Comparing biological effects and potencies of estrone and 17β-estradiol in mature fathead minnows, *Pimephales promelas*

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**Abstract**

The presence of endocrine active compounds such as estrogens in treated wastewater effluent and their effects on aquatic life are causing concern among aquatic resource managers. In contrast to 17β-estradiol (E2), the steroid hormone produced by all vertebrates, the biological effects of estrone (E1) one of its breakdown products are less understood, even though the aquatic concentrations of E1 are often higher than those of E2. The central hypothesis of this study was that at environmental concentrations, E1 has estrogenic effects in fish, with increased vitellogenin concentrations and decreased reproductive success in both male and female fathead minnows, as found with E2. In two replicate experiments, we exposed mature fathead minnows to three concentrations of each estrogen for 21 days in a flow-through exposure system and measured a broad suite of anatomical (body indices, histopathology), physiological (plasma vitellogenin), behavioral (nest defense), and reproductive (fecundity, fertility, hatching) endpoints. These endpoints have previously been associated with adverse effects of estrogenic exposures. While body length and weight parameters were unaltered by exposure, secondary sex characteristics exhibited an exposure concentration-related decline in male fathead minnows. Interestingly, low concentrations of estrone (15 ng/L) enhanced the aggressiveness of male fathead minnows in a behavioral assay. Vitellogenin concentrations in male fish increased with higher concentrations of both estrogens, but remained unchanged in all female treatments. A decrease in fecundity was observed at high concentrations of E2 as compared with control minnows. These results suggest that E1, at concentrations previously found in waters receiving wastewater effluent, can have reproductive effects on fish.

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1. Introduction

Understanding the contributions of biological breakdown products of steroid hormones commonly found in aquatic environments is needed to fully assess the effects of estrogenic endocrine active compounds. Steroidal estrogens are a potent subset of this group of aquatic contaminants and can be characterized as either natural (for example, 17β-estradiol [E2] and estrone [E1]) or synthetic (for example, ethinylestradiol) based on their origin. Environmental estrogens have the ability to bind and activate estrogen receptors, thereby mimicking a normal endocrine response (Jobling and Tyler, 2003) resulting in changes to organismal homeostasis (Norris, 2007) and potentially reproductive impairment. Numerous studies have reported adverse effects when fish were exposed to steroidal estrogens. These effects include increased plasma vitellogenin (an egg yolk precursor normally found in females, Panter et al., 1998) concentrations in males, increased rates of intersex (Jobling et al., 1998), altered behavioral responses (Murphy et al., 2001; Belanger et al., 2007; Schoenfuss et al., 2008; McGee et al., 2009; Hyndman et al., 2010) and decreased reproductive success (Panter et al., 1998; Bjerselius et al., 2001; Thorpe et al., 2003; Parrott and Blunt, 2005; Thorpe et al., 2009; Paulos et al., 2010). On an ecological scale, exposure to endocrine active compounds can lead to decreased sustainability of fish populations (Kidd et al., 2007; Palace et al., 2009).

