Comparative biological effects and potency of 17α- and 17β-estradiol in fathead minnows

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A B S T R A C T

17β-Estradiol is the most potent natural estrogen commonly found in anthropogenically altered environments and has been the focus of many toxicological laboratory studies. However, fewer aquatic toxicological data on the effects of 17α-estradiol, a diastereoisomer of 17β-estradiol, exists in the literature even though it has been found in the aquatic environment, sometimes at higher concentrations than 17β-estradiol. The central objective of this study was to determine how the anatomical, physiological, and behavioral effects of exposure to 17α-estradiol compare to the well-documented effects of 17β-estradiol exposures in aquatic vertebrates. A 21-day flow-through exposure of mature male and female fathead minnows to three concentrations each of 17α- and 17β-estradiol (averaged measured concentrations 27, 72, and 150 ng/L for 17α-estradiol, and 9, 20, and 44 ng/L for β-estradiol, respectively) yielded significant, concentration-dependent differences in plasma vitellogenin concentrations among estradiol-exposed males when compared to fish from an ethanol carrier control. Interstitial cell prominence in the testis of fish was elevated in all estradiol treatments. Aggressiveness of male fish to defend nest sites appeared depressed in many of the higher concentration estradiol treatments (albeit not significantly). No clear effects were observed in female fish. Based on plasma vitellogenin data, it appears that 17β-estradiol is 8–9 times more potent than 17α-estradiol and that the lowest observable effect concentration (LOEC) for 17α-estradiol in fathead minnows is greater than 25 ng/L and may be less than 75 ng/L.

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1. Introduction

The presence of endocrine active compounds (EACs) in aquatic environments has been a growing concern due to evidence indicating disruption of normal reproductive function in male fish. Studies have shown increased vitellogenin production in males (an egg yolk precursor protein normally found in female fish, Hemmer et al., 2002), impaired spermatogenesis (Jobling et al., 1998), disrupted gonadal morphology (Purdom et al., 1994; Harries et al., 1997; Jobling et al., 1998), and impaired reproductive behavior (Bayley et al., 1999; Bjerselius et al., 2001; Schoenfuss et al., 2002) in fishes exposed to EACs. Most dramatically, the exposure of an entire lake in Canada for three summers to the synthetic estrogen ethinylestradiol resulted in the collapse of the resident fathead minnow population (Kidd et al., 2007).

One of the most common chemicals used in EAC exposure studies is 17β-estradiol. This is due in large part because there is a large database on 17β-estradiol, it is potent and invokes biological responses in fish, and it can be easily and inexpensively assayed for by enzyme-linked immunosorbent assay (ELISA). However, little aquatic toxicological data on the effects of 17α-estradiol, a stereoisomer of 17β-estradiol, exists in the literature even though it has been found in the aquatic environment, sometimes at higher concentrations than 17β-estradiol (Velicu and Suri, 2009). The α isomer has been found in beef, dairy and swine waste (Gadd et al., 2010; Zheng et al., 2008; Shappell et al., 2010; Hutchins et al., 2007), posing a potential risk to surface waters and its biota. A cursory review of mammalian toxicological literature found few studies assessing the effects of 17α-estradiol. One study on neonatal mice showed that the effects of 17α-estradiol (vaginal cornification, hypospadia, vaginal concretions, and hyperproliferation) were similar to effects seen in mice treated with 17β-estradiol (Hajek et al., 1997). No studies could be found that investigated the effects of 17α-estradiol on fish.

In order to fully assess the potency of 17α-estradiol and its ability to elicit endocrine disruption in an aquatic environment, a range of anatomical, physiological, and behavioral endpoints were

Abbreviations: E2, estradiol; 17α-, 17α-estradiol; 17β-, 17β-estradiol; Ab, antibody; SSSc, secondary sex characteristics.
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assessed. Previously, field and laboratory EAC exposures using fish have reported changes in various anatomical endpoints including secondary sex characteristics, organ indices, and histopathology (Jobling et al., 1998; Purdom et al., 1994; Harries et al., 1997). Measurement of plasma vitellogenin concentrations in male fish has become a standard physiological endpoint in assessment of acute exposure to estrogenic compounds. Behavioral endpoints have often been found to be most sensitive to EACs effects, with several studies demonstrating impaired reproductive behaviors following exposure to EACs (Bayley et al., 1999; Bjerselius et al., 2001; Schoenfuss et al., 2002; Martinovic et al., 2008).

