/L treatment group, compared to control (Table 1 and S1). There were no

371   significant differences between treatment groups for length or weight in males.

372

373   **Gene expression analysis**

374   *Differential expression*

375   RNAseq analysis identified 41,565 total transcripts and 28,314 total DE transcripts

376   among all comparisons (BioProject Accession PRJNA485177). Multidimensional scaling of DE

377   transcripts revealed clustering of expression patterns in the 1 ng EE2/L and 100 µg ATR/L

378   treated male fish (Fig 3). In females, expression patterns in both atrazine treatments clustered

379   with the controls. Many transcripts were significantly differentially expressed between sexes in

380   the controls, with slightly more male-biased (expression significantly greater in males) than

381   female-biased (expression significantly greater in females; Fig 4). Overall, males exhibited more

382   DE transcripts in response to treatments than females (Fig 4). Few DE transcripts were common

383   between ATR-treated males and females. Most responsive transcripts in males were down-

384   regulated. In females, most responsive transcripts were upregulated, particularly male-biased

385   transcripts (M>F; Fig 4). In the 1 ng/L EE2-exposed fish, of those transcripts differentially

386   expressed in gonad (3811 in males, 2120 in females), 721 were identified in both sexes. There

387   was a strong correlation of log-fold changes between males and females among these 721

388   transcripts ($R^2 = 0.92$).

389

390   *GO analysis*

391   We developed three gene sets of interest based on intersections among gene sets

392   responsive to different conditions. To test the hypothesis that atrazine exposure had estrogenic

393   effects on gene expression in male gonad, we examined gene set 1: female-biased DE genes

394   (female expression greater than male in controls) that were also differentially expressed in

395   response to both the 100 µg ATR/L and 1 ng EE2/L treatments in males. We predicted that more

396   genes with higher expression in ovary would be upregulated in response to both 1 ng EE2/L and

397   100 µg ATR/L exposures in males. To investigate atrazine-specific gene expression fingerprints

398   in males, we examined gene set 2: DE genes in common among ATR treatments at both 1 and

399   100 µg/L in males. Finally, to investigate atrazine-specific gene expression fingerprints in

400   females we examined gene set 3: DE genes in common among ATR treatments at both 1 and 100

401   µg/L in females. Gene ontology (GO) analysis of differentially expressed annotated transcripts

402   (based on *Danio rerio* annotation) identified several significantly overrepresented terms among

403   the three gene sets of interest tested. Among DE genes responsive to both ATR and EE2

404   treatments in males, there was an overall trend of down-regulation of female-biased genes,

405   contrary to our prediction of a trend for upregulation of genes in this set (Table 2). Two GO

406   terms that were enriched among DE gene sets from males of all treatment groups were ribosome

407   biogenesis and small molecule metabolic process (Table 2). Common GO terms among male and

408   female groups were transport and transmembrane transport. Whereas the male response was

409   typically down-regulation of female-biased genes, the female response was typically

410   upregulation of genes in overrepresented GO terms (Table 2).

411

412   *Comparative meta-analysis*

413   The comparative meta-analysis identified specific genes of interest that have been shown

414   to respond to both ATR and EE2 exposure (S1 File). This analysis also provided a summary of

415   literature in which those contaminant gene interactions could be found. Of our identified genes

416   of interest, *cytochrome P450, family 11, subfamily A, polypeptide 2* (*cyp11a2*), *steroidogenic*

417   *acute regulatory protein* (*star*), *cytochrome P450, family 1, subfamily A* (*cyp1a*), *DEAD (Asp-*

418   *Glu-Ala-Asp) box polypeptide 4* (*ddx4* (previously *vasa*)), *SAM domain and HD domain 1*

419   (*samhd1*), and *wingless-type MMTV integration site family, member 5b* (*wnt5b*) showed

420   differential expression from the control group in at least one treatment group in males (Fig 5).

421

422   **Discussion**

423   In this study we examined the potential endocrine disrupting effects of a common use

424   herbicide (ATR) and a model estrogen (EE2) in developing largemouth bass. We evaluated both

425   somatic growth and gonad development, as well as global gene expression in isolated developing

426   gonad tissue. Our observation of near-complete sex reversal in the 10 ng EE2/L treatment

427   confirmed that early development was a sensitive window for estrogenic endocrine disruption in

428   LMB and our gene expression results were consistent with the hypothesis that ATR exposure

429   induces some estrogenic responses in the developing gonad.

430

431   **Sex reversal, gonad growth, and somatic growth**

432   Overall, there was an absence of response to ATR exposure in growth, sex ratio, and

433   gonad morphology, while EE2 exposure elicited effects on all three. An increase in growth was

434   observed in the EE2-treated female LMB. In the literature there have been both increased and

435   decreased growth observed in fish exposed to EE2. However, low concentration exposures

436   during development have been shown to act like a growth promoter (Chen et al. 2017; Luzio et

437   al. 2015; Örn et al. 2003), as was observed in the current study. All but one fish (94/95) in the 10

438   ng EE2/L treatment were histologically identified as female. Sex reversal resulting from

439 exposure to high concentrations of EE2 has been observed in other species at exposure

440 concentrations of 3 ng EE2/L and above (Caldwell et al. 2008). The LMB under the current

441 exposure conditions appear to be as sensitive to sex reversal resulting from developmental EE2

442 exposure as the small fish model species zebrafish (Hill & Janz 2003; Weber et al. 2003) and

443 fathead minnow (van Aerle et al. 2002). LMB appear to be more sensitive to sex reversing

444 effects than what has been observed in three-spined stickleback (Peters et al. 2010), medaka

445 (Scholz & Gutzeit 2000), and sheepshead minnow (Zillioux et al. 2001). Previous studies have

446 shown that exposure to a concentration of EE2 over a species-specific threshold for sex reversal

447 throughout early development led to organizational changes, and although the sex-reversed

448 phenotype reverted to a phenotype that matched genetic sex in some individuals after a recovery

449 period during adulthood, reproductive dysfunction persisted (Larsen et al. 2009). In wild

450 populations of LMB sex differentiation occurs in spring and summer, which coincides with

451 storm events and typically greater exposures of EDCs in surface water, particularly in areas

452 dominated by agricultural land use (Gall et al. 2011). The mechanisms of sex differentiation in

453 this species have not been characterized well. Since the window of sex differentiation is

454 extremely sensitive to endocrine disruptors in all the vertebrate species tested so far, the EDC

455 effects in LMB germ cells may lead to reproductive impairment later in life, which we did not

456 examine. Such far-reaching effects would be detrimental for LMB populations in the

457 contaminated sites.

458

459 **Gene expression alterations**

460 *Differential expression*

461   The male LMB gonads were sensitive to estrogenic exposures, as observed by the

462   apparent sex reversal in the 10 ng EE2/L treatment and the overall response in gene expression in

463   the 1 ng EE2/L treatment. We had hypothesized upregulation of female-specific genes in testes

464   among males exposed to EE2. The most differentially expressed genes were those more highly

465   expressed in females, however, most DE genes were downregulated. This counterintuitive result

466   may be caused by negative feedback, leading to downregulation of the female-biased genes in

467   the 1 ng EE2/L treatment.

468   During this period of gonad development, sex differentiation is just beginning in the

469   males, so effects on gene expression patterns during this stage could lead to alterations in later

470   gonad morphology and possibly function. The earlier stage of gonad differentiation in males

471   compared to females may possibly account for some of the greater responsiveness of gene

472   expression in males, as the fish may be more resistant to the influence of exogenous hormones as

473   gonads become more developed (Blázqueza et al. 1995). The relationship between the observed

474   sex reversal in males and gene expression changes, to intersex formation in adults was not tested

475   in this study. Due to the undifferentiated testes at the sampling time point intersex was not able

476   to be identified. No morphological abnormalities were observed in either treated or controlled

477   fish during the histological analysis and sex identification.