One of the most commonly studied steroidal estrogens is E2. The extensive existing literature database for this compound allows for a benchmark comparison with less understood steroid estrogens such as E1. Estradiol can be metabolized or degraded to E1, and E1 is often found at higher concentrations than E2 in the environment (Kolpin et al., 2002; Vajda et al., 2008; Writer et al., 2010). Estradiol, however, is assumed to be more potent than E1 at similar concentrations. For example, Van den Belt et al. (2004) utilized a combination of in vitro and in vivo assays to determine estrogenic potencies across a range of concentrations at or above environmental concentrations (Fig. 1), which revealed E1 to be half the potency...
of E2 for causing ovarian somatic index reduction. However, E1 and E2 were found to have equal potencies for vitellogenin induction in zebrafish (Van den Belt et al., 2004). In contrast, Panter et al. (1998) found that at equal concentrations, E2 exposure resulted in significantly greater plasma vitellogenin concentrations than a similar amount of E1. Estradiol was also more effective in reducing the gonadosomatic index compared with similar concentrations of E1. Previous comparisons of estrogenic effects on male fathead minnows (Pimephales promelas) have shown significant increases in plasma vitellogenin levels when exposed in a flow-through system to nominal concentrations (not confirmed through analytical chemistry) of ≥32 ng/L E1 (Panter et al., 1998). Significant increases in vitellogenin were not observed until >100 ng/L E2. In conjunction with raised plasma vitellogenin, rate of testicular growth decreased upon E1 exposure. Interestingly, an increase in male gonadosomatic index was also observed (Panter et al., 1998). While larval fish behavior has been affected by E1 exposure, little is known about its effects on mature fish. Larval fathead minnows exposed for 12 days in a 50% static renewal system exhibited reduced predator avoidance behavior, including a delayed latency period and a decrease in the total escape response at a concentration of 50 ng/L E1 (McGee et al., 2009). It is noteworthy, that high concentrations of E1 (307 and 781 ng/L) caused increased mortality in both male and female fathead minnows, in addition to a significant decrease in female fecundity (Thorpe et al., 2003). Specifically, the number of eggs spawned decreased by 51% (at 307 ng/L) and 66% (at 781 ng/L) due to a decrease in the number of spawning events over the 21-day exposure period. In males, gonadosomatic index decreased by 23% (307 ng/L) and 30% (781 ng/L) (Thorpe et al., 2007). A mixture of E1 and E2 (62.5 ng/L E1 and 4.4 ng/L E2) inhibited cumulative egg production by 28–33% during a 21-day exposure (Thorpe et al., 2008).

An examination of the pertinent literature revealed that previous laboratory exposure experiments assessing the biological effects of natural steroidal endocrine active compounds frequently used estrogenic concentrations that were higher than those found in the environment (Fig. 1) or examined effects of exposure only in basal organismal endpoints such as vitellogenin induction rather than in whole-organism exposure experiments. Therefore, the purpose of this study was to assess organismal effects at estrogen concentrations that were environmentally relevant and provide a continuum with previous laboratory exposures (Fig. 1). To test the hypothesis that at environmental concentrations, E1 contributes substantially to overall aquatic estrogenicity, we exposed reproducibly mature male and female fathead minnows for 21 days to either E1 or E2 and assessed exposure effects on a series of anatomical, physiological, and behavioral endpoints. The degree of potency of E1 when compared to E2 was assessed by an evaluation of gonad indices, histopathology, and sex characteristics all of which have been found to differ with exposure to estrogens (Panter et al., 1998; Shappell et al., 2010). Vitellogenin concentrations have become a standard physiological indicator to assess estrogenic effects (Jobling et al., 1998; Panter et al., 1998; Kidd et al., 2007; Thorpe et al., 2007; Shappell et al., 2010). The effects of E2 on behavior (Bayley et al., 1999; Bjersellius et al., 2001), and adverse effects on reproductive success (Bjersellius et al., 2001; Thorpe et al., 2003, 2007) have been reported previously, yet similar studies on E1 have not been conducted. The central hypothesis of this study was that at environmental concentrations, E1 has estrogenic effects in fish, with increased vitellogenin concentrations and decreased reproductive success in both male and female fathead minnows, as found with E2. This study will provide agencies responsible for protecting aquatic systems with additional information to better assess the relative endocrine disrupting potential of E1 to fish.

2. Materials and methods

2.1. Experimental design

Two replicate exposure experiments were conducted at the St. Cloud State University Aquatic Toxicology Laboratory in St. Cloud, MN using published flow-through exposure protocols (Schoenfuss et al., 2008; Hyndman et al., 2010; Shappell et al., 2010) in November and December 2010. In each exposure experiment, six-month-old mature male and female fathead minnows (n = 10/tank per treatment/sex) were separated by sex and exposed to one of three concentrations of E1 or E2 for 21 days in 16L aquaria (2 aquaria/sex/treatment). The experiment was conducted in replicate to assess the repeatability of effects at very low exposure concentrations. On day 0, prior to the exposure, a subset of 10 males and 10 females fathead minnows were dissected to provide a baseline data set for all endpoints (except reproduction). On day 22, a subset of exposed fish (10 males and 10 females randomly obtained per treatment) were sacrificed and assessed for anatomical (secondary sex characteristics, body condition factor, somatic indices) and physiological endpoints (plasma vitellogenin concentrations). The remaining fish were paired up, one male and one female from the same treatment, and randomly distributed to 7L aquaria of estrogen-free water for a 12-day observation of female fecundity and male aggression. Three behavioral assays were conducted (days 3, 5 and 9) to assess aggression of male fish toward an imitation fish (described below). Results from the three assays were combined for statistical analysis.

