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1 Effects of 17α -ethynylestradiol on early-life development, sex differentiation and
2 vitellogenin induction in mummichog (Fundulus heteroclitus)

3

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36 **Abstract**

37 Fertilized mummichog eggs retrieved from 17 α -ethynylestradiol (EE₂) exposed
38 adult fish were raised in concentrations of EE₂ ranging from 0 to 100ng/L (100ng/L EE₂
39 estimated to have actual average exposure concentrations of 30% of nominal; 0.1 to
40 10ng/L were below detect throughout 24-h exposure period) for 61 weeks post-hatch.
41 Eggs exposed at 100ng/L hatched sooner, the larvae were longer, and survival of juvenile
42 fish from hatch to study termination was greater than all other treatments, though fewer
43 hatched at this treatment. Sex ratios were skewed (>80% female phenotype) at 100ng/L
44 EE₂, and some gonadal male fish displayed female secondary sex characteristics.
45 Condition factor, gonadosomatic index (GSI), and liver somatic index (LSI) were found
46 to decrease in both sexes between 52 and 61 weeks post-hatch. Female fish had
47 increased hepatic vitellogenin (VTG) at 52 weeks post-hatch. When exposed to 1, 10 and
48 100ng/L EE₂, female fish had a higher proportion of vitellogenic follicles in the ovarian
49 tissue. Males exposed at 100ng/L may have had disruption at some endpoints (GSI,
50 VTG) that is masked due to reduced sample size compared to other treatments. Fish
51 exposed to concentrations of EE₂ at or below 10ng/L showed inconsistent effects on
52 development and reproductive potential. This study indicates the potential for
53 population-level effects at the high range of environmental EE₂ at concentrations
54 equivalent to those at which consistent effects in fecundity in the adult mummichog
55 reproductive test have been measured. This work demonstrates that chronic EE₂
56 exposure causes developmental effects at concentrations similar to those which cause
57 effects in the shorter-term adult mummichog reproductive test. Effects are at higher
58 concentrations than have been noted for freshwater model species. Whether this is

- 59 because of species sensitivity or due to differences between freshwater and saltwater
- 60 availability of EE₂ or its uptake requires further study.

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61 Key Words

62 Ethynylestradiol, *Fundulus heteroclitus*, vitellogenin, sex differentiation, development,

63 lifecycle, bioassay, endocrine disruption

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64 **Introduction**

65 Endocrine disrupting substances (EDSs) are exogenous substances or mixtures
66 that alter function of the endocrine system and consequently cause adverse effects in
67 individuals or their progeny (OECD, 1999). Effects of pulp mill effluents in Canada
68 (Hewitt et al. 2008) and sewage in Britain (Jobling and Tyler 2003) provide some of the
69 best examples of endocrine disruption in wild fish. Recently, in a whole-lake experiment,
70 chronic exposure of fathead minnow to low concentrations (5-6ng/L) of 17 α -
71 ethynylestradiol (EE₂) caused feminization and intersex in males, induced vitellogenesis,
72 altered oogenesis in females, and caused a near collapse of the population (Kidd et al.
73 2007). EDSs may interfere with any part of endocrine control, including production,
74 release, transport, metabolism, receptor binding, action or elimination. The sum total of
75 these effects can be measured in fish laboratory bioassays designed to assess
76 reproduction, development and growth (Patyna et al., 1999; Zillioux et al., 2001; Parrott
77 and Wood, 2002; Seki et al., 2004). Chronic exposure to xenobiotics can induce changes
78 to organisms not noted by shorter-term exposures (Parrott and Blunt, 2005), including
79 factors that may be manifested at the population level, such as lower recruitment (Ankley
80 et al., 2001).

81 Lifecycle bioassays or multigenerational bioassays have been developed in
82 freshwater fish species, including the fathead minnow, *Pimephales promelas* (Ankley et
83 al., 2001; Länge et al., 2001), Japanese medaka, *Oryzias latipes* (Seki et al., 2003) and
84 zebrafish, *Danio rerio* (Olsson et al., 1999). Partial lifecycle and short-term bioassays
85 have been developed for estuarine and marine species; examples include sheepshead
86 minnow, *Cyprinodon variegates* (Folmar et al., 2000; Zillioux et al., 2001; Hemmer et

87 al., 2008) and mummichog, *Fundulus heteroclitus* (MacLatchy et al., 2003; Boudreau et
88 al., 2004; Peters et al. 2007; Bosker et al., 2009). Effects of EDSs on species living in
89 salt water may be quite different than those in fresh water because of differences in
90 biological availability of the contaminants based on physical water chemistry and fish
91 physiology (e.g., in contaminant uptake). Therefore, studies focused on the effects of
92 EDSs on estuarine species are warranted, especially given the extent of coastal activity by
93 humans and the importance of estuaries as spawning and nursery grounds (Oberdörster
94 and Cheek, 2001).

95 Mummichog are the numerically dominant fish species in salt marshes along the
96 east coast of Canada and the United States (Armstrong and Child, 1965), and have
97 demonstrated sensitivity to EDSs in laboratory (MacLatchy et al., 2003; Peters et al.,
98 2007), artificial stream (Dubé et al., 2002), and field (Leblanc et al., 1997) assessments.
99 The mummichog is a good candidate for lifecycle bioassay development due to its size,
100 ease of husbandry and ability to manipulate its reproductive cycles. Mummichog are
101 relatively sedentary, exhibiting small home ranges in the wild (Skinner et al. 2005) and
102 they are potentially exposed to environmental EDSs throughout their life cycle.

103 EE₂ has been chosen as a model EDS for developing bioassays due to its
104 environmental relevance as well as its confirmed effects on the reproductive endocrine
105 system via estrogen receptor-mediated pathways (OECD, 1999; Ankley et al., 2001;
106 Metcalfe et al., 2001; MacLatchy et al., 2003). EE₂ is one component of sewage effluent
107 associated with increased incidence of intersex in male fish exposed downstream of
108 sewage treatment plants (STPs) (Jobling and Tyler, 2003). It is a synthetic
109 pharmaceutical (birth control pill and hormone replacement therapy) that is not broken

110 down in sewage treatment; concentrations of EE₂ present in Canadian STPs are usually
111 between 1-10ng/L EE₂, although levels have been documented as high as 42ng/L
112 (Desbrow et al., 1998; Ternes et al., 1999). In earlier short-term (7- or 15-day) exposure
113 studies using EE₂, adult mummichog displayed endocrine impacts at low,
114 environmentally-relevant concentrations, as well as similar responses at higher
115 pharmaceutical concentrations (MacLatchy et al., 2003). In longer-term (21- or 28-day)
116 EE₂ exposures, reproductive cycling was shifted in females, sex steroid production and
117 circulating levels were altered and at environmentally-relevant (approximately 20% of
118 nominal 100ng/L exposures), fecundity and fertility were reduced (Peters et al., 2007).