478   Liver gene expression of *vitellogenin 1* (*vtg1*), a commonly assayed biomarker of

479   estrogen exposure in fish (Bowman & Denslow 1999), was not measured in this study, and

480   expression was not observed in gonad. We observed sex-specific expression of three zona

481   pellucida genes, *zona pellucida glycoprotein 3a, tandem duplicate 2* (*zp3a.2*), *zona pellucida*

482   *glycoprotein 3b* (*zp3b*), and *zona pellucida glycoprotein 3d tandem duplicate 2* (*zp3d.2*), which

483   are generally estrogen-regulated and expressed in ovary and/or liver in adults (Onichtchouk et al.

484   2003). Expression of the three zp genes was female-biased, but there were no significant effects

485   of treatment on expression.

486

487   *GO analysis*

488   Many of the GO terms overrepresented in this analysis were in general involving protein

489   synthesis and transport. Those terms that were found to be common among the sexes had

490   responses in opposite directions, with differentially expressed genes in those categories being

491   largely downregulated in males and upregulated in females exposed to ATR. Downregulation of

492   these processes in developing males may lead to effects later in gonad development.

493   A GO term of particular interest that was overrepresented for transcripts altered in

494   females in both 1 and 100 μg/L ATR treatments was immune system process. All of the DE

495   genes were upregulated in this term. ATR has been shown to alter immune function in juvenile

496   fish (Kreutz et al. 2012). Estrogen is also known to interfere with immune function in fish

497   (Burgos-Aceves et al. 2016). There are still gaps in our knowledge of the potential effects of

498   these contaminants on juvenile LMB and potential effects on disease susceptibility. There is even

499   less known about how contaminants of interest act in real world mixtures and what role they

500   potentially play in concert to modulate the immune function and disease-resistance of wild

501   populations.

502

503   *Comparative meta-analysis*

504   The annotation used for the analysis was *Danio rerio*, as this is the taxonomically closest

505   species to LMB that has an annotated genome. There are, and will continue to be, limitations in

506   this kind of study until the LMB genome can be fully sequenced and annotated. A reciprocal

507   best-match criterion between *D. rerio* and LMB transcripts was used to strengthen annotation

508   inferences, which reduced the number of gene proxies to 11,916 out of approximately 25,549

509   coding genes in *D.rerio*. The number of genes in LMB is unknown, but the N50 of the assembly

510   was not low by transcriptomic standards (3,545 bp) and only one tissue was analyzed.

511   Nonetheless, partial capture of coding sequences, retention of non-coding sequence, and

512   alternative splicing remain challenges to annotation. We did not attempt to assess alternative

513   splicing with these data, as robust identification of splice variants requires a genomic reference.

514   In developing fish, genes involved in steroidogenesis and gonad development have been

515   shown to either be upregulated or downregulated with EDC exposure depending on the timing,

516   duration, and concentration of the exposure (Leet et al. 2011). In the current study there was a

517   general trend of downregulation of DE genes in males, including *cyp11a2*, *star*, and *ddx4* (Fig

518   5), possibly indicating a negative feedback from EE2 and low concentrations of ATR (Baron et

519   al. 2005; Filby et al. 2007; Leet et al. 2015). The genes of interest identified by the comparative

520   meta-analysis are involved in steroidogenesis, metabolism, and gonad development. The gene

521   products of *star* and *cyp11a2* are required for production of all steroid hormones; star encodes a

522   cholesterol transporter which is rate-limiting for steroidogenesis, and *cyp11a2* encodes a

523   cholesterol side-chain cleavase (Arukwe 2008). Downregulation of *star* has been observed in

524   adult human granulosa cells, mediated by an atrazine-induced increase in phosphodiesterase

525   activity (Pogrmic-Majkic et al. 2018). The enzyme encoded by *cyp1a* is involved in metabolic

526   breakdown of xenobiotics and steroids (Otte et al. 2017). The RNA helicase encoded by *ddx4*

527   (previously *vasa*) is a regulator of translation and is required for primordial germ cell migration

528   (Li et al. 2009). *Samhd*, an immune-related gene, was also downregulated in the current study. In

529   contrast, a previous study in early life stage zebrafish showed *samhd* to be upregulated in

530   response to exposure to ATR (Weber et al. 2013). *Wnt5b* is an extracellular signaling molecule

531   and morphogen involved in cell differentiation and formation and maintenance of tissues and

532   organs (Yang 2012).

533   However, a few of the DE genes of interest were upregulated in males. *Wnt5b* was

534   upregulated in the EE2 treatment, and C*yp1a* was also upregulated in EE2 and high ATR

535   treatment males. *Cyp1a* has previously been shown to upregulate in fish where ATR exposure

536   lead to DNA strand breaks and damaged blood cells (Chang et al. 2005).

537

538   **Conclusion**

539   The early stage in LMB gonad development assessed in the current study was seen to be

540   sensitive to molecular responses to EDCs, and organizational alterations in the form of sex

541   reversal with exposure to a high concentration of a potent estrogen. Exposure to ATR resulted in

542   changes in gene expression, with both similarities and differences compared to pathways

543   activated by estrogen. Exposure to the strong estrogenic EDC EE2 set developing fish on a path

544   to physiological change that was observed across multiple levels of biological organization:

545   changes in gene expression, changes in histology, and changes in morphology. Thus, our results

546   delineate pathways from estrogenic exposures to adverse outcomes in a major sport fish. The

547   role of ATZ in population impairments observed in the field remains unclear. It is possible that

548   the combined impacts of mixtures of contaminants including atrazine lead to population-level

549   effects on reproduction and disease resistance (Berninger et al. 2019). Investigation of effects of

550   mixtures at multiple levels of biological organization may help reveal diagnostic biomarkers of

551   pathways leading to adverse population-level effects. In wild populations early sex

552   differentiation occurs in the spring and summer, which coincides with storm events and typically

553   higher concentrations of EDCs in surface water, particularly in areas dominated by agricultural

554   land use. To our knowledge this is the first examination of developmental and molecular

555   responses to EDCs in juvenile LMB. This study can serve as an initial piece of the larger picture

556   of the sensitivity of developing bass to contaminants of interest in the Chesapeake Bay.

557   Additional studies are being conducted in adult LMB exposed to mixtures of contaminants of

558   interest. Controlled laboratory exposures with field-relevant sport fish can provide a basis for

559   identification of specific mechanisms of action, determination of effect concentrations, and

560   establishment of cause and effect linkages for contaminants and other stressors that may limit the

561   health and growth of wild populations.

562

563   Acknowledgements

564   We are grateful for the assistance of R. Claunch, J. Candrl, and V. Velez (Columbia

565   Environmental Research Center, US Geological Survey, Columbia, MO). The present study was

566   supported by the US Geological Survey, Contaminants Biology Program, Environmental Health

567   Mission Area. The authors declare no conflicts of interest. Any use of trade, product, or firm

568   names is for descriptive purposes only and does not imply endorsement by the US government.