2.2. Exposure chemicals

Stock solutions of E1 and E2 in 100% ethanol were prepared at a concentration of 72 µg/L. Daily solutions for dosing were prepared from these stocks, brought up to a 2 mL final volume with 100% ethanol (ensuring equivalent carrier concentrations of 0.0001% (v/v) in all treatments and controls), and stored at 4°C until use.

2.3. Exposure organisms

Sexually mature male and female fathead minnows were obtained from a laboratory fish supplier (Environmental Consulting and Testing, Superior, WI). Fish were maintained following
US EPA guidelines (Denny, 1987) throughout the experiments at constant environmental conditions (16 h:8 h light:dark, 21 °C water temperature; 4.5 mg/L dissolved oxygen; circum neutral pH, all monitored daily). Fish were fed frozen brine shrimp (Artemia franciscana, San Francisco Bay Brand, Inc, Newark, CA) as well as frozen blood worms (Glyceria spp., Hikari Sales, Hayward, CA) once daily ad libitum. Animal use and care in all experiments was approved by the St. Cloud State University Animal Care and Use Committee (IACUC). Tanks were monitored daily for mortality.

2.4. Analysis

2.4.1. Water quality

Chemical concentration and purity of E1 and E2 stock solutions were analyzed by LC–MSMS prior to the exposure experiments. Duplicate water samples were collected on days 3, 5, 10, 15 and 20 of the exposure period from the outflow of the stainless steel mixing chambers where ground water and the treatment-specific E1 and E2 concentrations were mixed prior to delivery to the aquaria. Water samples in HDPE bottles were frozen until estrogen analysis was performed essentially as previously described (Shappell et al., 2010). Samples were extracted and analyzed by set, which consisted of all samples from that day of exposure (all treatments, including solvent control) from both experiments, plus one fresh nanopure water sample. Briefly, 100 mL of water sample was fortified with 3 ng of δ4 estrone and δ4 17β-estradiol in 30 μL of EtOH, mixed, extracted on an Oasis HLB SPE column (Waters Corp, Milford, MA), and re-suspended in 150 μL 1:1 acetonitrile:water for LC–MSMS analysis as previously described (Shappell et al., 2008). Parent and three fragment ions were monitored in the ESI mode using a quadrupole-time-of-flight mass spectrometer (Waters, Beverly, MA). Sample sets from each experimental day were run in triplicate (20 μL injection volume) bracketed between runs of standard curves. Concentrations were determined using standard curves (9 points from 0.5 to 200 pg/μL for both compounds) quantitation based on 0–50 pg/μL standards for all but day 5 E1 treatments, when the curve was extended to 200 pg/μL. Final concentrations were internal standard corrected (20 pg/μL).

2.4.2. Biological endpoints

2.4.2.1. Plasma vitellogenin analysis. After fish were anesthetized in 0.1% MS-222 (Argent Laboratories, Redmond, WA), blood was harvested via tail severance in heparinized capillary tubes and centrifuged at (5000 × g for 5 min at 20 °C). Plasma vitellogenin was measured using a competitive antibody-capture ELISA following Parks et al. (1999). Standard vitellogenin was purified by anion-exchange chromatography (Parks et al., 1999) from estradiol-exposed fathead minnows. Primary antibody was rabbit polyclonal anti-vitellogenin antiserum. The standard curve was prepared as an eight-step two-fold serial dilution with a range of 5.0–0.039 μg/mL. Microtiter wells were coated with purified fathead minnow vitellogenin (200 μL of 250 ng/mL) in a coating buffer of 0.35 M sodium bicarbonate, 0.15 M sodium carbonate, pH 9.6. Plasma samples/standards were pre-incubated at a ratio of 1:1 sample dilution to 1/ Ab (1:20,000 final dilution) at 25 °C for 2 h. Plate incubation times were 1 h at 25 °C with wash cycles of 3 × 300 μL using an automated plate washer. Secondary antibody was horseradish peroxidase labeled anti-rabbit IgG (200 μL/well) (Sigma, St. Louis, USA). Tetramethylbenzidine (Sigma, St. Louis, USA) was used as substrate (200 μL/well). Absorbance was read at 620 nm on a Multiskan EX (Thermo Fisher, Waltham, MA) and sample values were calculated using the accompanying Multiskan Ascent software.