119 The objective of this study was to determine the impact of chronic EE₂ exposure
120 on offspring development. Embryos derived from mummichog parents exposed during
121 pre-spawning and spawning phases to EE₂ were continuously exposed to EE₂ for 15
122 months, through their development to pre-spawning juveniles/adults. Embryonic/larval
123 endpoints (time to hatch, hatch success, length at hatch), larval/juvenile endpoints
124 (growth, survival, vertebral abnormalities) and yearling endpoints (liver vitellogenin,
125 gonad and liversomatic indices, condition factor, sex ratios) were evaluated for
126 anomalies. This study, in conjunction with our previous studies (MacLatchy et al., 2003,
127 Boudreau et al., 2004; MacLatchy et al., 2005; Boudreau et al., 2005; Sharpe et al., 2004;
128 Peters et al., 2007) furthers our ability to understand the effects of EDSs on various life
129 stages of mummichog. This study demonstrates that developmental stages of
130 mummichog are sensitive to EE₂ at exposure levels similar to those that interfere with
131 reproduction in adult mummichog (Peters et al. 2007), and that the concentrations at

132 which effects occur are higher than those noted in freshwater model species (Länge et al.
133 2001; Andersen et al., 2003).

134

135 **Materials and Methods**

136 *Chemicals*

137 The 17 α -ethynylestradiol (EE₂; 98% purity) was purchased from Sigma-Aldrich
138 Canada (Oakville, ON, Canada). EE₂ was stored at -20°C in 100% ethanol (Les Alcools
139 de Commerce, Boucherville, QC, Canada) at stock concentrations of 3ng/mL, 30ng/mL,
140 300ng/mL and 3000ng/mL EE₂ for adult exposures and 10ng/mL, 100ng/mL, 1000ng/mL
141 and 10000ng/mL EE₂ for larval and juvenile exposures. Unless otherwise indicated,
142 chemicals and reagents were purchased from Sigma-Aldrich and laboratory supplies from
143 Fisher Scientific (Nepean, ON, Canada).

144

145 *Experimental Conditions*

146 Collection and breeding protocols for adult mummichog used in this study have
147 previously been described (Peters et al., 2007). Offspring were maintained at the same
148 exposure conditions as their parental groups at 0, 0.1, 1, 10, or 100ng/L EE₂, in static
149 conditions with daily water changes and treatment renewal for the 61-week study period.
150 As developing embryos, larvae, fry and juveniles grew, photoperiod was adjusted to
151 simulate seasonal day length: 16:8 h light:dark at initiation (July); 14:10 h light:dark at
152 week 14 (October); 12:12; light:dark at week 20 (November); 14:10 light:dark at week 32
153 (February); 15:9 light:dark at week 40 (April); and 16:8 light:dark from week 46 (May)
154 to termination of the experiment. Temperature was held at room temperature, which

155 decreased slightly from 18-21°C in the summer (weeks 1-20) to 16-18°C for the winter
156 (weeks 21-40), returning to 18-21°C for the remainder of the study.

157 Larvae were fed live, newly hatched *Artemia* sp. nauplii (Bohai Bay Salt Ponds
158 *Artemia* Cysts, Aquatic Ecosystems, Apopka, FL, USA) enriched with Roti-rich™
159 (Aquatic Ecosystems) twice daily (1 mL of concentrated *Artemia* per L of water) and Fry
160 Food (Rolf C. Hagen, Montreal, QC, Canada) to satiation once daily for 14 weeks.
161 Beginning at 8 weeks, freeze-dried Red Grubs (Rolf C. Hagen) were used to supplement
162 the Fry Food diet as the juveniles were weaned from the *Artemia*. Flaked Staple Food
163 (Rolf C. Hagen) was introduced as the primary feed at 22 weeks, fed 2-3 times daily,
164 supplemented by Red Grubs or Cichlid Food (Rolf C. Hagen) once daily.

165

166 *Exposures*

167 Naturally-spawned, fertilized mummichog eggs were collected from adults
168 exposed to nominal exposure concentrations of 0, 0.1, 1, 10, or 100ng/L EE₂ for 21 and
169 28-days as previously reported (Peters et al., 2007). The fertilized eggs were transferred
170 to glass Petri dishes at an initial density of 30 eggs per dish and held in 50 mL of 20‰
171 salinity EE₂-treated water. Each dish was examined daily at 4X magnification, and dead
172 embryos and hatched larvae were removed. The water was then removed from each dish
173 and replaced with 50 mL of water treated with the appropriate amount of EE₂. Time to
174 hatch, survival to hatch and length at hatch were monitored over the hatching period.

175 Upon hatch, larvae were maintained in 50mL beakers containing the appropriate
176 EE₂ concentrations and held at a maximum density of 10 larvae per beaker. Once the
177 yolk sac was absorbed (1-3 days) and swimming began, the larvae were randomly

178 allocated to one of four aerated, static 37-L aquaria per treatment. Daily water renewals
179 were done by completely replacing the water and adding new treatment solution in each
180 tank after removing fish with a dip net and placing them in a temporary holding
181 aquarium; fish were returned to their original treatment tanks following the water change.
182 Upon initiation of the growout phase, each aquarium contained 5 L of EE₂-treated 20‰
183 saline water (dissolved oxygen > 80% saturation). At five weeks, the volume of water in
184 each aquarium was increased to 10 L to accommodate growing larvae. Beginning at 10
185 weeks, volumes of water were adjusted separately for each tank to minimize the effect of
186 density differences among the tanks due to differential survival. Water volume was set to
187 1 L per 1 g total wet weight of the fish in the aquarium and was adjusted every 3-4 weeks
188 for the remainder of the experiment. Each tank was replaced 100% daily throughout the
189 exposure period. Due to a dosing error in week 14, replicate tanks for 0 ng/L and 100 ng/L
190 EE₂ were reduced to three tanks for subsequent endpoints.

191 Growth was determined weekly for the initial seven-week period, and then
192 approximately every three weeks for the remainder of the experiment by collecting total
193 length measurements (to the nearest mm) from 25 fish removed, measured and returned
194 to each aquarium. Survival was determined from the number of fish in each tank on each
195 measurement day, and calculated as the proportion of the original number of fish in the
196 tank. Vertebral abnormalities were assessed at weeks 15, 48 and 61 and ranged from
197 mild (one or two slight bends in the spine) to severe (one or more bends in the spine that
198 dramatically altered body shape and/or affected swimming ability) and included scoliosis
199 (lateral curvature) and lordosis (dorsoventral curvature) (Boudreau et al., 2004).