The central objective of this study was to determine how the anatomical, physiological, and behavioral effects of exposure to 17α-estradiol compare to the well-documented effects of 17β-estradiol exposures in aquatic vertebrates. This comparison will provide much needed information about the contribution of 17α-estradiol to the overall estrogenicity of aquatic environments impacted by anthropogenic activities.

2. Materials and methods

2.1. Experimental design

Randomized complete block design exposure of fathead minnows to 17α- and 17β-estradiol exposure was conducted at the St. Cloud State University Aquatic Toxicology Laboratory in St. Cloud, Minnesota following established and published flow-through exposure protocols (Schoenfuss et al., 2008; Hyndman et al., 2010). Mature male and female fathead minnows (separated by sex based on secondary sex characteristics, replicated in two tanks, n = 10/tank/treatment/sex) were exposed to three concentrations of the two chemicals for 21 days in 16-L aquaria. Immediately following the exposures, a subset of fish from each treatment (10 males and 10 females randomly netted from all aquaria within one treatment) were sacrificed and assessed for anatomical and physiological endpoints. The remaining fish were paired up, one male and one female from the same treatment, placed in a 7-L aquarium, and observed over a 13-day depuration period, to document female fecundity and male aggression. Two behavioral assays were performed on the 3rd and 12th days to assess aggression of the male toward an imitation fish. On the 13th day, the remaining fish were sacrificed and analyzed for plasma vitellogenin concentrations, organosomatic indices, and secondary sex characteristics to determine whether the 13-day depuration period altered any biomarkers of estradiol exposure when compared to fish in the same treatment analyzed immediately following the 21-day exposure.

2.2. Exposure chemicals

Stock solutions of 17α- and 17β-estradiol (α-Steraloids, Newport, RI; β-Sigma, St. Louis, MO) in 100% ethanol were prepared at concentrations of 0.432 mg/mL and 0.072 mg/mL, respectively. Daily solutions for dosing were prepared from these stocks, brought up to a 2 mL final volume with 100% ethanol (ensuring equivalent carrier concentrations in all treatments and controls), and stored at 4 °C until use. A low (1×), medium (3× for α, 2.5× for β), and high concentration (6× for α, 5× for β) of each isomer was used to allow for the comparison of biological effects resulting from 17α- and 17β-estradiol exposures. 17β-Estradiol concentrations were chosen to be in the range of those reported in runoff post-poultry litter application (Finlay-Moore et al., 2006), but below concentrations previously found to result in toxicity in fathead minnows (unpublished data). 17α-estradiol concentrations were based on the range reported by Zheng et al., 2008 for dairy wastewater, and in vitro cellular assay (E-Screen) activity that indicated the α isomer to be ~1/100th as active as the β isomer. An ethanol solvent control (Control) was also included.

For all treatments, fresh aqueous exposure solutions were prepared daily by mixing a spike of the appropriate concentrated stock solution in 10 L of deionized water in an amber glass bottle. Solvent concentrations did not exceed 1.8 µL ethanol/L, well below solvent concentrations used in previous experiments, where no effect had been found (Schoenfuss et al., 2002; Bistodeau et al., 2006; Barber et al., 2007). After spike addition, each amber bottle was gently agitated for 10 s, and the neck of the bottle was covered with aluminum foil. A stainless steel tube was used to draw the daily exposure solution into a stainless steel mixing chamber located above the treatment aquaria at a nominal rate of 0.008 L/min using a Cole-Palmer Masterflex 7523–40 peristaltic pump (Vernon Hills, IL). In the stainless steel mixing chamber, ground water from a dedicated well was added to the continuous flow of the daily exposure solution to achieve the final aquarium concentration of estradiol. This solution was then gravity fed to the treatment aquaria at a rate of 0.2 L/min/aquarium. The flow rate resulted in approximately 18 water exchanges each day.