2.4.2.2. Organosomatic indices. Whole body weights were measured for each fish (0.01 g precision, Acculab Vicon, Edgewood, NY). Gonads and livers from each fish were excised and immediately weighed (0.001 g precision, Mettler Toledo AG245, Columbus, OH). Liver and whole body weights were used to calculate the hepatosomatic index = (liver weight/whole body weight × 100). Gonad and whole body weights were used to calculate the gonadosomatic index = (gonad weight/whole body weight × 100). Body weight and total length were used to calculate the body condition factor = (body weight/total length3) × 100,000 (Fulton, 1904).

2.4.2.3. Secondary sex characteristics. Prior to organ excision, secondary sex characteristics of the male fish were evaluated. This evaluation used a simple, blind scoring system modified after Smith (1978). The prominence of the tubercles was scored on a scale of 0–3 with 0 indicating no expression and 3 prominent expression of this secondary sex characteristic. The dorsal pad and color/banding intensity were evaluated by a similar method and scored on a scale of 0–3. For statistical analysis, the sum of all three secondary sex characteristics was calculated and compared between treatments.

2.4.2.4. Histopathology. Histological protocols have been described in detail elsewhere (Hyndman et al., 2010; Shappell et al., 2010). Briefly, liver and gonads were fixed in 10% neutral buffered formalin for 24 h (Gabe, 1976) and then were dehydrated in a series of ethanol and xylene baths before being embedded in paraffin. Embedded tissues were sectioned at approximately 1/3 and 2/3 of the depth of the gonads (resulting in tissues slices ~100 μm apart) using a Reichert-Jung cassette microtome (4 μm sections). Sectioned tissues were stained using a standard haematoxylin and eosin counter stain protocol modified after Gabe (1976) and Carson (1997). Histological sections were ranked on semi-quantitative scales (0–4) for vacuolization of the liver (0, no vacuoles visible; 1, <5% of total area; 2, vacuoles small but throughout image <25% of area; 3, broad presence of large vacuoles 25–50% of area; 4, >50% of area vacuolated) and the presence/absence of eosin staining/proteinaceous fluid. The developmental stage of the gonad (testis or ovary) and the proliferation of interstitial cells between seminiferous tubules of the testis substructure were also evaluated histologically. Random slides (10%) were ranked for a second time to determine between analysis variance, which was found to be less than 1%.

2.4.3. Egg count and aggression assays – in absence of E1/E2

On day 22, the remaining male fish not used for anatomical assessment (10 per treatment) were individually paired with females from the same treatment in 7 L aquaria in estrogen-free water. As a result of mortality, the number of available males per treatment ranged from 5 to 9 of the original 10. Each aquarium contained a nest site made of a short section of 8 cm diameter PVC pipe cut in half. These nest sites were monitored for eggs daily for 11 days. Nest sites found to contain eggs during the daily inspection were replaced with new nest substrate. Nest sites containing eggs were placed into keepers with water from the appropriate tank and a low flow of air. Three days following egg production, fertilization success was measured by counting eggs with eye spots. Five to eight days following egg production, hatching success was measured (number of larvae hatched).

Behavioral aggression assays were performed on the 3rd, 5th, and 9th day following the pairings to assess aggression of the male toward an imitation fish. During the assay, the male fish was observed for aggressive behavior toward the fish mimic in efforts to protect the nest site, including butting with head or tubercles (Unger, 1983). The fish mimic was mounted on a short flexible plastic tube, which in turn was centered on a dowel. The dowel was placed into a groove in each aquarium to ensure that the fish mimic would be placed in the same position and at the same height above the aquarium floor directly in front of the nest site, which was
also controlled in placement. The introduction of the fish mimic was quick with the observer out of sight of the fish and minimal disturbance of the aquarium. Both latency period before the first attack (count started immediately upon introduction of the fish mimic) and number of attacks in the first 60 s after the encounter was initiated were used as endpoints in this assay. If the male fish failed to attack the fish mimic within 5 min of its introduction to the tank, the male fish was assigned a latency of 300 s. The totality of the aggressive behavior was described as the Total Aggression Index and calculated by dividing the number of attacks (multiplied by a factor of 10 to equally weigh both endpoints) by the latency of the response to the fish mimic (seconds to first contact with dummy fish). A higher index score is indicative of a more pronounced response to the introduction of the fish mimic. The sum of observations on days 3, 5 and 9 were used for the statistical analysis.