200 At week 48 (May 2004), all fish were assessed for weight (to 0.001g), standard
201 and total length (mm), vertebral abnormalities and external sex. Sex was determined
202 based upon colouration and secondary sex characteristics including yellow bellies, dark
203 dorsal fin spot, silver vertical stripes, white or yellow spots in males, and brown or green
204 backs and pale bellies in females. At week 52 (22 June 2004), less than a week following
205 the full moon, 20 fish were randomly sampled from each aquarium except one 1ng/L
206 aquarium and one 100ng/L aquarium where 0 and 15 fish were selected respectively, due
207 to low numbers of fish in these tanks. These fish were anaesthetized (buffered 0.05%
208 tricaine methane sulfonate; Syndel Laboratories, Vancouver, BC, Canada), assessed for
209 weight (to 0.001g), standard and total length (mm), vertebral abnormalities and sex based
210 upon secondary characteristics and gonadal assessment at 4X magnification.

211 Vitellogenin (VTG) in the liver was assessed at week 52 using an ELISA
212 modified from MacLatchy et al. (2003). Liver tissue was dissected out, weighed, frozen
213 on dry ice, and then stored at -80°C. For each mg of liver tissue, 1 µL of aprotinin
214 (1KIU/µL) was added to the microfuge tube. Tissue was thawed and homogenized with a
215 Kontes Pellet Pestle hand-held homogenizer. A small amount of the slurry (5 µL) was
216 diluted 100X with Tris buffered saline containing Tween and bovine serum albumin and
217 assayed as per MacLatchy et al. (2003). Prior to analysis, the method was tested by
218 spiking liver slurry samples with a known concentration of VTG. Recovery of the spiked
219 amount of VTG [recovered VTG = 0.549 + 1.071 * (spiked VTG); R²=0.774; p<0.001]
220 was 88%. Interassay variability was 7% and intra-assay variability was 6%.

221 Gonadal tissue collected at week 52 was fixed in 10% buffered formalin and
222 stored in 70% ethanol. Tissues were embedded in paraplant and sectioned at 7 µm using

223 a rotary microtome. Tissue sections were stained with Mallory stain and examined with a
224 light microscope at 10X magnification for female tissue and 40X magnification for male
225 tissue. For each fish that had sufficient tissue, six fields of view (at different depths into
226 the gonad) were assessed for intersex and to determine developmental stage at week 52.
227 Using the terminology for developmental stage of Blazer (2002), the number of
228 previtellogenic and vitellogenic follicles were counted in females and the developmental
229 stage(s) of the testes were determined for each field of view.

230 At week 61 (August 2004), four days prior to the full moon, all remaining fish
231 were sampled and assessed for weight (0.001g), standard and total length (mm), vertebral
232 abnormalities and sex based upon secondary characteristics and gonadal assessment at
233 4X magnification. At 52 weeks, the fish were not reproductive and by 61 weeks were
234 beginning to regress; therefore, a breeding trial could not be carried out and the
235 experiment was terminated.

237 *EE₂ Analysis*

238 Analysis of the concentration of EE₂ in the water was unavailable for this study
239 due to insufficient water volumes collected for analysis. Therefore, a subsequent two-
240 week exposure of adult mummichog (with equivalent volume of water and mass of fish
241 as per juvenile exposures) was conducted in duplicate aquaria at nominal concentrations
242 of 0 and 100ng/L EE₂. Volumes of water extracted and analyzed were 1L. EE₂ levels
243 were extracted and analyzed as previously described (Peters et al., 2007) with additional
244 method standardization to ensure EE₂ recovery in salt water. The water concentrations
245 for the 100ng/L EE₂ treatment aquaria were highest at the point of delivery (74.7ng/L)

246 and decreased with time to 66.8ng/L at 1 h and 30.7ng/L at 6 h; at 12 and 24 h EE₂ levels
247 were undetectable (detection limit at 10ng/L). Because of the inability to determine
248 exposure concentrations at exposures <10ng/L nominal, all exposure concentrations are
249 given as nominal values.

250

251 *Statistics*

252 Statistical analyses were conducted using SigmaStat 3.0 (SPSS, Chicago, IL,
253 USA) or Systat 9.0 (Systat Software Inc., Richmond, CA, USA). Significant differences
254 ($p < 0.05$) among the data were assessed separately for each sex when it was possible to
255 determine gonadal sex. Prior to parametric analysis, assumptions of normality and
256 variance homogeneity were tested on morphological data using normal probability plots
257 and Levene's test. Where the data failed to meet the assumptions, they were \log_{10}
258 transformed and the assumptions retested. If the data still failed to meet the assumptions,
259 an equivalent non-parametric test was used. Measures of time to hatch, hatch success
260 rate, and length at hatch were assessed using single factor ANOVA, using Petri dishes as
261 the replication units. Mortality, vertebral abnormalities and liver VTG were assessed
262 using single factor ANOVA, with aquarium as the unit of replication. Vitellogenin data
263 were examined for outliers using Dixon's test prior to analysis. Analyses of weight and
264 length were performed using a nested analysis of variance (ANOVA) followed by
265 Tukey's or Dunn's tests, with aquarium as the factor nested within treatment group.
266 Kruskal-Wallis was used when the data failed to meet parametric assumptions.
267 Differences in gonadal and liver weights relative to body weights (gonadosomatic index;
268 GSI; [gonad wt/body wt]·100; and liversomatic index; LSI; [liver wt/body wt]·100), body

269 weight to standard length (condition factor; CF; $[\text{body wt}/\text{standard length}^3] \cdot 100$), and
270 total length over time (growth) were determined using analysis of covariance
271 (ANCOVA). The interaction term significance was set at $p=0.1$ (Environment Canada,
272 2005). Differences in morphological variables between sampling dates were assessed
273 using A. Student's t-test. Sex ratios and gonadal development ratios were assessed using
274 a chi-square analysis for proportions, with expected proportions set at 50:50, followed by
275 an angular transformation and Tukey-type multiple comparison (Zar, 1999). Values are
276 reported as means \pm standard error (SE).

277

278 **Results**

279 Eggs exposed to 10ng/L and 100ng/L EE₂ hatched sooner than eggs exposed to
280 0ng/L EE₂ ($p=0.018$; Fig. 1). Eggs exposed to 100ng/L EE₂ had significantly lower hatch
281 success than those in 0, 0.1, and 1ng/L ($p<0.001$; Fig. 1). Larvae hatched at 1 and
282 100ng/L EE₂ were significantly longer than those at 0, 0.1 and 10ng/L ($p<0.001$; Fig. 1).

283 Weight at length (condition factor) for both sexes decreased between week 48
284 (data not shown) and week 61 in all treatment groups. There were no differences in
285 weight at length in male or female fish between 0ng/L and any other treatment at weeks
286 48, 52 (data not shown) or 61 (Table 1).