2.3. Exposure organisms

Six-month-old sexually mature male and female fathead minnows were obtained from a laboratory fish supplier (Environmental Testing and Consulting, Superior, WI). Fish were maintained following US EPA guidelines (Denny, 1987) throughout the experiments at constant environmental conditions (16:8 h light:dark, 21 °C water temperature; for detailed environmental information see Supplement Table 1) and fed frozen brine shrimp (Artemia franciscana, San Francisco Bay Brand Inc., Newark, CA) twice daily ad libitum. Animal use and care in all experiments was approved by the St. Cloud State University Animal Use and Care Committee (IACUC). Tanks were monitored daily for mortality.

2.4. Analysis

2.4.1. Water quality

Water samples were collected on days 5, 10, 13, and 20 of the exposure period from the outflow of the stainless steel mixing chambers where ground water and the treatment specific estradiol concentrations were mixed prior to delivery to the aquarium. Water samples were stored frozen. In addition to the solvent control samples, a nano-pure water sample was also included for each day of analyses. Due to the hardness of the source water, samples required extraction using SPE. A mixture of deuterated standards (2 ng each of d4 estrone, d4 17β-estradiol, and d2 estriol in 20 µL EtOH) was added to 45 mL of the sample water and mixed prior to loading on an OASIS HLB SPE column (3cc, 60 mg, Waters Corp., Milford, MA). Internal standards for estrone and estriol were included to quantitate any possible commercial contamination or experimental degradation of estradiol in water samples. Solid phase extraction was performed as described by Waters (2002). Briefly the column was conditioned with acetone, methanol, and water prior to loading sample; then post-loading washed with water, 40% MeOH/water, 10% MeOH/2% ammonium hydroxide; and sample eluted with 100% MeOH. Eluent was taken to dryness under N2 and resuspended in 100 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 duplicate (20 µL injection volume) bracketed between runs of standard curves. Concentrations were
determined using standard curves (0–20 pg/μL for 17β-estradiol, 0–100 pg/μL for 17α-estradiol) with internal standard correction (20 pg/μL).

2.4.2. Biological endpoints

2.4.2.1. Plasma vitellogenin analysis. After fish were deeply anesthetized in 0.1% MS-222 (Argent Laboratories, Redmond, WA), fish tails were severed to harvest blood using a heparinized capillary tube. Blood was immediately centrifuged to isolate plasma (5900 × g for 5 min at 20 °C), and the plasma was stored at −80 °C until analysis. Plasma vitellogenin levels were measured via a competitive antibody-capture ELISA. The polyclonal anti-fathead minnow vitellogenin antiserum (1:20,000 final dilution) at 25 °C with 200 ng/mL to 37 mg/mL. Microtiter wells were coated with the purified fathead minnow vitellogenin (200 μL of 600 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 in microcentrifuge tubes at a ratio of 1:1 sample dilution to 1 Ab (1:20,000 final dilution) at 25 °C for 2 h. Just prior to the completion of the pre-incubation, microtiter plates were washed three times with wash buffer in an automated plate washer, then 200 μL of each pre-incubation mix (1 Ab + sample or standard) was loaded into the microtiter wells of the assay plate and incubated 1 h at room temperature. Plates were washed and incubated for 1 h at room temperature with 200 μL of horseradish peroxidase labeled anti-rabbit IgG 2 Ab (Sigma, St. Louis, USA). Plates were again washed and incubated with 200 μL of TMB substrate (Sigma, St. Louis, USA) for 15 min in the dark. Absorbance was read at 620 nm on a Multiskan EX (Thermo Electron). Standard curves were constructed and sample values calculated using the accompanying Multiskan Ascent software. The standard curves produced were robust, with \( r^2 \) values routinely higher than 0.99. The lowest standard was periodically removed from the curve to maintain linearity. The samples were diluted 1:75, 1:825 and 1:7700 in 0.075 M PBS assay buffer, giving an assay quantitation range of 5.6 μg/mL to 37 mg/mL.

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 (HSI = liver weight/whole body weight × 100). Gonad and whole body weights were used to calculate the gonadosomatic index (GSI = gonad weight/whole body weight × 100). Body weight and total length was used to calculate the body condition factor (BCF = body weight/total length)\(^3\) (Fulton, 1904).