569

570   References

571   Abdel-Moneim A, Coulter DP, Mahapatra CT, and Sepulveda MS. 2015. Intersex in fishes and
572   amphibians: population implications, prevalence, mechanisms and molecular biomarkers.
573   *Journal of Applied Toxicology*. 10.1002/jat.3204

574   Abdelmoneim A, Abdu A, Chen S, and Sepulveda MS. 2019. Molecular signaling pathways
575   elicited by 17alpha-ethinylestradiol in Japanese medaka male larvae undergoing gonadal
576   differentiation. *Aquatic Toxicology* 208:187-195. 10.1016/j.aquatox.2019.01.013

577 American Society for Testing and Materials (ASTM). 2004. *Standard guide for conducting acute*
578   *toxicity tests on test materials with fishes, macroinvertebrates, and amphibians (ASTM*
579   *E729-96)*. West Conshohocken, PA: ASTM.

580 Arukwe A. 2008. Steroidogenic acute regulatory (StAR) protein and cholesterol side-chain
581   cleavage (P450scc)-regulated steroidogenesis as an organ-specific molecular and cellular
582   target for endocrine disrupting chemicals in fish. *Cell Biology and Toxicology* 24:527-
583   540.

584 Baron D, Houlgatte R, Fostier A, and Guiguen Y. 2005. Large-scale temporal gene expression
585   profiling during gonadal differentiation and early gametogenesis in rainbow trout.
586   *Biology of Reproduction* 73:959-966.

587 Benjamini Y, and Hochberg Y. 1995. Controlling the False Discovery Rate: A Practical and
588   Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B*
589   *(Methodological)* 57:289-300.

590 Berninger JP, DeMarini DM, Warren SH, Simmons JE, Wilson VS, Conley JM, Armstrong MD,
591   Iwanowicz LR, Kolpin DW, Kuivila KM, Reilly TJ, Romanok KM, Villeneuve DL, and
592   Bradley PM. 2019. Predictive analysis using chemical-gene interaction networks
593   consistent with observed endocrine activity and mutagenicity of U.S. streams.
594   *Environmental Science & Technology* 53:8611-8620. 10.1021/acs.est.9b02990

595 Blazer VS, Iwanowicz DD, Walsh HL, Sperry AJ, Iwanowicz LR, Alvarez DA, Brightbill RA,
596   Smith G, Foreman WT, and Manning R. 2014. Reproductive health indicators of fishes
597   from Pennsylvania watersheds: association with chemicals of emerging concern.
598   *Environmental Monitoring and Assessment* 186:6471-6491. 10.1007/s10661-014-3868-5

599 Blazer VS, Iwanowicz LR, Iwanowicz DD, Smith DR, Young JA, Hedrick JD, Foster SW, and
600   Reeser SJ. 2007. Intersex (testicular oocytes) in smallmouth bass from the Potomac River
601   and selected nearby drainages. *Journal of Aquatic Animal Health* 19:242–253.

602 Blazer VS, Iwanowicz LR, Starliper CE, Iwanowicz DD, Barbash P, Hedrick JD, Reeser SJ,
603   Mullican JE, Zaugg SD, Burkhardt MR, and Kelble J. 2010. Mortality of centrarchid
604   fishes in the Potomac drainage: Survey results and overview of potential contributing
605   factors. *Journal of Aquatic Animal Health* 22:190-218. 10.1577/h10-002.1

606 Blazer VS, Walsh HL, Shaw CH, Iwanowicz LR, Braham RP, and Mazik PM. 2018. Indicators
607   of exposure to estrogenic compounds at Great Lakes Areas of Concern: species and site
608   comparisons. *Environmental Monitoring and Assessment* 190:577. 10.1007/s10661-018-
609   6943-5

610 Blázqueza M, Piferrer F, Zanuy S, Carrillo M, Donaldson EM. 1995. Development of sex control
611   techniques for European sea bass (Dicentrarchus labrax L.) aquaculture: effects of dietary
612   17 α-methyltestosterone prior to sex differentiation. *Aquaculture* 135:329-342.
613   10.1016/0044-8486(95)01030-0

614   Bolger AM, Lohse M, and Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina
615   sequence data. *Bioinformatics* 30:2114-2120. 10.1093/bioinformatics/btu170

616   Bowman CJ, and Denslow ND. 1999. Development and validation of a species- and gene-
617   specific molecular biomarker: Vitellogenin mRNA in largemouth bass (*Micropterus*
618   *salmoides*). *Ecotoxicology* 8:399-416.

619   Bray NL, Pimentel H, Melsted P, and Pachter L. 2016. Near-optimal probabilistic RNA-seq
620   quantification. *Nature Biotechnology* 34:525. 10.1038/nbt.3519

621   Brian JV, Harris CA, Scholze M, Backhaus T, Booy P, Lamoree M, Pojana G, Jonkers N,
622   Runnalls T, Bonfà A, Marcomini A, and Sumpter JP. 2005. Accurate Prediction of the
623   Response of Freshwater Fish to a Mixture of Estrogenic Chemicals. *Environmental*
624   *Health Perspectives* 113:721-728. 10.1289/ehp.7598

625   Burgos-Aceves MA, Cohen A, Smith Y, and Faggio C. 2016. Estrogen regulation of gene
626   expression in the teleost fish immune system. *Fish & Shellfish Immunology* 58:42-49.
627   10.1016/j.fsi.2016.09.006

628   Caldwell DJ, Mastrocco F, Hutchinson TH, Lange R, Heijerick D, Janssen C, Anderson PD, and
629   Sumpter JP. 2008. Derivation of an aquatic predicted no-effect concentration for the
630   synthetic hormone, 17 alpha-ethinyl estradiol. *Environ Sci Technol* 42:7046-7054.
631   10.1021/es800633q

632   Chang LW, Toth GP, Gordon DA, Graham DW, Meier JR, Knapp CW, deNoyelles FJ, Jr. ,
633   Campbell S, and Lattier DL. 2005. Responses of molecular indicators of exposure in
634   mesocosms: common carp (*Cyprinus carpio*) exposed to the herbicides alachlor and
635   atrazine. *Environmental Toxicology and Chemistry* 24:190–197.

636   Chen Y, Li M, Yuan L, Xie Y, Li B, Xu W, Meng F, and Wang R. 2017. Growth, blood health,
637   antioxidant status and immune response in juvenile yellow catfish *Pelteobagrus*
638   *fulvidraco* exposed to α-ethinylestradiol (EE2). *Fish & Shellfish Immunology* 69:1-5.
639   10.1016/j.fsi.2017.08.003

640   Filby AL, Thorpe KL, Maack G, and Tyler CR. 2007. Gene expression profiles revealing the
641   mechanisms of anti-androgen- and estrogen-induced feminization in fish. *Aquatic*
642   *Toxicology* 81:219-231.

643   Gall HE, Sassman SA, Lee LS, and Jafvert CT. 2011. Hormone discharges from a midwest tile-
644   drained agroecosystem receiving animal wastes. *Environmental Science & Technology*
645   45:8755-8764. 10.1021/es2011435

646   Grieshaber CA, Penland TN, Kwak TJ, Cope WG, Heise RJ, Law JM, Shea D, Aday DD, Rice
647   JA, and Kullman SW. 2018. Relation of contaminants to fish intersex in riverine sport
648   fishes. *Science of the Total Environment* 643:73-89. 10.1016/j.scitotenv.2018.06.071

649   Hill RL, Jr., and Janz DM. 2003. Developmental estrogenic exposure in zebrafish (*Danio rerio*):
650   I. Effects on sex ratio and breeding success. *Aquatic Toxicology* 63:417-429.

651 Hinck JE, Blazer VS, Schmitt CJ, Papoulias DM, and Tillitt DE. 2009. Widespread occurrence
652   of intersex in black basses (*Micropterus* spp.) from U.S. rivers, 1995-2004. *Aquatic*
653   *Toxicology* 95:60-70.