287 Post-hatch survival was found to be significantly higher for 100ng/L exposed fish
288 than any other treatment ($p<0.001$; Fig. 2). At week 15 the proportion of fish exhibiting
289 vertebral abnormalities at 0.1, 10, and 100ng/L were significantly lower than at 0 and
290 1ng/L ($p=0.021$; Fig.3). These differences among treatments were not retained at 48 nor
291 61 weeks (data not shown).

292 Male fish exposed to 100ng/L EE₂ had significantly lower GSI at 52 weeks
293 ($p=0.016$; 1.42 ± 0.15 at 0ng/L vs 0.60 ± 0.23 ng/L at 100ng/L EE₂) but not at 61 weeks
294 post-hatch (Table 1) and between week 52 and week 61, male GSI decreased in all
295 treatments except 100ng/L EE₂ (data not shown). No differences were found in male LSI
296 among treatments at weeks 48, 52 (data not shown) or 61 (Table 1). There was no
297 difference in female GSI among treatments at 52 weeks ($p=0.641$; range of 2.55 ± 0.14 at
298 0.1 ng/L to 4.81 ± 0.99 at 100ng/L) or 61 weeks (Table 1) though female GSI decreased
299 between week 52 and week 61 in fish exposed to 0, 0.1, and 1ng/L EE₂. No differences
300 in female LSI were found among fish exposed at 0ng/L and any other treatment (weeks
301 48 and 52, data not shown; week 61, Table 1).

302 At 52 weeks post-hatch, there was no difference among treatments in liver VTG
303 in males ($p=0.732$; data not shown). There was an increase in liver VTG in females
304 exposed to 100ng/L EE₂ compared to all other treatments ($p<0.001$; approximately 10-
305 fold increase from 1500-2500 μ g/mg VTG at 0-10ng/L EE₂ to 14,000 to 17,000 μ g/mg
306 VTG at 100ng/L EE₂).

307 At 52 weeks post-hatch, sex ratios based on secondary sexual characteristics were
308 significantly different at 100ng/L, with females accounting for greater than 90% of the
309 fish at this concentration ($p<0.001$; Table 2). Secondary sex characteristics differed in a
310 similar fashion at the highest exposure at 48 and 61-weeks post-hatch. Visual
311 examination of the gonads at 52 weeks post-hatch found sex ratios to be significantly
312 different at 100ng/L EE₂; the proportion of females was 86.8% ($p<0.001$; Table 2).

313 Histological analysis of the gonads found sex ratios to be significantly different at
314 100ng/L EE₂; the proportion of females was 86.1% ($p<0.001$; Table 2). There were

315 significantly more vitellogenic oocytes in female fish exposed to 1, 10 and 100ng/L EE₂
316 when compared to the group exposed to 0ng/L EE₂ (p<0.001; Table 3). There was only a
317 single case of intersex, where a male fish (tissue was primarily prespermatogenic)
318 exposed to 0.1ng/L EE₂ had three previtellogenic oocytes within the tissue. The
319 proportion of male gonadal tissue at each maturation stage did not differ among
320 treatments (p>0.05; Table 3).

321

322 Discussion

323 Previous studies in our laboratories (MacLatchy et al., 2003; Boudreau et al.,
324 2004; Peters et al., 2007) examined the effects of EE₂ exposure on adult and larval
325 mummichog. This present work addresses questions that arose from these studies,
326 including whether a standardized, multigenerational protocol could be developed for EDS
327 studies to correlate physiological effects with population and/or reproductive impacts in
328 mummichog as has been suggested for other fish species (Patyna et al., 1999; Ankley et
329 al., 2001). The eggs used in this study were spawned and fertilized in a short-term
330 reproductive bioassay in which adult mummichog were exposed to concentrations of EE₂
331 ranging from 0 to 100ng/L (nominal) for 21 and 28 days (Peters et al., 2007). The rearing
332 of these eggs to sexual maturity provides a more complete understanding of the total
333 impact of chronic and lifecycle EE₂ exposure in an estuarine species. Long-term
334 exposure to the highest concentration (100ng/L nominal; approximately 30% of exposure
335 concentration) resulted in a higher proportion of fish with female secondary sex
336 characteristics and gonads. As well, female livers contained higher VTG levels at this
337 concentration. These EE₂-induced changes occurred only at the highest concentrations

338 and parallel the physiological and reproductive effects observed in the parents of these
339 offspring (decreased circulating steroids, increased VTG, decreased egg production and
340 decreased fecundity/fertility; Peters et al., 2007) during shorter exposure periods.

341 In the progeny generation, hatching success was reduced when parents and eggs
342 were exposed to 100ng/L EE₂. Decreases in both spawning output and fertilization
343 success during adult exposure to 100ng/L EE₂ have been demonstrated in mummichog in
344 our laboratory (Peters et al., 2007). The effects on progeny may be a threshold effect, as
345 there is no indication of similar difficulties in spawning, fertilization or hatching at EE₂
346 levels <10ng/L (this study; Peters et al., 2007). However, a concentration-dependent
347 response may exist between exposure concentrations of 10 and 100ng/L EE₂.

348 Sheepshead minnow exposed to EE₂ showed reduced egg production at 20 and 200ng/L
349 and reduced hatching at 200ng/L (Zillioux et al., 2001). Hatching success decreased in a
350 dose-dependent manner in larval mummichog exposed to estradiol (E₂) (Urushitani et al.,
351 2002) and estrogenic alkylphenols caused complete larval mortality at concentrations
352 $\geq 10\mu\text{M}$ and hatching success was <10% below this concentration (Kelly and Di Giulio,
353 2000).

354 As EE₂ concentrations increased, there was a decrease in the egg incubation
355 period prior to hatch. Shortened time to hatch has been documented in zebrafish exposed
356 to the weakly estrogenic pesticide methoxychlor (Versonnen et al., 2004) and brown trout
357 (*Salmo trutta*) exposed to wood-sterols (Lehtinen, et al., 1999). Larvae that emerged in
358 the present study were significantly longer when exposed to 1 and 100ng/L EE₂.

359 Exposure to EE₂ prior to spawning and fertilization may affect the developing oocyte
360 before the development of the protective chorion and associated membranes of the egg

361 that prevent exogenous compounds from reaching the developing embryo (Anadu et al.,
362 1999).

363 Survival from hatch to the conclusion of the study was found to be significantly
364 higher for 100ng/L EE₂-exposed fish than any other treatment. Fathead minnow exposed
365 to EE₂ from fertilization showed no differences in survival among treatments (Länge et
366 al., 2001). Mummichog exposed for 60 days and reared in clean water showed increased
367 survival at 10ng/L, no change in survival at 1000ng/L and decreased survival at
368 10,000ng/L EE₂ (Boudreau et al., 2004). After 10 weeks of the growout period, volumes
369 in all the aquaria were adjusted to compensate for fish numbers in an attempt to eliminate
370 potential density-dependent effects. Boudreau et al. (2004) suggested that decreasing
371 density by increasing volume of water and/or decreasing number of fish reduced
372 mortalities in mummichog. It is also possible that the decreased mortality post-hatch in
373 the 100ng/L treatment was the result of increased embryonic mortality, which weeded-
374 out weaker fish.