2.4.2.3. Secondary sex characteristics. Prior to organ excision, secondary sex characteristics (SSC) 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 was 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. Following removal, 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 tissue 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 assessed by an experienced histologist (HLS) and ranked on a standard haematoxylin and eosin staining/proteinaeous fluid. Developmental stage of the gonad (testis or ovary) and the proliferation of interstitial cells between seminiferous tubules of the testis substructure was 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 E2

After the 21-day exposure period, 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. As a result of mortality, the number of available males per treatment ranged from 2 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 13 days. Nest sites found to contain eggs during the daily inspection were replaced with new nest substrate. Behavioral aggression assays were performed on the 3rd and 12th days 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 minimal disturbance of the aquarium and with maintaining the observer out of sight of the fish. 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 latency period by the number of contacts in the ensuing minute (fish not initiating an attack were arbitrarily assigned a divisor of 0.5 for the calculation of the index). A higher index score is indicative of a less pronounced response to the introduction of an imitation male which should induce an aggressive nest defending response in the treatment male. The sum of observations on day 3 and 12 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 4.01 statistical package, GraphPad Software Inc., Oxnard, CA). As the majority of data were found not to 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 in each treatment were combined for subsequent statistical analysis. A probability of \( p < 0.05 \) was set as level of significance for all comparisons.

3. Results and discussion

3.1. Aqueous estradiol concentrations and survival rates

Estradiol isomer concentrations by LC–MSMS analyses of aquaria water are reported in Fig. 1. Concentrations averaged 27, 72, and 150 ng/L for \( 17\alpha \)-estradiol, and 9, 20, and 44 ng/L for \( \beta \)-estradiol. Relative to the low concentration, the medium and high concentrations were 2.4/2.6 and 5.1/5.5 fold higher for \( \alpha \)- and \( \beta \)-estradiol, respectively. Overall relative variance of concentration was 19\%. The highest COVs were found at the lower doses (maximum of 30\% variation by day) and lowest COVs with the highest doses (8\% minimum) in agreement with the Horwitz curve (Albert and Horwitz, 1997). Based on the performance of standards, this variability is related to variability in LC–MSMS performance rather than to actual changes in concentrations. No estriol or estrone was detected in samples, indicating purity of chemicals and lack of chemical degradation.

Survival across the experiment was 88\%, with no significant difference by sex or treatment (Fig. 2). The lowest survival rate, 75\%, occurred with the \( 17\alpha \)-high treatment (151 ng/L E2), with most mortality occurring within the first week of exposure (7 of 9 deaths). Fathead minnow survival values in this laboratory typically average 95\%, and solvent controls from this experiment were 93\%. Others reported 50\% mortality in a 14-day exposure of male fathead minnows to 23 ng/L \( 17\beta \)-estradiol, while mortality was 33\% for females exposed to 91 ng/L (Miles-Richardson et al., 1999). Environmental conditions were stable throughout the experiments (temperature = 21.2 ± 0.2 °C; conductivity = 0.95 ± 0.02 mS/cm\(^2\); dissolved oxygen: 7.6 ± 0.01 mg/L; pH = 8.0 ± 0.2; hardness as CaCO3 = 15 ± 0 mg/L). These conditions are reflective of rearing conditions described by Denny (1987) and environmental conditions for fathead minnows during the reproductive season, as well as those previously reported by Hyndman et al. (2010). Average pH of 8.0, was slightly higher than Hyndman’s average of 7.8.

3.2. Physiological endpoints

Plasma vitellogenin concentrations did not differ between treatments for female fish, with mean concentrations for all treatments after a 21-day exposure or 13-day depuration of ~30,000 µg/mL (Fig. 3a). For male fish, however, there were several significant differences between treatments when compared to EtOH solvent control male fish (Fig. 3b). Plasma vitellogenin concentrations were significantly increased for \( 17\alpha \)-high (151 ng/L E2, \( p < 0.05 \)), \( 17\beta \)-med (16 ng/L E2, \( p < 0.01 \)), and \( 17\beta \)-high (44 ng/L E2, \( p < 0.01 \)), compared to the EtOH control. There were no statistical differences in vitellogenin response among EtOH control, \( 17\alpha \)-low (24 ng/L), \( 17\alpha \)-med (73 ng/L), and \( 17\beta \)-low (8 ng/L) treatments. The highest mean vitellogenin concentration, ~29,500 µg/mL, was found with \( 17\beta \)-high, and was similar to the plasma concentration found in females. In this study, 18 ng/L of the \( 17\beta \) isomer increased vitellogenin, while the \( \alpha \) isomer evoked a significant increase.
at 151 ng/L. Consistent with published literature (Hemmer et al., 2002; Hyndman et al., 2010) even after 13 days in blank well water, vitellogenin concentrations for male fish within treatments remained elevated and statistically similar to samples taken immediately following exposure.

