Aquatic Toxicology 101 (2011) 521–528

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Behavioral responses of adult male and female fathead minnows to a model estrogenic effluent and its effects on exposure regime and reproductive success

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ARTICLE INFO

Article history:
Received 20 July 2010
Received in revised form 1 December 2010
Accepted 11 December 2010

Keywords:
Behavior
Competitive mating
Natural selection
Temperature
Environmental estrogen

ABSTRACT

Laboratory studies of adult male fathead minnows have shown that when they are exposed to estrogens, they lose their ability to compete for access to females and sire young, suggesting that estrogenic effluents may reduce the genetic fitness of populations of wild fishes. However, it is unknown whether wild fish which are exposed to effluent actually compete with unexposed fishes, how long effects of estrogen exposure last, and whether females are affected by estrogens. This study addressed these issues using the fathead minnow (FHM) and effluent from the Metropolitan Wastewater Treatment Plant (MWTP) a well-studied source of environmental estrogens (EEs) in the Mississippi River. Maze tests found that adult FHMs are neither attracted nor repelled by MWTP effluent while previous studies have shown that minnows are attracted to the warmer waters which characterize effluents; it is realistic that previously unexposed fish enter MWTP effluent in the spring and then compete with exposed individuals. Competitive spawning experiments showed that male FHMs exposed to 44 ng E2/l (a high but realistic level) for three weeks failed to compete with unexposed males while males exposed to 4 ng E2/l outcompeted and sired more young than unexposed males ($p < 0.05$). The effects of estrogen exposure disappeared within a week of moving fish into uncontaminated water. Female FHM reproductive output and behavior were unaffected by exposure to estrogen. Taken together, these experiments suggest that the behavior of wild fishes likely determines their exposure to EEs and that while the effects of this exposure are likely significant to populations of wild fish, they will be location specific because of factors which determine the duration and intensity of male exposure. We conclude that the role of fish behavior in endocrine disruption strongly warrants additional consideration.

1. Introduction

Endocrine active chemicals (EACs) are exogenous chemicals that alter endocrine function in exposed animals by either mimicking or antagonizing the actions of endogenous hormones (Colborn et al., 1993). One class of EACs of special concern to aquatic life are the environmental estrogens (EEs) (Sonnenschein and Soto, 1998). These compounds can be either natural or synthetic estrogens (Blair et al., 2000) and are commonly found in effluents released by municipal wastewater treatment plant effluents, paper mills and feedlots (Kolpin et al., 2002; Tyler et al., 2008). Little is known about how EEs affect wild fish populations and while male fishes captured in their vicinity frequently possess measurable levels of plasma vitellogenin (VTG, the egg yolk protein precursor) (Sumpter and Jobling, 1995; Cheek et al., 2001), explicit evidence of reduced fertility (sperm number and motility) in wild fishes does not seem to have been reported (Mills and Chichester, 2005). However, the reproductive success (tendency to produce young) of wild exposed fish also does not appear to have been studied directly and the possibility that EEs exert sub-lethal effects on population remains.

In spite of a lack of evidence from the field, numerous laboratory studies suggest that the reproductive success of wild fishes exposed to MWTP is probably affected by exposure to EEs. These studies fall into two camps. First, numerous long-term experiments have demonstrated that development of young fish can be severely impacted by continuous exposure to even low levels of EEs (Lange et al., 2001; Metcalfe et al., 2001; Segner et al., 2003; Andersen et al., 2003). Second, short-term experiments have consistently shown that adult male fish exposed to EEs experience increased VTG expression, suppressed hormone levels, and a decreased ability to compete with previously unexposed males for mating opportunities although fertility does not appear to be directly impacted (Panter et al., 1998; Schoenfuss et al., 2002; Martinović et al., 2007, 2008). If true, the later phenomenon would have long-term consequences on population health and viability because any decrease in the ability of exposed but otherwise fit males to compete for opportunities to contribute to the gene pool would disrupt natural selection. However, whether wild adult male fish move into and

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doi:10.1016/j.aquatox.2010.12.007

out of estrogenic effluents in manners that might cause previously unexposed and exposed fish to interact is not known. It is also not known how long the effects of EE exposure might last in males or whether female fishes might be similarly impacted.