375 There were no differences in male or female condition (length at weight)
376 throughout the study and differences in vertebral abnormalities observed among
377 treatments at week 15 were not present at week 48 or 61. The proportion of 0ng/L fish
378 exhibiting vertebral abnormalities decreased over time, and may be attributable to the
379 mortality of these fish. The growth of soft-tissue that hides underlying abnormalities has
380 previously been suggested as a mechanism that decreases observable deformities
381 (Boudreau et al., 2004). Whether any of the endpoints measured in relation to time to
382 hatch, size at hatch, juvenile growth, abnormalities, etc., are significant from a
383 population/biological standpoint requires more study, especially in a field situation. It

384 has long been known that size at hatch, growing season length, and food availability all
385 significantly affect long-term survival in wild fish populations (Andersen, 1988;
386 Cargnelli and Gross, 1996) making any size deficiency an issue in the wild.

387 VTG production in the liver naturally occurs in female fish once a threshold level
388 of E_2 is reached in the hepatic estrogen receptors (Denslow et al., 1999). VTG is a
389 sensitive biomarker of estrogen exposure; exogenous estrogen induces VTG synthesis in
390 female and male and/or juvenile fish (Denslow et al., 1999) in both freshwater (Panter et
391 al., 2002) and saltwater (MacLatchy et al., 2003) species. VTG induction as an indicator
392 of decreased reproductive potential has recently been assessed in fathead minnow (Miller
393 et al., 2007) and high levels can cause kidney problems in fish (Folmar et al., 2001).

394 There was no difference in liver VTG in male fish at week 52. However, the mean VTG
395 for males exposed to 100ng/L EE_2 was more than twice the mean of male fish exposed to
396 0ng/L EE_2 . There were only five fish identified as male in the 100ng/L EE_2 treatments,
397 compared to 23-39 fish in each of the other treatments. The small sample size for male
398 fish at this treatment level may be masking any effect on VTG. Alternatively, during a
399 long exposure, feedback and regulatory physiological mechanisms may allow the fish to
400 achieve homeostasis and prevent a VTG response (Länge et al., 2001). Females exposed
401 to 100ng/L EE_2 had increased liver vitellogenin compared to all other treatments.

402 Medaka exposed to two estrogenic alkylphenols for 60 days post-hatch had
403 concentration-dependent increases in hepatic VTG in both sexes (Seki et al., 2003).

404 Whole body VTG increased in fathead minnow exposed to EE_2 for 142 days post-hatch
405 (Länge et al., 2001). Adult female mummichog had elevated plasma VTG at 100ng/L
406 EE_2 in a 15-day exposure (MacLatchy et al., 2003), indicating consistent effects through

407 different life stages in this species. While liver sizes in fish generally increase during
408 vitellogenesis (Kleinkauf et al., 2004), there was no correlation between LSI and liver
409 VTG in this study (data not shown).

410 At 52 weeks post-hatch, males exposed to 100ng/L EE₂ had significantly lower
411 GSI than all other treatments and at week 61, males exposed to 100ng/L EE₂ had GSIs
412 less than 60% of each of the other treatments. Male GSI decreased between week 52 and
413 week 61 in 0, 0.1, 1 and 10ng/L EE₂ treatments. Few males showed the brilliant yellow
414 of sexual maturity and it is possible that male GSI was decreasing due to the annual
415 reproductive cycle (week 61 was 26 August 2004). GSI in mummichog decreases as the
416 breeding season ends (Cochran, 1987). Male fish exposed to 100ng/L EE₂ did not
417 demonstrate the reduction in GSI between weeks 52 and 61 observed in the other
418 treatments. Male zebrafish exposed for 24 days at 10 and 25ng/L EE₂ showed reductions
419 in GSI, due to a predominance of immature sperm (Van den Belt et al., 2002). No
420 differences among treatments were found within each male gonadal maturation stage.
421 However, the high variability, especially in the 100ng/L treatments, where only five male
422 fish had enough gonadal tissue to embed and section for analysis, may be masking an
423 effect.

424 There was no difference in GSI among treatments in female fish at 52 (June) or
425 61 (August) weeks post-hatch. However, female GSI decreased between week 52 and
426 week 61 in fish exposed to 0, 0.1, and 1ng/L EE₂, and did not change in female fish
427 exposed to 10 and 100ng/L EE₂. These changes in GSI, and the increased proportion of
428 vitellogenic oocytes in female fish exposed to 1, 10 and 100ng/L EE₂, indicate that higher
429 levels of EE₂ may cause differences in gonadal development during the spawning season.

430 In the parental generation, there was also indication that EE₂ exposure could cause
431 disruption to reproductive cycles in female mummichog. Adult females exposed to
432 higher concentrations of EE₂ (1-100ng/L) had altered gonadal steroid production and
433 completed the spawning cycle earlier than fish exposed to 0 and 0.1ng/L EE₂ (Peters et
434 al., 2007).

435 Sex ratios based upon secondary sexual characteristics, visual examination of the
436 gonads and histological analysis demonstrated skewed sex ratios at 100ng/L. Early life
437 exposure of mummichog to EE₂ weakly skewed the sex ratio towards females, even when
438 raised following 60 days of post-hatch EE₂ exposure in clean water (Boudreau et al.,
439 2004). Medaka eggs injected with EE₂ have shown sex reversal of genetic males upon
440 growout (Papoulias et al., 1999) while medaka exposed to EE₂ from two days post-hatch
441 had gonadal intersex at 2ng/L (Balch et al., 2004). Fathead minnow have been
442 completely feminized in lifecycle exposures to 4ng/L EE₂ (Länge et al., 2001) and
443 3.2ng/L (Parrott and Wood, 2002). Mummichog sex ratios appear to be affected at
444 higher exposure concentrations (>10ng/L EE₂) than other species (<5ng/L EE₂). Gonadal
445 sex change in fish can be induced by exposure to estrogens during windows of sensitivity
446 related to gonadal differentiation and development (Nakamura et al. 1998). The fathead
447 minnow has a period of enhanced sensitivity to EE₂ between 10 and 15 days post-hatch,
448 when male fish are more likely to develop female characteristics (Van Aerle et al., 2002)
449 and 6-day exposures with medaka have demonstrated that exposure while an embryo,
450 newly hatched fry or 1 week post-hatch are sufficient to alter sex ratios and/or develop
451 intersex gonads at sexual maturity (Koger et al., 2000). In mummichog, the window of

452 sensitivity to exogenous steroids during differentiation has not been precisely identified
453 but most likely occurs from 2-4 weeks after hatching (Urushitani et al., 2002).