2.5. Statistical analysis

The assumption of normality for all data sets was tested with the Kolmogorov–Smirnov test for normality prior to any additional analysis (Prism 5.0 statistical package, GraphPad Software Inc., Oxnard, CA). As the majority of data did not meet standards of homogeneity, data were analyzed using a Kruskal–Wallis analysis followed by a Dunn’s post-test. Aquarium effects were tested for and not found, consequently data from both aquaria containing the same fish sex in each treatment were combined for subsequent statistical analysis. To assess concentration-dependent effects of increasing concentrations of E1 and/or E2, data were further analyzed using the rank-based Jonckheere–Terpstra test (SAS 9.1). A probability of p < 0.05 was set as level of significance for all comparisons.

3. Results

3.1. Fish survival and chemical measurements

Fish survival was excellent at >93% across both experiments (lowest survival rate was 87.5% for E1-M in experiment 1 and E2-M in experiment 2). Environmental conditions remained stable throughout and across both experiments (experiment 1: temperature (mean ± S.D.) = 22.3 ± 0.2 °C; pH = 8.1 ± 0.3; dissolved oxygen = 5.4 ± 1.3 mg/L; hardness as CaCO₃ = 403 ± 65 mg/L; conductivity = 0.96 ± 0.02; experiment 2: temperature = 20.8 ± 0.5 °C; pH = 7.7 ± 0.2; dissolved oxygen = 4.7 ± 0.8 mg/L; hardness as CaCO₃ = 428 ± 0 mg/L; conductivity = 0.95 ± 0.02) and are reflective of rearing conditions described by Denny (1987) and for environmental conditions for fathead minnows during the reproductive season.

In experiment 1, concentrations of E1 and E2 met the goal to establish exposure scenarios with increasing concentrations of the compounds across treatments (Table 1), with final concentrations lower than target concentrations for the higher concentration treatments of E1 and E2. In experiment 2, concentrations varied more (both between samples and across treatments) than in experiment 1 with the variability attributed to one of the peristaltic pumps needing adjustment during several daily runs. As a result, the concentration gradient for E1 was not established and mean concentrations in the E1-M treatment were below the E1-L concentrations in experiment 2. As a consequence of pump issues and the greater variability of concentrations in experiment 2, the following discussion of the results will focus on experiment 1 and will use experiment 2 for comparison.

3.2. Physiological and anatomical endpoints

Plasma vitellogenin concentrations for male fathead minnows exposed to E1-M in experiment 1 and E1-H in experiment 2 were significantly different from control (Fig. 2). Mean vitellogenin in E2-M fish was also elevated in the first exposure, however, concentrations did not reach statistical significance. In contrast to male fish where most treatments tended to result in increase in mean plasma vitellogenin concentrations, vitellogenin concentrations for female fathead minnows did not differ statistically between treatments after the 21-day exposure to estrogens. However, a step-wise decrease in mean plasma vitellogenin concentrations of females was noticeable for E1 in both experiments, albeit not reaching the level of statistical significance.

There was no significant effect on the body condition factor (p > 0.05; Supplementary Fig. S1) while in experiment 1 the gonadosomatic index of fathead minnows of either sex exposed to E1 and in males exposed to E2 exhibited a strong concentration-dependent decline (from low to high treatments) in experiment 1 (Fig. 3). This decline was concomitant with a trend for decreased secondary sex characteristics expression in male fathead minnows in both experiments (Fig. 4). However, the decline in the relative size of the reproductive organ and of secondary sex characteristics in male fathead minnows was not accompanied by a change in the maturity of these organs as the developmental stage of testes (and ovaries) in both experiments did not differ between treatments (Supplementary Fig. S2).