This study utilized the fathead minnow model fish species (*Pimephales promelas;* FHM), to determine what effects EEs found in a representative wastewater effluent might have on the reproductive biology of populations of wild male and female fishes. This species was chosen because its reproductive behavior and physiology appear to be typical of many other nest guarding species in temperate waters. It is also known to range considerable distances (100 s of m) (Isaac, 1961; Wisenden et al., 1995), allowing for interactions between fish that have been exposed to pollutants and those that have not. The effluent released by the Metropolitan Wastewater Treatment Plant (MWTP; Saint Paul, MN, USA) was used as a model because its estrogenicity and its biological effects have been described and seem relatively typical of many locations (Martinović et al., 2007; Barber et al., 2007; Garcia-Reyero et al., 2011). Our study addressed three questions: (1) Is the reproductive biology of adult female FHM affected by the estrogens released by MWTP and similar plants? (2) What might the exposure patterns of wild, free-ranging FHM to MWTP effluent be? (3) What effect does a natural exposure regime have on the reproductive health of adult male FHM?

2. Methods and materials

2.1. Study site, effluent and design

The MWTP is located immediately downstream of St. Paul, MN and processes approximately 700 million l/day of domestic sewage and industrial waste using both primary and advanced secondary treatment with chlorination/dechlorination. It then discharges into the Mississippi River after first passing through a 10 m channel. The chemistry of its effluent has been extensively characterized and found to contain a wide range of pharmaceutical, pesticides, hormonal products, etc. with estrogenic properties (Lee et al., 2004; Barber et al., 2007). Wild male fish collected from this location have repeatedly been found to have elevated VTG (Folmar et al., 1996, 2001), while adult fathead minnows exposed to this effluent and fractions thereof show elevated levels of VTG, suppression of androgenic hormones, a reduced ability to compete with non-exposed males, and altered gene expression (Martinović et al., 2007; Garcia-Reyero et al., 2011). Winters are relatively severe at this location and effluent temperature is typically greater than that of the river during the winter.

This study examined the effects of this effluent and its characteristics on adult male and female FHM in three experiments. The first experiment addressed whether EEs found in MWTP effluent affect the reproductive biology of female FHM and tested both the effects of whole MWTP effluent on females (experiment 1A) and then the effects of the level of EEs found in it (experiment 1B). The second experiment sought to determine the exposure regimes experienced by FHM living in the Mississippi River by testing their propensity to enter effluent (experiment 2), examining extant data on the effects of the warmer temperatures with which it is associated in the spring, and then modeling their combined effects on minnow behavior/exposure regime. The third experiment concluded by examining the competitive reproductive success of adult male FHM exposed to the levels of EEs found in this effluent.

2.2. General methods

2.2.1. Fish

Fathead minnows were lab reared and raised to maturity in the laboratory in 25 °C flow-through 70-l glass aquaria held on a summertime light cycle (16 h of light: 8 h of darkness). Fish were fed a mixture of brine shrimp and flakes (Aquatic Ecosystems, Orlando, FL) daily. The fish used in experiment 1A came from the U.S. Environmental Protection Agency facility in Duluth, MN (EPA) while those used in experiments 1B and 2 were bred at the University of Minnesota using fish from the EPA and a bait fish hatchery (Elverson, PA) as brood stock. The fish used in the third experiment came from a local supplier (Environmental Consulting and Testing, Superior, WI, USA). Although it is not known whether laboratory conditions affect the behavior of this species, because both breeding facilities routinely replenish breeding stock with wild-caught individuals, the likelihood seems small.