454 EE₂ is typically found in low concentrations (0.1-10ng/L with amounts as high as
455 42ng/L in some cases) in Canadian sewage effluents (Desbrow et al., 1998; Ternes et al.,
456 1999). In the low range of environmentally-relevant concentrations, exposure to EE₂
457 during different life stages results in minimal, if any, adverse effects on reproductive
458 endpoints (this study; MacLatchy et al., 2003; Boudreau et al., 2004). However,
459 exposure at the high end of the range (30% of nominal 100ng/L EE₂ exposure) negatively
460 affected larval and juvenile endpoints, including hatch success, liver VTG, male GSI, sex
461 ratios and female gonadal development in mummichog. Because these effects occurred
462 at the high range (>10ng/L actual exposure), long-term full lifecycle exposures in
463 mummichog do not appear to offer advantages from a testing and sensitivity perspective
464 over the shorter-term adult reproductive test (e.g., Peters et al., 2007).

465 More studies are required to determine the relative sensitivities of different fish
466 species, including those typically classified as “model” species. In the present study, the
467 100ng/L exposure averaged approximately 30% (30ng/L) over the course of a 24-h day;
468 all other exposures were \leq 10ng/L nominal and therefore significantly less in actuality.
469 This makes comparison to other studies that had exposure concentrations <10ng/L or
470 >10ng/L possible. Fathead minnow have failed development of secondary sex
471 characteristics, altered mating behaviour, intersex and induced VTG in males at 1-5 ng/L
472 EE₂ (Länge et al., 2001; Lattier et al., 2002; Parrott and Blunt, 2005); Japanese medaka
473 display intersex and reduced copulations at 2-10ng/L EE₂ (Balch et al., 2004); and
474 zebrafish have reduced spawning, fertilization, intersex and GSI at 5-10ng/L EE₂ (Van

475 den Belt et al., 2001; Van den Belt et al., 2002; Nash et al., 2004). In contrast to
476 freshwater species, in which effects are observed at concentrations $\leq 10\text{ng/L}$ EE₂,
477 sheepshead minnow have reduced reproductive success at EE₂ between 20 and 200ng/L
478 (Zillioux et al., 2001) while mummichog have altered steroid synthesis, VTG induction,
479 reproductive success and sex differentiation at $>10\text{ng/L}$ EE₂ (MacLatchy et al., 2003;
480 Peters et al., 2007; this study). Clearly, all of these species show significant effects from
481 estrogen exposure during lifecycle exposure. Estuarine species, however, may have a
482 different level of sensitivity, and/or their uptake may be altered (e.g., differences in gill
483 vs. gut uptake), and/or the fate of contaminants may be different in saltwater than
484 freshwater systems. Clearly, further investigation is required to determine the roles of
485 physiology and physical environment on biological effects of EDSs in estuarine species
486 before determining whether zebrafish or other freshwater species with shorter life cycles
487 have significant test advantages and can be used as surrogate test species for regulating
488 EDS exposures in saltwater environments.

489 In mummichog, population-level adaptation to bio-accumulative, persistent
490 contaminants such as halogenated aryl hydrocarbons has been observed in field and lab
491 studies (Nacci et al., 1999); the mechanism for such adaptation is associated with a
492 consistent lack of inducibility of genes that are normally inducible by aryl hydrocarbon
493 receptor agonists (Meyer et al., 2003). In the highly-polluted New Bedford Harbor
494 (Massachusetts, USA), reproductively-active males and females demonstrate different
495 physiological (e.g., lower estrogen and androgen levels) and molecular (e.g., higher brain
496 P450 aromatase B expression) states compared to reference-site fish. Despite these
497 effects, gonadal sex differentiation, reproduction and embryonic/larval development are

498 within normal ranges even following multi-generational exposure to estrogenic
499 contaminants (Greytak et al., 2003). Further studies are required to complete the picture
500 linking physiological/molecular effects and population-level effects in fish exposed to
501 EDSs; better understanding of these links may help explain differences in species
502 sensitivities.

503 In conclusion, this is the first lifecycle exposure of mummichog to EE₂ and
504 demonstrates that continued development of bioassay protocols in this cyclical, lunar
505 spawner of the east coast of North America can provide useful models for the study of
506 EDSs. The concentration levels at which effects occurred in the offspring were greater
507 than have been shown for freshwater species and at the same approximate level shown to
508 have effects in the mummichog adult reproductive test (>10ng/L). Of most concern in
509 this study are endpoints with the potential to affect the population structure. Chronic
510 exposure of successive generations of mummichog to EE₂ resulted in reduced spawning
511 (Peters et al., 2007), fertilization and hatching, as well as altered sex ratios and gonadal
512 development. This implies fewer fish being added to the population with each successive
513 year; coupled with altered GSIs in adult and juvenile males, problems could compound
514 with time. Severe effects of EE₂ on a population of fathead minnow have recently been
515 demonstrated in a whole-lake experiment (Kidd et al. 2007), demonstrating cause for
516 concern of environmentally-relevant EE₂ concentrations on fish populations.

517

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519

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781 **Figures**

782

783 Figure 1. (A) Mean time to hatch of mummichog eggs exposed to 0, 0.1, 1, 10, or
784 100ng/L 17 α -ethynylestradiol (EE₂). (B) Mean hatch success of mummichog eggs
785 exposed to 0, 0.1, 1, 10, or 100ng/L EE₂. (C) Mean length at hatch of mummichog larvae
786 exposed to 0, 0.1, 1, 10, or 100ng/L EE₂. Bars represent means \pm SE. Means showing
787 different letters are significantly different ($p < 0.05$). $n = 39-51$ Petri dishes per treatment.

788

789 Figure 2. Mean survival of mummichog exposed to 0, 0.1, 1, 10, or 100ng/L 17 α -
790 ethynylestradiol from hatch to 61 weeks post-hatch. $n = 3-4$ tanks per treatment.

791

792 Figure 3. Mean prevalence of vertebral abnormalities in juvenile mummichog exposed to
793 0, 0.1, 1, 10, or 100ng/L 17 α -ethynylestradiol at week 15 post-hatch. Bars represent
794 means \pm SE. Means showing different letters are significantly different ($p < 0.05$). $n = 3-4$
795 tanks per treatment.

1 Table 1: Mean ($\pm 1SE$) weight, length, liver somatic index (LSI), gonadosomatic index
 2 (GSI), and condition factor for juvenile male and female *Fundulus heteroclitus* exposed
 3 to 17 α -ethynylestradiol (EE₂) from fertilization at 61 weeks after hatch. Different letters
 4 identify significantly different treatments within variables ($p < 0.05$).