654 Hubbard T, Barker D, Birney E, Cameron G, Chen Y, Clark L, Cox T, Cuff J, Curwen V, Down
655   T, Durbin R, Eyras E, Gilbert J, Hammond M, Huminiecki L, Kasprzyk A, Lehvaslaiho
656   H, Lijnzaad P, Melsopp C, Mongin E, Pettett R, Pocock M, Potter S, Rust A, Schmidt E,
657   Searle S, Slater G, Smith J, Spooner W, Stabenau A, Stalker J, Stupka E, Ureta-Vidal A,
658   Vastrik I, and Clamp M. 2002. The Ensembl genome database project. *Nucleic Acids*
659   *Research* 30:38-41. 10.1093/nar/30.1.38

660 Iwanowicz LR, Blazer VS, Guy CP, Pinkney AE, Mullican JE, and Alvarez DA. 2009.
661   Reproductive health of bass in the Potomac, USA, drainage: Part 1. Exploring the effects
662   of proximity to wastewater treatment plant discharge. *Environmental Toxicology and*
663   *Chemistry* 28:1072-1083.

664 Iwanowicz LR, Blazer VS, Pinkney AE, Guy CP, Major AM, Munney K, Mierzykowski S,
665   Lingenfelser S, Secord A, Patnode K, Kubiak TJ, Stern C, Hahn CM, Iwanowicz DD,
666   Walsh HL, and Sperry A. 2016. Evidence of estrogenic endocrine disruption in
667   smallmouth and largemouth bass inhabiting Northeast U.S. national wildlife refuge
668   waters: A reconnaissance study. *Ecotoxicology and Environmental Safety* 124:50-59.
669   10.1016/j.ecoenv.2015.09.035

670 Jiménez JJ, Bernal JL, del Nozal MJ, and Rivera JM. 1997. Determination of pesticide residues
671   in waters from small loughs by solid-phase extraction and combined use of gas
672   chromatography with electron-capture and nitrogen-phosphorus detection and high-
673   performance liquid chromatography with diode array detection. *Journal of*
674   *Chromatography A* 778:289-300.

675 Johnston PM. 1951. The embryonic history of the germ cells of the largemouth black bass,
676   Micropterus salmoides salmoides (Lacépède). *Journal of Morphology* 88:471-542.
677   10.1002/jmor.1050880304

678 Kellock KA, Trushel BE, Ely PC, Jennings CA, and Bringolf RB. 2014. Survey of intersex
679   largemouth bass from impoundments in Georgia USA. *Transactions of the American*
680   *Fisheries Society* 143:565-572. 10.1080/00028487.2013.824922

681 Kolpin DW, Blazer VS, Gray JL, Focazio MJ, Young JA, Alvarez DA, Iwanowicz LR, Foreman
682   WT, Furlong ET, Speiran GK, Zaugg SD, Hubbard LE, Meyer MT, Sandstrom MW, and
683   Barber LB. 2013. Chemical contaminants in water and sediment near fish nesting sites in
684   the Potomac River basin: Determining potential exposures to smallmouth bass
685   (*Micropterus dolomieu*). *Science of the Total Environment* 443:700-716.
686   10.1016/j.scitotenv.2012.09.063

687 Kreutz LC, Barcellos LJ, dos Santos ED, Pivato M, and Zanatta R. 2012. Innate immune
688   response of silver catfish (*Rhamdia quelen*) exposed to atrazine. *Fish and Shellfish*
689   *Immunology* 33:1055-1059. 10.1016/j.fsi.2012.08.006

690   Larsen MG, Bilberg K, and Baatrup E. 2009. Reversibility of estrogenic sex changes in zebrafish
691   (Danio rerio). *Environmental Toxicology and Chemistry* 28:1783-1785. 10.1897/08-563.1

692   Leet JK, Gall HE, and Sepulveda MS. 2011. A review of studies on androgen and estrogen
693   exposure in fish early life stages: effects on gene and hormonal control of sexual
694   differentiation. *Journal of Applied Toxicology* 31:379-398. 10.1002/jat.1682

695   Leet JK, Sassman S, Amberg JJ, Olmstead AW, Lee LS, Ankley GT, and Sepúlveda MS. 2015.
696   Environmental hormones and their impacts on sex differentiation in fathead minnows.
697   *Aquatic Toxicology* 158:98-107. 10.1016/j.aquatox.2014.10.022

698   Li M, Hong N, Xu H, Yi M, Li C, Gui J, and Hong Y. 2009. Medaka *vasa* is required for
699   migration but not survival of primordial germ cells. *Mechanisms of Development*
700   126:366-381. 10.1016/j.mod.2009.02.004

701   Li W, and Godzik A. 2006. Cd-hit: a fast program for clustering and comparing large sets of
702   protein or nucleotide sequences. *Bioinformatics* 22:1658-1659.
703   10.1093/bioinformatics/btl158

704   Luna LG. 1968. *Manual of histologic staining methods of the armed forces institute of*
705   *pathology, 3rd edn. McGraw- Hill, New York, NY.* New York, NY: McGraw- Hill.

706   Luzio A, Monteiro SM, Garcia-Santos S, Rocha E, Fontainhas-Fernandes AA, and Coimbra AM.
707   2015. Zebrafish sex differentiation and gonad development after exposure to 17alpha-
708   ethinylestradiol, fadrozole and their binary mixture: A stereological study. *Aquatic*
709   *Toxicology* 166:83-95. 10.1016/j.aquatox.2015.07.015

710   Örn S, Holbech H, Madsen TH, Norrgren L, and Petersen GI. 2003. Gonad development and
711   vitellogenin production in zebrafish (*Danio rerio*) exposed to ethinylestradiol and
712   methyltestosterone. *Aquatic Toxicology* 65:397-411.

713   Otte JC, Schultz B, Fruth D, Fabian E, van Ravenzwaay B, Hidding B, and Salinas ER. 2017.
714   Intrinsic xenobiotic metabolizing enzyme activities in early life stages of Zebrafish
715   (*Danio rerio*). *Toxicological Sciences* 159:86-93. 10.1093/toxsci/kfx116

716   Papoulias DM, Tillitt DE, Talykina MG, Whyte JJ, and Richter CA. 2014. Atrazine reduces
717   reproduction in Japanese medaka (*Oryzias latipes*). *Aquatic Toxicology* 154:230-239.
718   10.1016/j.aquatox.2014.05.022

719   Peters RE, Courtenay SC, Hewitt LM, and MacLatchy DL. 2010. Effects of 17alpha-
720   ethynylestradiol on early-life development, sex differentiation and vitellogenin induction
721   in mummichog (*Fundulus heteroclitus*). *Marine Environmental Research* 69:178-186.
722   10.1016/j.marenvres.2009.10.002

723   Pogrmic-Majkic K, Samardzija D, Stojkov-Mimic N, Vukosavljevic J, Trninic-Pjevic A,
724   Kopitovic V, and Andric N. 2018. Atrazine suppresses FSH-induced steroidogenesis and
725   LH-dependent expression of ovulatory genes through PDE-cAMP signaling pathway in

726   human cumulus granulosa cells. *Molecular and Cellular Endocrinology* 461:79-88.
727   10.1016/j.mce.2017.08.015

728   Richter CA, Papoulias DM, Whyte JJ, and Tillitt DE. 2016. Evaluation of potential mechanisms
729   of atrazine-induced reproductive impairment in fathead minnow (*Pimephales promelas*)
730   and Japanese medaka (*Oryzias latipes*). *Environmental Toxicology and Chemistry*
731   35:2230-2238. 10.1002/etc.3376

732   Roberge M, Hakk H, and Larsen G. 2004. Atrazine is a competitive inhibitor of
733   phosphodiesterase but does not affect the estrogen receptor. *Toxicology Letters* 154:61-
734   68.