A different pattern of effects emerged in the liver, where the relative size of the organ (hepatosomatic index) exhibited considerable variability among fish across treatments and was not altered statistically between treatments (Supplementary Fig. S3). In contrast, the severity of liver vacuolation differed statistically between treatments in female fathead minnows (Fig. 5; Supplementary Fig. S4). However, in experiment 1, a Dunn’s post-test was unable to resolve statistical differences observed in the Kruskal–Wallis non-parametric analysis, and in experiment 2, significant differences were only observed against baseline fish dissected prior to the exposure. Liver vacuolization was generally greater in male fish than in females of the same treatment; however, these differences may be related to the greater energetic needs of ovulating female fathead minnows, which also produce more vitellogenin in the liver than their male counterparts.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Concentrations (mean and standard error of five measurements) of estrone (E1) and 17β-estradiol (E2) concentrations in two replicate 21-day exposure experiments with mature fathead minnows.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (abbreviation)</td>
<td>Nominal concentration (ng/L)</td>
</tr>
<tr>
<td>Ethanol control (EtOH)</td>
<td>0</td>
</tr>
<tr>
<td>Estrone low (E1-L)</td>
<td>10</td>
</tr>
<tr>
<td>Estrone medium (E1-M)</td>
<td>50</td>
</tr>
<tr>
<td>Estrone high (E1-H)</td>
<td>100</td>
</tr>
<tr>
<td>Estradiol low (E2-L)</td>
<td>5</td>
</tr>
<tr>
<td>Estradiol medium (E2-M)</td>
<td>25</td>
</tr>
<tr>
<td>Estradiol high (E2-H)</td>
<td>50</td>
</tr>
</tbody>
</table>

No estrone detections.
3.3. Nest guarding behavior and reproductive success

Nest guarding behavior was more pronounced in the second experiment with control males exhibiting more aggressive nest defense as indicated by the higher Total Aggression Index. It is unclear why overall nest defense was more pronounced in experiment 2. The lowest measured concentrations of E1 in both experiments (experiment 1: E1-L statistically different; experiment 2: E1-M non-statistical increase) elicited a strong nest guarding response in male fathead minnows previously exposed to E1 (Fig. 6). Males from these treatments displayed a high degree of aggression as indicated by the significantly elevated Total Aggression Index. Higher concentrations of E1 and E2 seemed to negate these effects (Fig. 6).

Reproductive success was assessed by analyzing mean egg production per female, % fertilization, and % hatching success (Table 2). E1-M (nominal concentration of 50 ng/L) exposed females had the highest egg production in the first experiment, whereas, E1-L (nominal concentration of 10 ng/L) exposed females produced the greatest amount of eggs in experiment 2. In general, egg production was stimulated at lower concentrations of estrogens, and inhibited by higher concentrations of estrogens, and possibly delayed. The mean number of eggs produced per female in each treatment drove reproductive success as fertilization rates (Table 2) and hatching success did not alter reproductive success as much as the mean number of eggs produced by females in each treatment. However, fertilization success was conspicuously low in E2-H in experiment 1 and E1-H and E2-M in experiment 2.

4. Discussion

An increase in plasma vitellogenin for male fathead minnows upon exposure to estrogens is consistent with previous studies (Panter et al., 1998; Thorpe et al., 2007; Sowers et al., 2009; Shappell et al., 2010). Previously, our lab exposed fathead minnows to E2
Fig. 3. Gonadosomatic index for male and female fathead minnows exposed in a flow-through exposure system to one of three concentrations of estrone or 17β-estradiol in two replicate exposure experiments (see Fig. 2 for treatments and abbreviations). Columns represent means, bars indicate standard error. Sample size listed within columns. For explanation of variable sample size see Fig. 2. While ANOVA found no differences between treatments for either sex and experiment (p > 0.05), arrows with asterisk indicate significant concentration-dependent responses in relative gonad size by the rank-based Jonckheere–Terpstra test (p < 0.05).