5

Sex	Treatment (ng/L)	n	Weight (g)	Length (cm)	LSI (%)	GSI (%)	Condition (%)
Male	0	26	0.85 \pm 0.06 ^a	3.76 \pm 0.08 ^a	2.35 \pm 0.16 ^a	0.86 \pm 0.12 ^a	1.57 \pm 0.07 ^a
	0.1	37	0.71 \pm 0.05 ^a	3.55 \pm 0.08 ^a	1.97 \pm 0.12 ^a	0.77 \pm 0.08 ^a	1.50 \pm 0.05 ^a
	1	51	0.85 \pm 0.05 ^a	3.70 \pm 0.07 ^a	1.98 \pm 0.13 ^a	0.78 \pm 0.07 ^a	1.60 \pm 0.04 ^a
	10	34	0.83 \pm 0.06 ^a	3.72 \pm 0.07 ^a	2.33 \pm 0.17 ^a	0.82 \pm 0.10 ^a	1.54 \pm 0.06 ^a
	100	12	0.65 \pm 0.07 ^a	3.53 \pm 0.15 ^a	2.27 \pm 0.35 ^a	0.45 \pm 0.13 ^a	1.46 \pm 0.10 ^a
	p-value			0.155	0.497	0.242	0.395
Female	0	27	0.94 \pm 0.05 ^{ab}	3.91 \pm 0.08 ^a	2.45 \pm 0.19 ^{ab}	2.78 \pm 0.77 ^a	1.56 \pm 0.05 ^{ab}
	0.1	42	0.85 \pm 0.05 ^a	3.82 \pm 0.08 ^a	2.59 \pm 0.15 ^{ac}	1.78 \pm 0.09 ^a	1.49 \pm 0.07 ^a
	1	45	0.98 \pm 0.05 ^{ab}	3.93 \pm 0.08 ^a	2.05 \pm 0.14 ^b	2.07 \pm 0.19 ^a	1.58 \pm 0.04 ^{ab}
	10	38	0.91 \pm 0.06 ^{ab}	3.83 \pm 0.07 ^a	2.85 \pm 0.10 ^a	3.62 \pm 0.79 ^a	1.57 \pm 0.04 ^{ab}
	100	47	1.11 \pm 0.06 ^b	4.04 \pm 0.07 ^a	2.24 \pm 0.11 ^{bc}	3.56 \pm 0.81 ^a	1.65 \pm 0.06 ^b
	p-value			0.025	0.371	<0.001	0.364

1 Table 2. Proportion (mean \pm 1SE) of juvenile mummichog exhibiting female secondary sex
 2 characteristics and/or female gonads at 52 weeks post-hatch after exposure to 0, 0.1, 1, 10 or
 3 100ng/L EE₂. Differing superscripts identify significantly different treatments (p<0.05). n=3-4
 4 tanks per treatment.

5

Treatment (ng/L)	N	Mean % ♀ \pm SE		
		Secondary Sex Characteristics	Gonadal Sex	Histological Sex
0	3	0.514 \pm 0.104 ^a	0.495 \pm 0.095 ^a	0.583 \pm 0.084 ^a
0.1	4	0.462 \pm 0.078 ^a	0.480 \pm 0.045 ^a	0.430 \pm 0.043 ^a
1	3	0.594 \pm 0.075 ^a	0.567 \pm 0.058 ^a	0.602 \pm 0.033 ^{ab}
10	4	0.580 \pm 0.084 ^a	0.525 \pm 0.103 ^a	0.570 \pm 0.076 ^a
100	3	0.928 \pm 0.043 ^b	0.868 \pm 0.076 ^b	0.861 \pm 0.071 ^b

6

Table 3. Developmental stages of gonadal tissue for female and male juvenile mummichog exposed to 0, 0.1, 1, 10 or 100ng/L EE₂ at 52 weeks post-hatch. Proportion (mean \pm 1SE) of fish with vitellogenic gonads (females) and spermatogenic gonads (males) is shown. Differing superscripts identify significantly different treatments ($p < 0.05$) within each gonadal stage. n=2-4 tanks per treatment.

Treatment (ng/L)	n	Females	n	Males
		Vitellogenic		Spermatogenic
0	3	0.467 \pm 0.014 ^a	3	0.590 \pm 0.117 ^a
0.1	4	0.519 \pm 0.024 ^{ab}	3	0.703 \pm 0.104 ^a
1	3	0.526 \pm 0.040 ^{bc}	3	0.515 \pm 0.039 ^a
10	4	0.565 \pm 0.008 ^d	4	0.431 \pm 0.067 ^a
100	3	0.504 \pm 0.039 ^{bc}	2	0.195 \pm 0.195 ^a