735   Robinson MD, McCarthy DJ, and Smyth GK. 2009. edgeR: a Bioconductor package for
736   differential expression analysis of digital gene expression data. *Bioinformatics* 26:139-
737   140. 10.1093/bioinformatics/btp616

738   Sanderson JT, Letcher RJ, Heneweer M, Giesy JP, and van den Berg M. 2001. Effects of chloro-
739   s-triazine herbicides and metabolites on aromatase activity in various human cell lines
740   and on vitellogenin production in male carp hepatocytes. *Environmental Health*
741   *Perspectives* 109:1027-1031.

742   Scholz S, and Gutzeit HO. 2000. 17-a-ethinylestradiol affects reproduction, sexual differentiation
743   and aromatase gene expression of the medaka (*Oryzias latipes*). *Aquatic Toxicology*
744   50:363-373.

745   Smedley D, Haider S, Ballester B, Holland R, London D, Thorisson G, and Kasprzyk A. 2009.
746   BioMart – biological queries made easy. *BMC Genomics* 10:22. 10.1186/1471-2164-10-
747   22

748   Suzawa M, and Ingraham HA. 2008. The herbicide atrazine activates endocrine gene networks
749   via non-steroidal NR5A nuclear receptors in fish and mammalian cells. *PLoS ONE*
750   3:e2117.

751   Tillitt DE, Papoulias DM, Whyte JJ, and Richter CA. 2010. Atrazine reduces reproduction in
752   fathead minnow (*Pimephales promelas*). *Aquatic Toxicology* 99:149–159.
753   10.1016/j.aquatox.2010.04.011

754   van Aerle R, Pounds N, Hutchinson TH, Maddix S, and Tyler CR. 2002. Window of sensitivity
755   for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow,
756   *Pimephales promelas*. *Ecotoxicology* 11:423-434. 10.1023/a:1021053217513

757   Wang F, Yang QW, Zhao WJ, Du QY, and Chang ZJ. 2019. Effects of short-time exposure to
758   atrazine on miRNA expression profiles in the gonad of common carp (*Cyprinus carpio*).
759   *BMC Genomics* 20:587. 10.1186/s12864-019-5896-6

760   Weber GJ, Sepúlveda MS, Peterson SM, Lewis SS, and Freeman JL. 2013. Transcriptome
761   alterations following developmental atrazine exposure in zebrafish are associated with

762 disruption of neuroendocrine and reproductive system function, cell cycle, and
763 carcinogenesis. *Toxicological Sciences* 132:458-466. 10.1093/toxsci/kft017

764 Weber LP, Hill RL, Jr. , and Janz DM. 2003. Developmental estrogenic exposure in zebrafish
765 (*Danio rerio*): II. Histological evaluation of gametogenesis and organ toxicity. *Aquatic*
766 *Toxicology* 63:431-446.

767 Wirbisky S, and Freeman J. 2015. Atrazine exposure and reproductive dysfunction through the
768 hypothalamus-pituitary-gonadal (HPG) axis. *Toxics* 3:414-450. 10.3390/toxics3040414

769 Yang Y. 2012. Wnt signaling in development and disease. *Cell & Bioscience* 2:14.

770 Yonkos LT, Friedel EA, and Fisher DJ. 2014. Intersex (testicular oocytes) in largemouth bass
771 (*Micropterus salmoides*) on the Delmarva Peninsula, USA. *Environmental Toxicology*
772 *and Chemistry* 33:1163-1169. 10.1002/etc.2544

773 Young MD, Wakefield MJ, Smyth GK, and Oshlack A. 2010. Gene ontology analysis for RNA-
774 seq: accounting for selection bias. *Genome Biology* 11:R14. 10.1186/gb-2010-11-2-r14

775 Zillioux EJ, Johnson IC, Kiparissis Y, Metcalfe CD, Wheat JV, Ward SG, and Liu H. 2001. The
776 sheepshead minnow as an in vivo model for endocrine disruption in marine teleosts: A
777 partial life-cycle test with 17a-ethynylestradiol. *Environmental Toxicology and Chemistry*
778 20:1968-1978.

779

780

Figure 1

Chemical concentration data.

Concentrations of atrazine (ATR, [A]) and 17α-ethinylestradiol (EE2, [B]) at various time points during the 70 day flow-through exposure. All replicates measured at each time (n = 4) for all treatments, except for the solvent control (n = 1 for ATR, n = 2 for EE2). Error bars represent standard deviation.

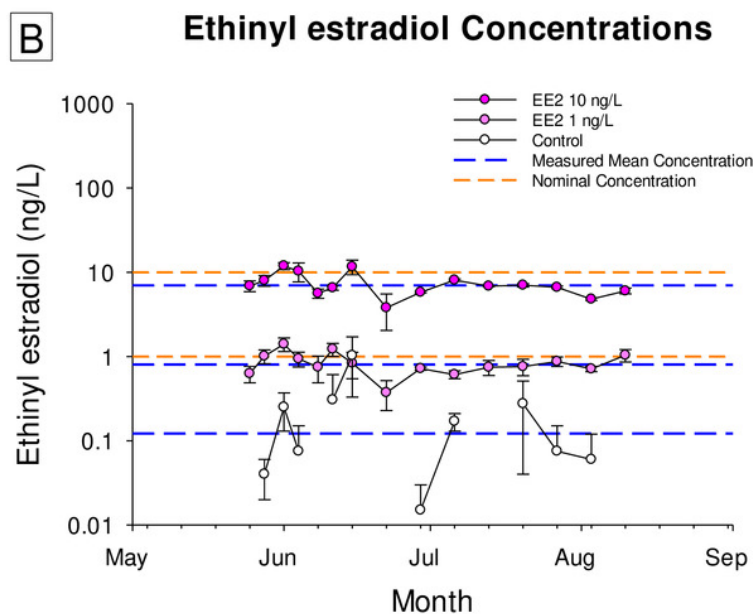
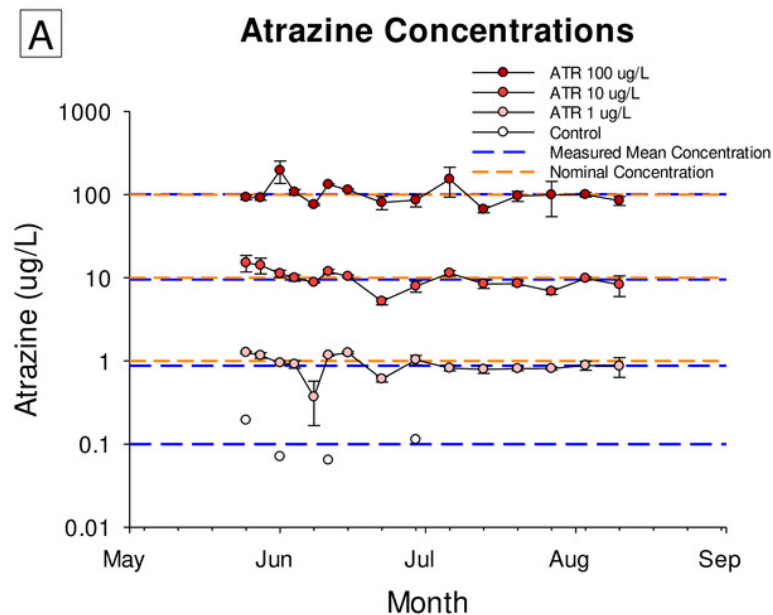



Figure 2

Gonad development.