2.2.2. Dosing protocols

Fish were exposed in flow-through glass aquaria equipped with air stones following established procedures (Martinović et al., 2007). For experiment 1A, MWTP effluent (14–22 °C, pH = 7.1) was collected in polypropylene containers (Nalgene, Rochester, NY, USA), heated to 25 °C (±1 °C) the day of collection, and then distributed to aquaria at 200 ml/min for 2-h intervals twice daily. This resulted in a 95% replacement of aquaria water by effluent twice daily. Control aquaria received well water only (pH = 7.9). For all other experiments (1B, 3A, and 3B) estrogen (17β-estradiol or E2; Sigma Co., St Louis, MI) was used instead of effluent. It was dissolved in ethanol to make a stock solution (9 μg/ml) that was further diluted (final ethanol concentration was 0.005%) and continuously metered into aquaria by pumping it into the well water flowing into aquaria (200 ml/min). Control aquaria received ethanol carrier alone (0.005% ethanol). The E2 concentrations in exposure aquaria were estimated by collecting 100 ml (experiment 1B) or 1L (experiment 3) samples of each, concentrating their steroid hormones by C18 extraction (Sep-pak, Waters Chromatography, Milford, MA) and then measuring E2 and related estrogens using enzyme linked immunoassay (ELISA, Cayman Chemical, Ann Arbor, MI) following established protocols (Martinović et al., 2008).

2.2.3. Vitellogenin analysis

To measure plasma vitellogenin (VTG), fish were euthanized in 0.05% MS-222 (Finquel, WA) and blood collected from their caudal vasculature using heparinized microhematocrit tubes after severing their tails. Serum was centrifuged for 1 min at 10,000 × g and plasma collected and frozen at −20 °C until it could be analyzed using a competitive binding ELISA that uses a FHM antibody (Korte et al., 2000; Martinović et al., 2008).

2.3. Experiment 1. Effects of environmental estrogens on female fecundity and behavior

2.3.1. Experimental design and rationale

Experiment 1A examined the effects of MWTP exposure on adult female FHM reproductive success while experiment 1B repeated this experiment using E2 alone to specifically address estrogenicity. This experimental design was based on a test that we previously used to evaluate male FHM reproductive success although for this experiment we replaced males weekly to reduce the possibility that male impacts might confound results and we started observations immediately upon the onset of dosing (Martinović et al., 2007). The dose of E2 used in the second experiment was that previously measured in MWTP effluent and found to reduce male reproductive success (Martinović et al., 2007).

2.3.2. Fish selection, exposure, and testing

Both male and female fish were pre-selected for this experiment to ensure they were sexually mature and responsive. This was accomplished by placing pairs of males and females into 5-l aquaria with nests (transected PVC pipes) and then removing egg-producing females and nest-guarding males to holding tanks every 2–3 days. For experiment 1A, 20 pairs of these pre-selected fish were placed into 70-l glass aquaria where half were exposed to effluent, and the other half to well water control for 21 days as described above. Nests were removed daily to measure egg production and replaced with new clean nests lacking eggs. Males were replaced weekly. Experiment 1B was identical to experiment 1A except that half the fish were exposed to either E2 (44 ng/l) or ethanol control. At the conclusion of both experiments, fish were sacrificed using an overdose of anesthesia and both their gonadosomatic indices (GSI = gonad weight/body weight × 100%), hepatosomatic indices (HSI = liver weight/body weight × 100%) measured, and blood samples taken for VTG determination as described above.

2.3.3. Data analysis

Experiments 1A and 1B were analyzed separately because the fish were from different sources. Parametric tests (paired *t*-tests) were used to compare experimental and control values for each set of variables because all data passed the Kolmogorov–Smirnov test for normality (InStat3, Graphpad Software, San Diego, CA).

2.4. Experiment 2. Determining the possible exposure regimes of fathead minnows living near a model effluent by examining and modeling their tendencies to enter effluent

2.4.1. Experimental design and rationale

To determine whether adult FHM living in the Mississippi River might move in and out of MWTP effluent – and thus how they might be exposed to its EEs and interact with exposed fish – we tested their behavioral preferences. Because we could not test all aspects of this effluent, or all effluents, we focused on two variables that seemed most important: chemistry and temperature. The former was evaluated using a two-choice laboratory maze maintained at springtime spawning temperatures. Because the responses of FHM to temperature changes had previously been studied (Cherry et al., 1977), we used previously published data to evaluate this topic. A hypothetical model of riverine fish movement in and out of the effluent was then created using these datasets and comparing them with the spawning temperature of this species.