Fig. 4. Sum of secondary sex characteristics for male fathead minnows (tubercle and dorsal pad size and color/banding intensity) exposed in a flow-through exposure system to one of three concentrations of estrone or 17β-estradiol in two replicate exposure experiments (see Fig. 2 for treatments and abbreviations). Columns represent means, bars indicate standard error. Sample size listed within columns. For explanation of variable sample size see Fig. 2. Letters indicate statistically significant differences (p < 0.05) obtained through Kruskal–Wallis analysis with Dunn’s post-test. Arrows with asterisk indicate significant concentration-dependent declines in secondary sex characteristics by the rank-based Jonckheere–Terpstra test (*p < 0.05; **p < 0.01).
and its isomer 17α-estradiol, which produced a significant increase in male plasma vitellogenin concentrations when compared with control fish but did not result in differences in female vitellogenin levels (Shappell et al., 2010). These results are consistent with the findings of the current study. Although no significant differences were observed in female vitellogenin induction, noteworthy is the dose-dependent decrease of vitellogenin induction with exposure to E1 (Fig. 2). When considering potency, E2 did not seem to have a stronger effect on vitellogenin concentrations than E1; indeed, no plateau effect in which male plasma vitellogenin concentrations reach mean female concentrations (Hyndman et al., 2010) was observed in the current study (Fig. 2). Previous studies have

<table>
<thead>
<tr>
<th>Table 2</th>
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<tr>
<td><strong>Reproductive success in fathead minnow breeding pairs previously exposed to estrogens.</strong> Percentage of fertilized eggs and percentage of eggs hatched compared to number of eggs produced for each treatment in experiments 1 and 2.</td>
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</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th></th>
<th></th>
<th>Experiment 2</th>
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<tr>
<td></td>
<td># of eggs/female</td>
<td>% Fertilized</td>
<td>% Hatched</td>
<td># of eggs/female</td>
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<tr>
<td>Control</td>
<td>72</td>
<td>73.5</td>
<td>42.2</td>
<td>205</td>
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<tr>
<td>E1-L</td>
<td>114</td>
<td>63.0</td>
<td>85.2</td>
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</tr>
<tr>
<td>E1-M</td>
<td>244</td>
<td>84.9</td>
<td>59.4</td>
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</tr>
<tr>
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<td>37.3</td>
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<tr>
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<td>57</td>
<td>33.3</td>
<td>36.8</td>
<td>56</td>
</tr>
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</table>
shown E1 to be less potent than E2 (Panter et al., 1998), while the current study approximates more closely findings of Van den Belt et al. (2004), which indicated that E1 and E2 were similar in potencies with respect to vitellogenin induction. It is noteworthy in this context to highlight the measured concentrations of both estrogens in this study, which are at or below, often substantially, concentrations used in previous laboratory exposure studies with teleost fishes.

The morphological and histological indices in this study were also assessed as indicators of estrogenic potency. Among the morphological indices, body condition factor and hepatosomatic index, did not differ significantly between treatments. In contrast, concentration-dependent responses in the gonadosomatic index were observed in the first experiment in male fathead minnows exposed to E1 (high) or E2 (medium and high) and in female exposed to E1 (Fig. 3). A decrease in both gonadosomatic index and secondary sex characteristics in male fathead minnows is consistent with feminizing effects found in previous studies (Shappek et al., 2010; Vajda et al., 2011). Previous studies used high concentrations (non-environmentally relevant) of estrogens to accomplish similar declines in the gonadosomatic index (>100 ng/L, E2, Panter et al., 1998; Metcalfe et al., 2001). Assessment of histological endpoints on reproductive organs (gonad stage) revealed no differences between treatments. The lack of difference in gonadal development and incidence of intersex was inconsistent with previous literature (Kidd et al., 2007; Woodling et al., 2006; Vajda et al., 2008; Jobling et al., 1998) and length of exposures is not likely to explain the results as several studies have noted reduction in gonadal development in as little as 14 days during a field exposure of fathead minnows to treated wastewater effluent (i.e., Vajda et al., 2011). Thus, the concentrations of estrogens in this study may have been below a threshold level required to cause such effects. In contrast, liver vacuolization differed statistically among females in both experiments, but without a clear directionality making this endpoint less useful for study. As a result, little information regarding the potency of E1 and E2 could be gleaned from morphological and histological endpoints for this 21-day exposure study. While these indices may be appropriate when using higher estrogenic concentrations, and in life cycle exposures (Kidd et al., 2007; Sowers et al., 2009), they may not be pertinent in short-term (<21-day) exposures.