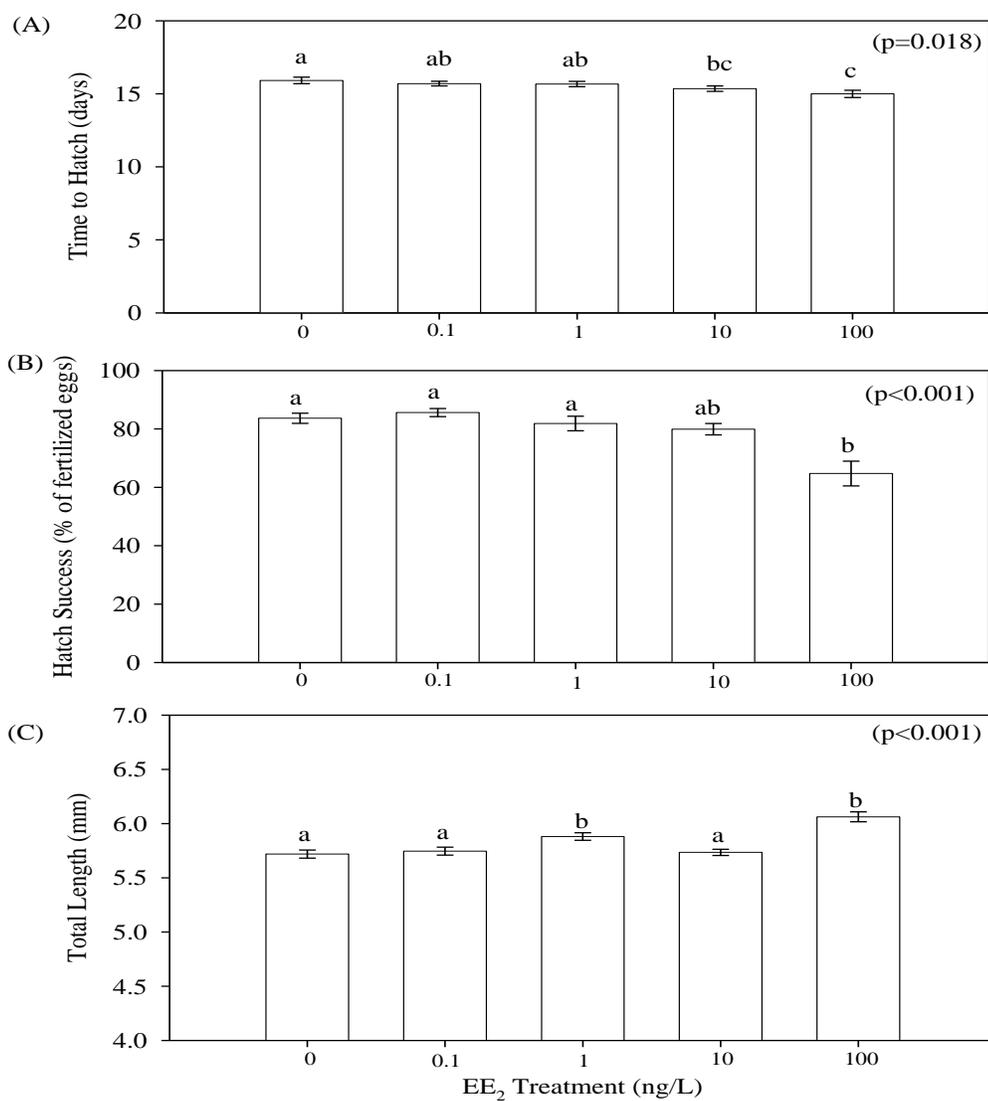


Figure 1

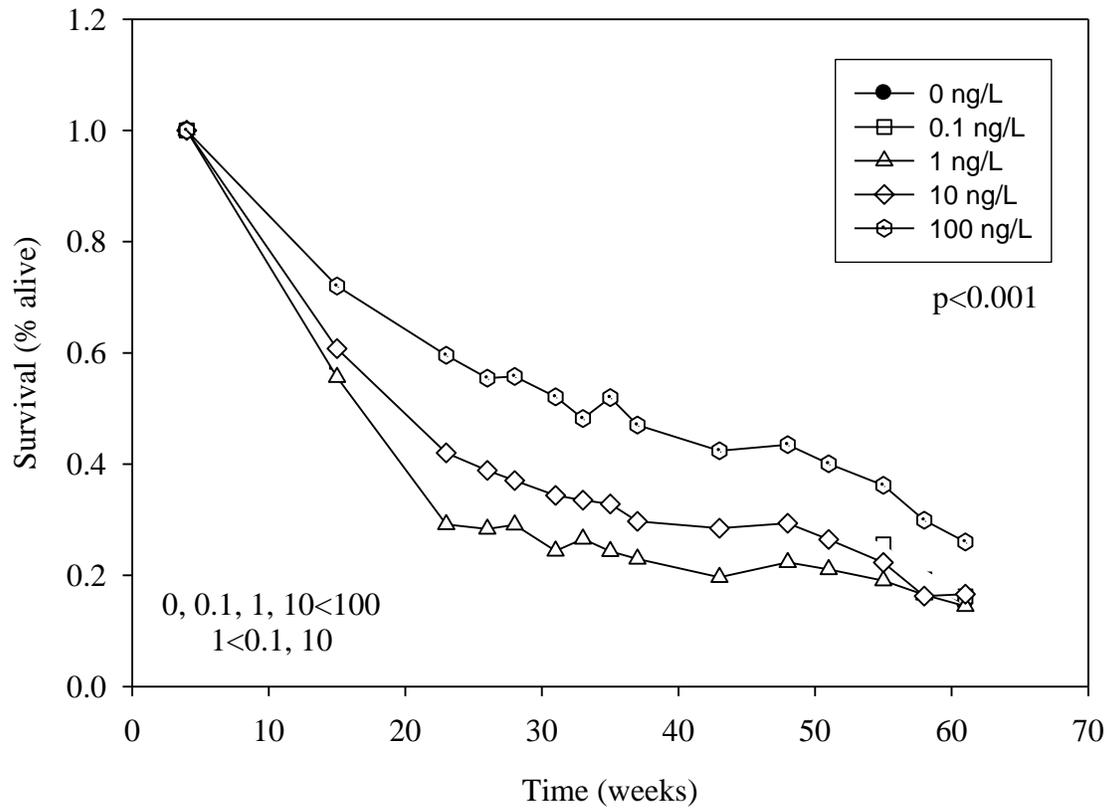


Figure 2

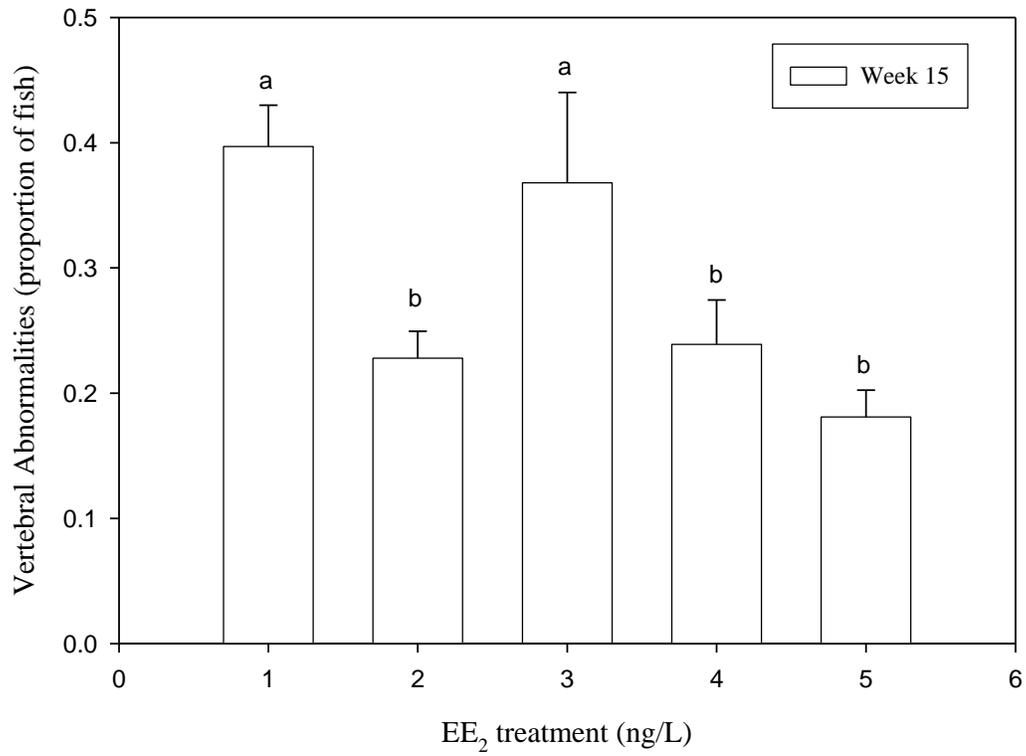


Figure 3