2.4.2. Testing the effects of effluent chemistry

Behavioral responses of FHM were tested in a custom-built maze which had a neutral arm where waters mixed and two inlet arms which received effluent or well water control (175 ml/min, 25 °C) and contained 5 nests (Fig. 1). Water chemistry analysis of our well water (pH 7.9, hardness of 220 mg $CaCO_3$/l) showed it to closely resemble river water (pH of 8.1, hardness of 230 mg $CaCO_3$/l), so it was used as surrogate. For tests, four mature male (fin clipped to identify individuals) and four mature females were placed into the maze for one day to acclimate while 50% effluent and 50% well water was added down both arms. After this period, the nests were removed and a shade cloth cover placed over the third arm for 1-h to encourage fish to return to this neutral region after which time the nests were replaced and full strength MWTP effluent (25 °C) was added down one arm and 25 °C well water control down the other. The distribution of fish was observed every 10-min for four 2-h intervals over the next 24-h, after which the nests were removed, the neutral end re-covered, water inputs reversed, and the fish observed for another 24-h period. This three day test was repeated 6 times using a new set of fish for each replicate. For data analysis, the total number of male and female fish in each arm was determined for each 24-h period and compared using a Fisher exact test (R, version 2.7.1; http://www.r-project.org/). By analyzing the test periods independently of each other we also evaluated side bias.

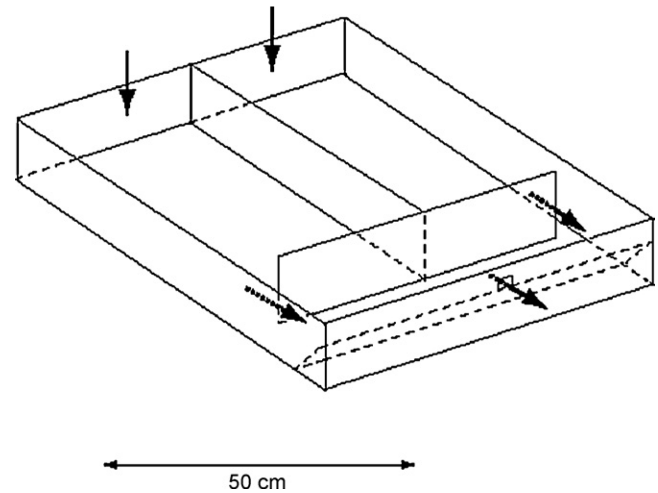

Fig. 1. Schematic diagram of the two-choice maze used in experiment 2A. The arrows denote waters flowing through the apparatus, mixing, and then exiting through the neutral area where FHMs were added.

2.4.3. Modeling the possible effects of temperature on FHM distribution

A literature review discovered the temperature preferences of adult FHM had already been studied in a large maze (Cherry et al., 1977). Local authorities provided us with effluent and local Mississippi River temperatures (personal communication, Terrie O'Dea, Environmental Information Management System, MWTP, St. Paul, MN; http://es.metc.state.mn.us/eims/index.asp) while data on FHM spawning temperature in the region was available from Isaac (1961). Temperature data for both the effluent and river water were averaged by month for those periods for which continuous records were available, plotted, and then compared to published temperature preferences to estimate when FHM located at the edge of MWTP effluent might be expected to enter and leave the effluent and spawn. These tendencies were then modeled and represented using a graphical figure.

2.5. Experiment 3. Testing the effects of spring-time exposure regimes on the relative competitive reproductive success of small populations of fathead minnows

2.5.1. Experimental design and rationale

This experiment used an established competitive male spawning assay (Martinović et al., 2007) to examine the effects of two exposure conditions on the competitive spawning abilities of adult male FHM. Exposure regimes identified by experiment 2 were recreated. The possibility that effluent-exposed males might recover after moving into effluent-free river water for 1–3 weeks (a possibility suggested by experiment 2) was tested. Male FHMs were exposed to E2 alone because it is easier to test than effluent and Martinović et al. (2007) found that it mimics the entire effect of MWTP effluent on male FHM. Effluent estrogenicity was estimated using a rainbow trout estrogen receptor assay (rtERA) which does not differentiate between receptor agonists and antagonists but has been validated for this effluent (Martinović et al., 2007, 2008). The estimated concentration was tested. In addition we tested the effects of exposing males to 44 ng E2/l, the concentration of estrogens which had previously been measured at this site (Martinović et al., 2007).