Example of gonad development from the control treatment at 17 days post-spawn (dps) at 100X (A) and 400X (B) magnification, 33 dps at 40X (C) and 400X (D) magnification, 80 dps male at 40X (E) and 400X (F) magnification, and 80 dps female at 40X (G) and 100X (H) magnification. The bar in the upper right of each panel equals 50 μm. PGC, primordial germ cell; PO, primary oocyte; OC, ovarian cavity.

Time 1 (17 dps)

A

B

PGC

Time 2 (33 dps)

C

D

PGC

Time 3 (80 dps)
Male

E

F

PGC

Time 3 (80 dps)
Female

G

H

PGC   PO

OC

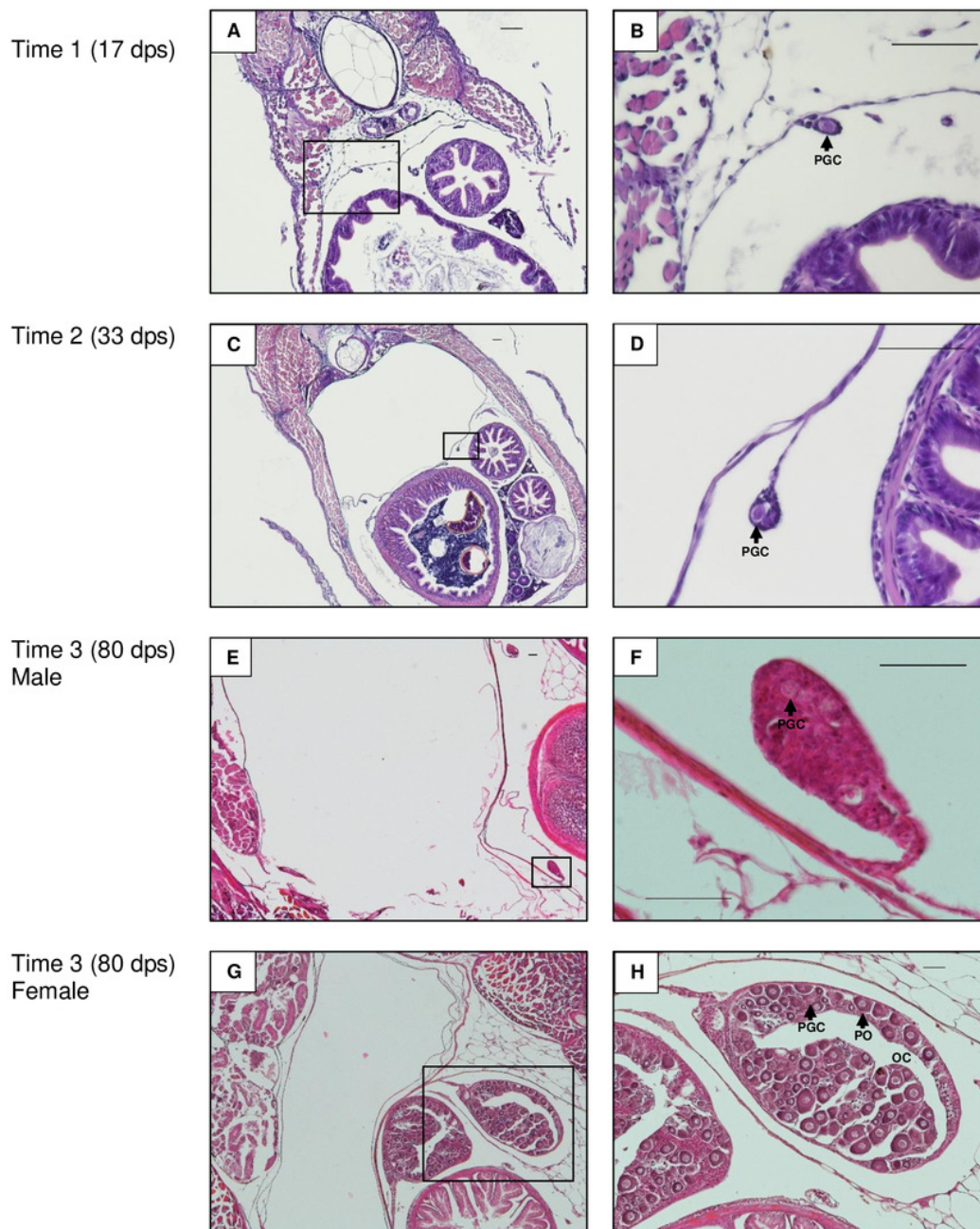



Figure 3

Multidimensional scaling (MDS) with normalized counts.

MDS of transcript expression patterns in juvenile largemouth bass gonads. Individual samples are represented by triangles (males) or circles (females). 1-ATR = 1 µg/L Atrazine; 100-ATR = 100 µg/L Atrazine; 1-EE2 = 1 ng/L 17α-ethinylestradiol.

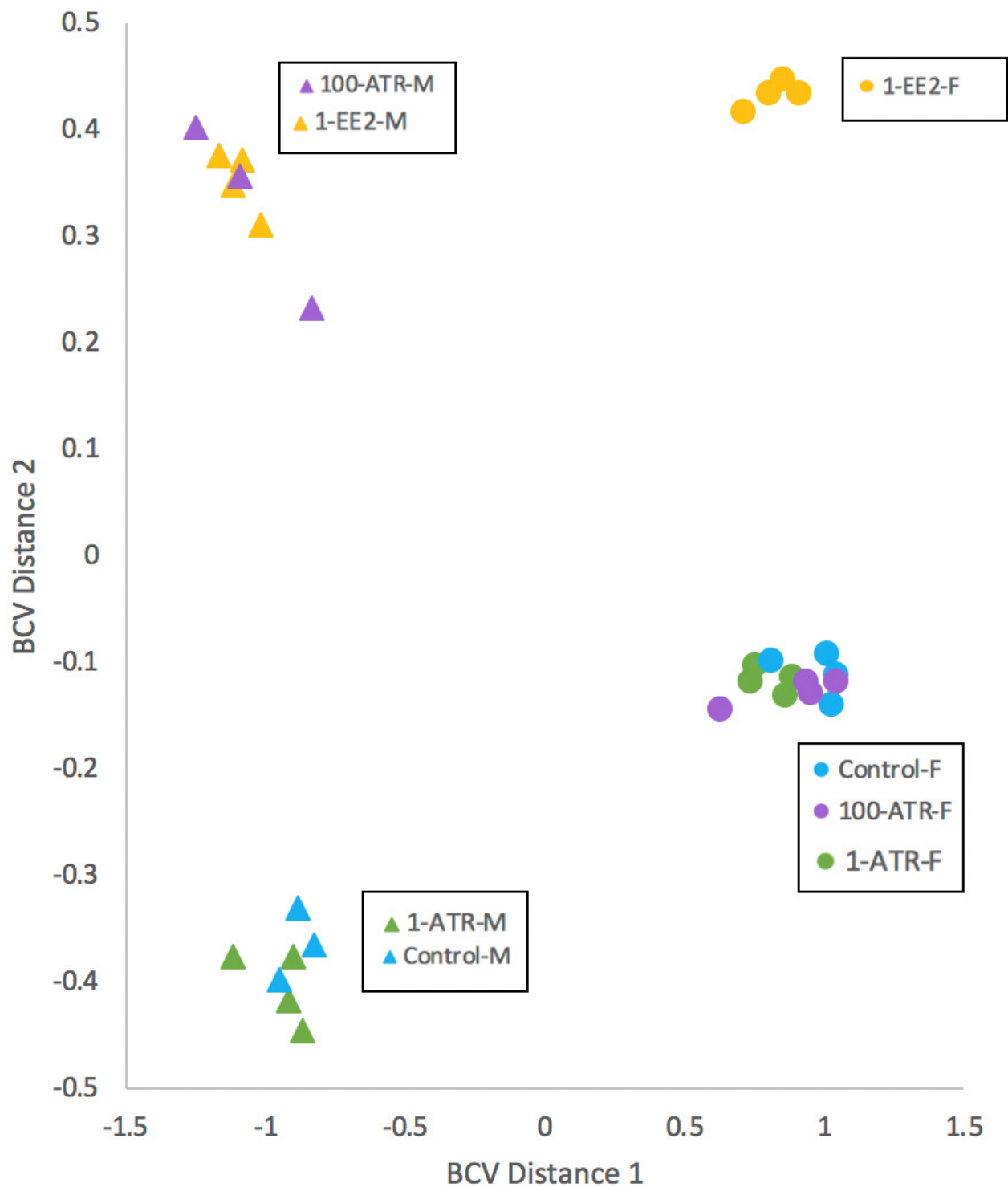



Figure 4

Comparison of differentially expressed transcripts in each test group by sex.