Male vitellogenin production was not significantly different between 17α-high (151 ng/L E2) and 17β-high groups (44 ng/L E2), indicating that both chemicals at these high concentrations yield similar results for vitellogenin production. It should be noted, however, that some studies have reported a ceiling effect for vitellogenin production with increased exposure to estradiol (Thorpe et al., 2007; Schmid et al., 2002; Panter et al., 2000; Korte et al., 2000; Hyndman et al., 2010). Therefore, the lack of significant differences between these two groups may be the result of reaching a ceiling in vitellogenin production. Evaluating other non-significant results, male fish from α-low (24 ng/L E2), α-med (73 ng/L E2), and β-low (8 ng/L E2) had similar vitellogenin production compared to the solvent control group suggesting that concentrations at these levels have little physiological effect on the fish.

As a general trend, 73 ng/L of 17α-estradiol (α-med) seemed to invoke similar effects as 8 ng/L 17β-estradiol (β-low). Similarly, α-high (151 ng/L E2) and β-med (18 ng/L E2) treatments had statistically the same vitellogenin production, as did the α-med (73 ng/L E2) and β-low (8 ng/L E2) groups. These results imply that 17β-estradiol is β–9x more potent than 17α-estradiol and also supports the likelihood of the 17β-high result being influenced by a ceiling effect for vitellogenin production.

While this is the first report of 17α-estradiol effects in fish, studies have evaluated 17β-estradiol effects on several species of fish. In addition to the studies cited above, a similar quantitative response using fathead minnows was reported for 31 ng/L of 17β-estradiol in a 21-day exposure, with a plasma vitellogenin concentration of 18,000 μg/mL (Martinovic et al., 2007). An EC50 of 25 ng/L was reported for plasma vitellogenin response in a 14-day fathead exposure, compared to 0.9 ng/L for the synthetic estrogen ethinylestradiol (EE2, Brian et al., 2005). Similarly in zebrafish, EC50 were 41.2 and 2.51 ng/L for 17β-estradiol and EE2, respectively, based on total body vitellogenin and an 8-day exposure period (Rose et al., 2002). Zebrafish appear similar to fathead minnows in that plasma vitellogenin was not significantly different from solvent controls in fathead minnows that received 8 ng/L, and zebrafish exposed to 5 ng/L for up to 40 days, show no change in plasma vitellogenin (Nash et al., 2004).

3.3. Anatomical endpoints

Neither organosomatic indices (hepatosomatic index, gonadosomatic index; Supplemental Tables 2 and 3) nor body condition factor (Supplemental Table 4) differed between treatments or sexes, with the expected exception of sex difference for gonadosomatic index. Previously, a 21-day exposure of male fathead minnows to nominal concentrations of 320 ng/L 17β-estradiol or estrone was required to decrease the gonadosomatic index (Panter et al., 1998). Similarly, in Japanese medaka, a change in condition factor (weight/length) was measured after 100 days of exposure to 100 ng/L 17β-estradiol, but not to 10 ng/L (nominal concentrations, Metcalfe et al., 2001).

Secondary sex characteristics of male fathead minnows differed among treatments immediately following the 21-day exposure period (Fig. 4). Males in the α-low (not significant) and α-medium (p < 0.05) groups had higher scores for secondary sex characteristics, an effect also noted in previous studies with β-estradiol (Schoenfuss et al., 2008). The 17α-estradiol treatment may be mimicking the “priming” effect reported in conspecific male fish exposed to natural steroids released by female fish (Zheng and Stacy, 1997). In a previous study from our laboratory using fathead minnows and a 21-day exposure period, 12 or 17 ng/L of 17β-estradiol failed to cause a decrease in SSC (Hyndman et al., 2010). In contrast, the expected reduction in SSC was found in the β-high group (44 ng/L in comparison to control fish. Similarly, a 14-day exposure of male fathead minnows to ~272 ng/L resulted in an atrophy of breeding tubercles (Miles-Richardson et al., 1999). Following the transfer into the paired spawning tanks for the 13-day depuration period, constant or increased expression of secondary sex characteristics was observed in males, the expected response to the presence of a female fish (Fig. 4). Previous estradiol treatment did not result in any statistical difference in secondary sex characteristics scores post-depuration.