Secondary sex characteristics of male fathead minnows were a valuable indicator of anatomical effects of estrogenic exposure and the decline in the expression of these markers followed closely the decline in the gonadosomatic index. Exposures to high concentrations of E2 have been previously shown to decrease secondary sex characteristics (Hyndman et al., 2010; Shappek et al., 2010). Again, with respect to secondary sex characteristics, these results indicate E1 and E2 seem to have somewhat similar potencies at low concentrations.

When subject to a behavioral challenge, male fathead minnows increased aggression with exposure to low concentrations of estrogens (Fig. 6). The higher concentrations did not elicit a difference in aggression compared with control fish. This may be due to the higher concentrations of plasma vitellogenin and a decrease in secondary sex characteristics at high concentrations, which are both indicators of feminization (Smith, 1978). The increase in aggression in males exposed to low levels of estrogens may be explained by the artificial separation of male and female minnows in captivity. Female fish naturally release steroid hormones at the time of ovulation as a cue to male fathead minnows, which, in turn will dramatically increase sperm production and aggressive behavior. This effect may be simulated by the exposure to low concentrations of estrogens, which may mimic the presence of a female. Previous studies (for example, Schoenfuss et al., 2008) have documented similar stimulatory effects of low concentrations of other estrogenic endocrine active compounds. Interestingly, the enhanced aggressiveness of male fathead minnows exposed to the E2-L treatment was only observed in experiment 2 but not in experiment 1. Although examining the mode of action of these effects was beyond the scope of this study, subtle differences in exposures or reproductive conditions of fish in the two experiments may have led to the discrepancies in the aggressive response of male fish in the E2-L treatment in experiment 2. The observed differences in response between the replicate experiments may hint at the lower limits of sensitivity of this behavioral endpoint.

**Fig. 6.** Behavioral findings. Sum of mean Total Aggression Index and standard error for male fish as assessed on days 3, 5 and 9 of depuration period following the treatment-specific exposures. A higher index score is indicative of a more aggressive nest defending response. See Section 2.4 for description of calculation of aggression index. p-values derived from ANOVA; asterisks indicate significant differences between treatments (Tukey’s post-test) and control “p < 0.05; **p < 0.01.”
The reproductive activity of female fathead minnows was inhibited with high concentrations of estrogens and possibly delayed, yet stimulated with low concentrations of estrogens (Table 2). Egg production was most directly affected by estrogen exposure. High concentrations of E1 (100 ng/L) and E2 (50 ng/L) were shown to decrease egg production in female fathead minnows. Thorpe et al. (2007) conducted a reproductive assessment using E1, and found inhibition of egg production at ≥307 ng/L. Our study suggests that concentration as low as ~50 ng/L E1 and ~25 ng/L E2 can diminish egg production. Interestingly, fertilization rates (number of eggs that developed eye spots) and hatching success (number of eggs with eye spots from which larvae hatched) did not markedly alter overall reproductive success that was driven by the initial number of eggs deposited by each female in a treatment. These findings are novel and suggest that the exposure effect on reproductive success is driven by the exposure of females as any effect on the male should have decreased fertilization success among treatments. Similarly, the differing reproductive outcomes were also not driven by the quality of the developing embryos (which were not exposed as exposure ceased prior to the reproductive component of the study) as hatching success was not different among treatments. However, if the hatched larvae were exposed to estrogenic compounds (similar to the parent generation), effects may have been elicited later in life (Sowers et al., 2009).

Estrone and E2 were both found to increase vitellogenin in males, but not in females. Yet these estrogens appeared to impair reproductive success more through rearrangement in the female’s capacity to produce eggs, than through the male’s inability to fertilize. These data indicate relatively low concentrations of E1 can have reproductive consequences in fish. Such E1 concentrations have typically been reported in impacted surface waters, where little to no E2 was detected, yet relatively little biological significance was assumed. Based on the multitude of endpoints affected by E1 exposure in this study, the overall potency of E1 at environmentally relevant concentrations appears to approximate that of E2. In the past, attention has been focused almost exclusively on the presence or absence of E2. These results should change that practice.

Disclosure

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2011.08.011.

References