Diagram of overlapping sets of differentially expressed (DE) transcripts that were common between sample groups and treatments. M = male, F = female; F>M = female-biased transcripts that had significantly greater expression in females than males in the controls; M>F = male-biased transcripts that had significantly greater expression in males than females in the controls; 1-ATR = 1 µg/L Atrazine treatment; 100-ATR = 100 µg/L Atrazine treatment; 1-EE2 = 1 ng/L 17α-ethinylestradiol treatment. DE genes that did not have uniform regulation responses between treatments were not represented in the number of up- and downregulated genes in bar graphs A-H, but were included in the total number of DE genes in the Venn diagrams. There are 28,314 total DE transcripts. Conditions used to frame the three gene ontology (GO) queries are highlighted.

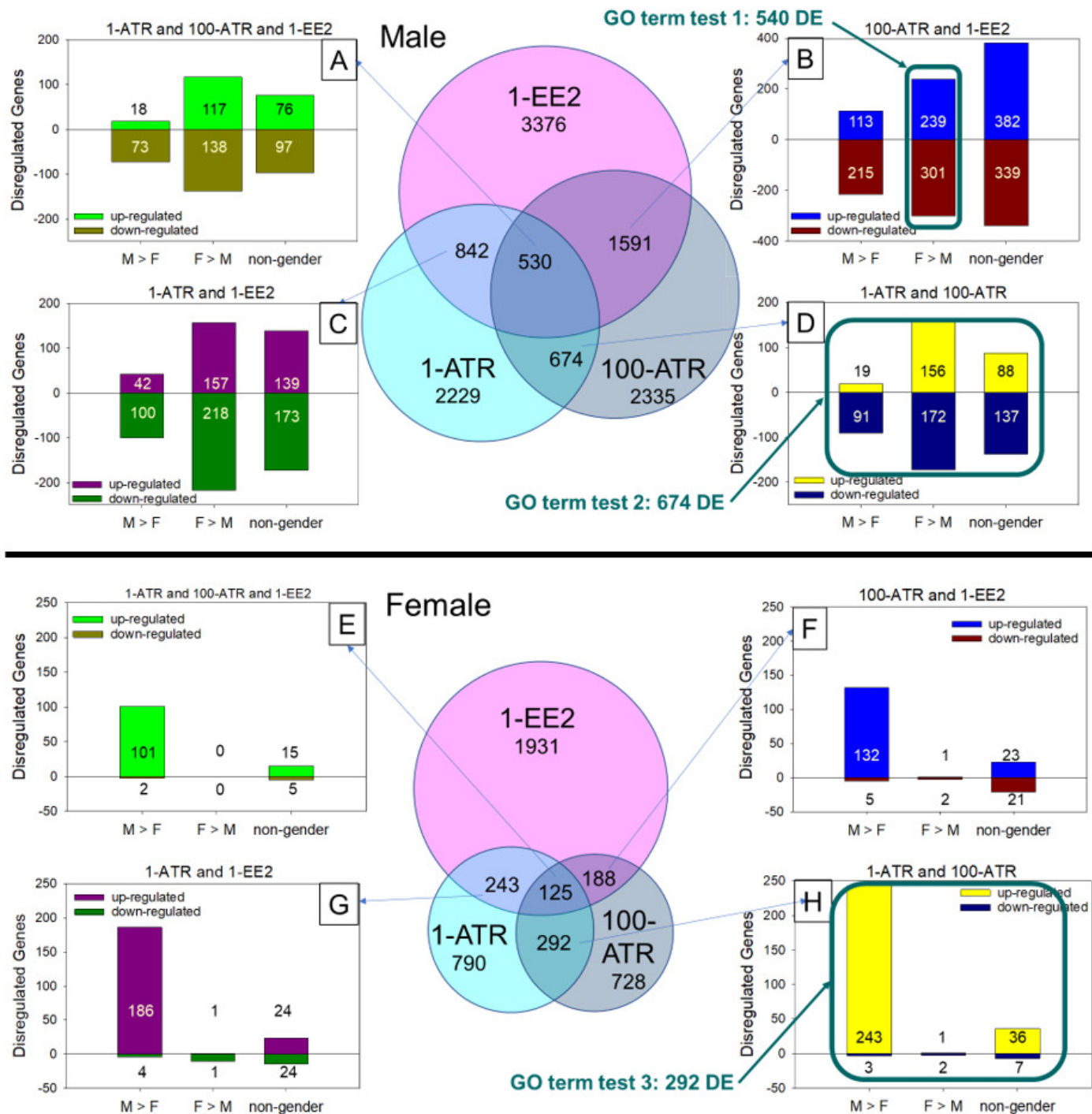
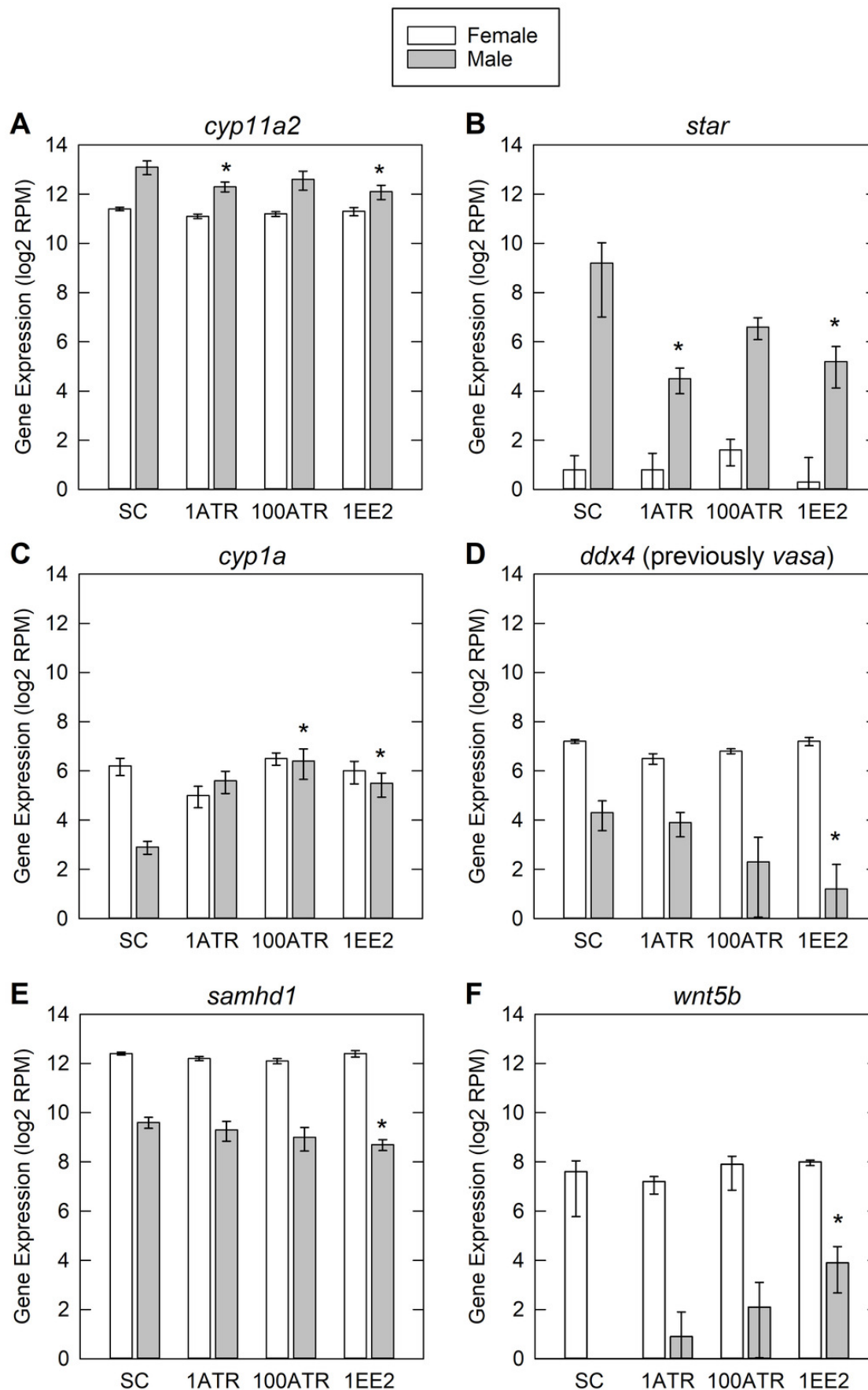