Histological assessment of ovaries did not yield any significant differences among treatments (data not shown). Ovaries of the majority of female fish across all treatments were staged as mid-to late-development with >50% of follicles presumed vitellogenic (dark staining material-identified previously through immunohistochemistry as vitellogenin, Miles-Richardson et al., 1999). Others found female fathead minnows exposed to 17β-estradiol for 14 days exhibited an increased percent of immature follicles in the ovaries with concentrations of >27 ng/L (Miles-Richardson et al., 1999), while follicles/oocytes of zebrafish became atretic after 21 days at doses of ≥2700 ng/L (van der Ven et al., 2007).

Male fish were mostly mid-spermatogoniac with a balanced presence of all spermatogenic stages. Few gross-histopathological abnormalities of testicular tissues were noted. One male from the β-medium dose exhibited oocytes within seminiferous tubules (“intersex”). Japanese medaka exposed from hatch to 100 days with 10 ng/L 17β-estradiol had 10% intersex males (3 of 30, Metcalfe et al., 2001). The prominence of interstitial cells, including the testosteron producing Leydig cells, varied significantly among treatments (Fig. 5c). All estradiol treatments exhibited greater prominence of interstitial cells (means) than control fish. These differences were significant for α-low, α-medium and β-high (Kruskal–Wallis analysis with Dunn’s post-test, p < 0.01). An increase in Sertoli or interstitial cells was previously reported for fathead minnows exposed to higher doses of 17β-estradiol (136 ng/L, 14 days, Miles-Richardson et al., 1999) and in male zebrafish exposed to the pharmaceutical estrogen receptor-blocker tamoxifen (van der Ven et al., 2007). Differences (albeit not resolved statistically) were also observed among liver tissues (Fig. 5a and b). For both male and female fathead minnows, the occurrence of eosin staining/proteinaceous fluids in the liver was more common in treated fish than control fish. This was especially pronounced in 17β-estradiol treatments at medium and high concentrations. Similar findings were found in liver of Japanese medaka (100 day exposure, 87 ng/L 17β-estradiol, van der Ven et al., 2007).

3.4. Egg count and aggression assay

Egg counts did not differ significantly among treatments (data not shown), which can partially be attributed to the randomness of egg deposition and number of eggs laid by each female. When challenged by a model of a conspecific male, the aggressive nest defense and egg count and aggression assay

3.5. Relevance in environmental monitoring

The advances in chemical analyses have just recently led to the identification of the 17α isomer of estradiol in wastewaters.
Fig. 4. Sum of secondary sex characteristics in solvent control and treatments. Mean ± standard error; number indicates sample size. Vertical line separates treatments analyzed following exposure and depuration period, respectively. Number along x-axis indicates sample size, with differences between treatments following the behavioral challenge assay due to mortality and/or the number of opposite sex fish available for pairing with a treatment. See Fig. 1 for mean estradiol treatment concentrations. Letters indicate statistically significant differences (p < 0.05) obtained through Kruskal–Wallis analysis with Dunn’s post-test. No differences between treatments were observed following the depuration period.

Fig. 5. Histological findings. (a) Observation frequency of proteinaceous fluids in the liver of male fish, as % of total number of fish in treatment (stippled/presence, solid/absence); (b) observation frequency of proteinaceous fluids in the liver of female as described for (a); (c) prominence of interstitial cells (0–4 scale) in male fathead minnows. Sample size listed along x-axis. P-values derived from Kruskal–Wallis analysis with Dunn’s post-test; *Significance at p < 0.05; **p < 0.01.
While this isomer has not typically been found in treated human wastewater (Zuehlke et al., 2005), it has been found in swine, beef, and dairy wastewater at concentrations higher than the β isomer, ranging from 8 to 2000 ng/L (Hanselman et al., 2006; Hutchins et al., 2007). When evaluating the estrogenicity of samples, whether surface or wastewater samples, three assays have commonly been used. The Yeast Estrogenicity Screening assay (YES) is based on a compound’s ability to bind to the estrogen receptor that has been transfected into the yeast cell and initiate signal transduction. The E-Screen uses estrogen-dependent human mammary epithelial cells, monitoring proliferation as an end point. The in vivo screen requires the most time and an aquaculture facility—yet is the most biologically relevant, exposing male fish to the chemicals or environmental samples (typically 14–21 days) followed by quantitation of plasma vitellogenin. The relative potency factors for the 17α-estradiol isomer, 1 and 10 ng/L have been proposed as PNEC and LOEC, respectively, for 17β-estradiol by the Environment Agency, U.K. (Young et al., 2002; Gross-Sorokin et al., 2006). Based on plasma vitellogenin data, it appears that a LOEC for 17α-estradiol in fathead minnow lies between 25 ng/L and 75 ng/L.

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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.2010.07.005.

References


Fig. 6. Behavioral findings. Sum of mean total aggression index ± standard error for male fish as assessed twice during the 13 day depuration period following the treatment-specific exposures. A higher index score is indicative of a less pronounced nest defending response in the treatment male. Index is calculated by dividing the time to respond by the number of attacks in 60s following the initial response to the imitation competitor.

While this isomer has not typically been found in treated human wastewater (Zuehlke et al., 2005), it has been found in swine, beef, and dairy wastewater at concentrations higher than the β isomer, ranging from 8 to 2000 ng/L (Hanselman et al., 2006; Hutchins et al., 2007). When evaluating the estrogenicity of samples, whether surface or wastewater samples, three assays have commonly been used. The Yeast Estrogenicity Screening assay (YES) is based on a compound’s ability to bind to the estrogen receptor that has been transfected into the yeast cell and initiate signal transduction. The E-Screen uses estrogen-dependent human mammary epithelial cells, monitoring proliferation as an end point. The in vivo screen requires the most time and an aquaculture facility—yet is the most biologically relevant, exposing male fish to the chemicals or environmental samples (typically 14–21 days) followed by quantitation of plasma vitellogenin. The relative potency factors for the 17α versus the 17β isomer is 0.01–0.02 by E-Screen (Gadd et al., 2010 and author’s unpublished data) and 0.45 for the Yeast Estrogen Assay (personal communication, Erin Yost). From the data presented here, the plasma vitellogenin response of fathead minnows would indicate that these screening assays may over— or underestimate the in vivo biological potency of the 17α isomer. Our data indicate the relative potency factor of 17α isomer for fathead minnows is approximately one order of magnitude greater than for the mammalian proliferation based cell screening assay. In contrast, the relative estrogenicity of estrone by E-Screen (Gadd et al., 2010; Soto et al., 1995) and the in vivo assay using plasma vitellogenin in juvenile trout (14 day, Thorpe et al., 2006) are similar, but about ten fold less than value reported for the YES assay.

4. Conclusion

This experiment was conducted to investigate how the anatomical, physiological, and behavioral effects of exposure to 17α-estradiol compare to the effects of exposure to 17β-estradiol. At the doses tested, neither alterations in organ indices nor metabolic impairment were observed. However, a concentration-dependent trend to less vigorous nest defense was observed among fish treated with both estradiol isomers. Furthermore, and only in male fish, 17β-estradiol was about eight times more effective than the 17α isomer in increasing plasma vitellogenin concentrations. The highest dose of 17β-estradiol (44 ng/L) resulted in a significant decrease in secondary sex characteristics (males) and suggest that exposure to high concentrations of 17β-estradiol may place these fish at a reproductive disadvantage when compared to fish that have not been exposed or have been exposed to chemicals at lower concentrations or potency levels. To date, a predicted no effect concentration or lowest observable effect (PNEC or LOEC) has not been established for 17α-estradiol, while 1 and 10 ng/L have been proposed as PNEC and LOEC, respectively, for 17β-estradiol by the Environment Agency, U.K. (Young et al., 2002; Gross-Sorokin et al., 2006). Based on plasma vitellogenin data, it appears that a LOEC for 17α-estradiol in fathead minnow lies between 25 ng/L and 75 ng/L.

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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.2010.07.005.