Figure 5

Differential expression data for genes of interest.

Genes of interest were based on literature search and comparative meta-analysis. Genes presented here are those that were significantly altered in one or more treatment groups. SC = solvent control; 1-ATR = 1 µg/L Atrazine treatment; 100-ATR = 100 µg/L Atrazine treatment; 1-EE2 = 1 ng/L 17α-ethinylestradiol treatment. Error bars are one standard deviation from the mean. The y-axis is in reads per million (RPM) on a log base 2 scale. Asterisk denotes significant differential expression compared to SC. Gene symbols and names: (A) *cyp11a2, cytochrome P450, family 11, subfamily A, polypeptide 2*; (B) *star, steroidogenic acute regulatory protein*; (C) *cyp1a, cytochrome P450, family 1, subfamily A*; (D) *ddx4* (previously *vasa*), *DEAD (Asp-Glu-Ala-Asp) box polypeptide 4*; (E) *samhd1, SAM domain and HD domain 1*; (F) *wnt5b, wingless-type MMTV integration site family, member 5b*.

A — *cyp11a2*
B — *star*
C — *cyp1a*
D — *ddx4* (previously *vasa*)
E — *samhd1*
F — *wnt5b*

Legend: Female (white), Male (grey)

x-axis categories: SC, 1ATR, 100ATR, 1EE2
y-axis: Gene Expression (log2 RPM)

Summary of growth and sex ratio data

Summary of Mean (SE) of growth and sex ratio data measured in 80 dps largemouth bass juveniles that were successfully identified as either female or male.

1 **Table 1. Summary of growth and sex ratio data.** Summary of Mean (SE) of growth and sex ratio data
2 measured in 80 dps largemouth bass juveniles that were successfully identified as either female or male.

Treatment	Survival (%)	n		Length (mm)		Weight (g)		Sex Ratio
		Female	Male	Female	Male	Female	Male	% Female
Control	58.4 (2.6)	57	52	54.2 (1.0)	54.1 (1.4)	1.70 (0.16)	1.62 (0.15)	52.5 (5.3)
1 ng/L EE2	55.6 (2.2)	42	61	60.3 (0.4)*	58.3 (1.4)	2.37 (0.15)*	1.95 (0.11)	40.3 (6.4)
10 ng/L EE2	52.9 (1.4)	94	1	59.5 (2.3)*	64.0	1.98 (0.22)	1.93	99.0 (1.0)** ††
1 µg/L ATR	54.0 (2.9)	44	59	55.8 (0.6)	57.1 (1.5)	1.68 (0.09)	1.48 (0.13)	42.7 (3.5)
10 µg/L ATR	58.2 (2.3)	38	56	58.8 (1.3)	56.8 (2.3)	2.15 (0.18)	1.80 (0.23)	39.7 (5.4)
100 µg/L ATR	54.1 (2.3)	55	58	58.7 (1.1)	60.1 (1.2)	2.08 (0.17)	2.23 (0.16)	48.4 (4.6)

4 * = $p < 0.05$, ** = $p < 0.01$ for treatment means significantly different from control tested by Dunnett's
5 post hoc
6 † = $p < 0.05$, †† = $p < 0.01$ for sex ratios significantly different than expected 1:1 female:male tested by
7 chi-square analysis
8

Overrepresented GO terms for gene sets of interest

Gene ontology analysis of differentially expressed (DE) annotated transcripts identified by gene set 1, 2, or 3. Only GO terms that were significantly overrepresented (p-value < 0.05) are presented. Corresponding letters identify common GO terms between gene sets.

1   **Table 2. Overrepresented GO terms for gene sets of interest.** Gene ontology analysis of differentially expressed (DE) annotated transcripts
2   identified by gene set 1, 2, or 3. Only GO terms that were significantly overrepresented (p-value < 0.05) are presented. Corresponding letters
3   identify common GO terms between gene sets.
4

Gene sets	Category	p-value	# DE genes	# genes in category	GO term	# genes down-regulated	# genes up-regulated
1: Female specific transcripts altered in both the 1 ng/L EE2 and 100 µg/L Atrazine treatments	GO:0006605	0.0033	6	96	protein targeting	6	
	GO:0042254	0.00757	6	127	ribosome biogenesis [a]	6	
	GO:0007005	0.00975	5	89	mitochondrion organization	5	
	GO:0006913	0.0149	4	63	nucleocytoplasmic transport	4	
	GO:0006810	0.038	29	1352	transport [b]	16	13
	GO:0044281	0.0473	14	564	small molecule metabolic process [c]	12	2
2: Transcripts altered in males in both the 1 and 100 µg/L Atrazine treatments	GO:0006412	0.00131	14	282	translation	14	
	GO:0044281	0.00329	23	564	small molecule metabolic process [c]	21	2
	GO:0006520	0.00336	9	133	cellular amino acid metabolic process	8	1
	GO:0055085	0.00382	22	495	transmembrane transport [d]	15	7
	GO:0009058	0.0262	54	1916	biosynthetic process		
	GO:0042254	0.0396	6	127	ribosome biogenesis [a]	6	
	GO:0008150	0.0464	203	8605	Biological process		
3: Transcripts altered in females in both the 1 and 100 µg/L Atrazine treatments	GO:0006810	0.00196	18	1352	transport [b]	1	17
	GO:0007267	0.00211	6	249	cell-cell signaling		6
	GO:0055085	0.00708	9	495	transmembrane transport [d]		9
	GO:0007010	0.0189	7	370	cytoskeleton organization		7
	GO:0007165	0.026	19	1709	signal transduction	1	18
	GO:0002376	0.0271	7	428	immune system process		7
	GO:0051604	0.0345	2	42	protein maturation		2