2.5.2. Fish selection, exposure and testing

Mature male FHMs were pre-selected for this experiment based on their secondary sex characteristics (tubercles, dorsal pad) and by pre-testing pairs of fish as described above. Pre-selected fish

Table 1
Effects of exposing adult female fathead minnow to Metropolitan Wastewater Treatment Plant (MWTP) effluent or estradiol alone in experiment 1 on vitellogenin (VTG), gonadosomatic index (GSI), hepatosomatic index (HSI), and egg production. Data expressed as mean ± SEM.

	Experiment 1A		Experiment 1B	
	Control (n = 10)	Effluent (n = 10)	Control (n = 10)	Estradiol (n = 10)
VTG (mg/ml)				
Female	8.68 ± 1.96	10.8 ± 2.19	8.86 ± 1.59	12.2 ± 1.80
Male	0.003 ± 0.001	0.025 ± 0.004*	0.0 ± 0.0	3.52 ± 0.35*
GSI				
Female	NA[a]	NA	10.1 ± 0.92	10.4 ± 0.93
HSI				
Female	NA	NA	2.78 ± 0.45	2.91 ± 0.62
Fecundity (eggs/female/day)	42.8 ± 4.45	33.4 ± 3.72	25.7 ± 4.57	28.81 ± 3.84

[a] NA = not available.
* $p < 0.05$.

were then exposed to either 44 ng E2/l, 4 ng E2/l, or control (0.005% ethanol carrier) for three weeks in exposure aquaria. After exposure, males were either tested immediately or moved into aquaria supplied with well water and allowed to recover for either one or three weeks. Sample sizes for these 6 experiments ranged between 16 and 27 depending on fish availability. For each test, exposed and control males were matched by size (±3 mm total length and 0.1 g total mass), given distinctive fin clips and then placed with two mature females into 5-l glass aquaria containing a single nest (thus creating competition) and supplied with flowing well water (see Martinović et al., 2007). Experiments lasted 5 days during which time each aquarium was observed for 5-min each day by an observer who was unaware of fish treatment. The observer identified nestholders (NH; males that spent more than 50% of their time in nests while performing nest tending or exhibiting territorial displays) and NH with eggs. At the end of the experiment, males and nests were both removed. The eggs found in each nest were then counted and the males were euthanized (0.05% MS-222), plasma samples taken, their livers and gonads weighed to determine GSI and HSI, and their secondary sex characteristics (SSC) scored following Danylchuk and Tonn (2001).

2.5.3. Statistical analysis

Each of the six experiments was analyzed independently of the others but in the same manner. Analyses varied on whether data were ordinal or continuous, and/or met the assumption of normality (Kolmogorov–Smirnov test). Thus, while the competitive abilities of male FHMs were evaluated by *t*-test, the number of nestholding males noted in experimental tanks was compared with controls using Fishers Exact tests (R, version 2.7.1). The morphological and physiological characteristics of exposed and unexposed fish were analyzed by ANOVA using a randomized block design in which the blocking factor was the dosing tanks (personal communication, Sai Okabayahi, Statistics Clinic, University of Minnesota). A Tukey HSD post hoc test was subsequently used to test for significance amongst individual comparisons. ANOVA was also used to analyze the number of eggs per nest. The number of eggs per male was not normally distributed so this variable was analyzed with the Wilcoxin matched pair test (InStat 3, Graphpad Software, San Diego, USA). The relative number (%) of NH and total number of eggs were calculated as descriptors of fish performance only.

3. Results

3.1. Experiment 1

Female FHM exposed to effluent produced equivalent number of eggs as control females while possessing equivalent levels of VTG (Table 1). Similarly, females exposed to E2 also produced equivalent number of eggs as their controls and had similar VTG levels, GSIs, and HSIs ($p > 0.05$). In contrast, males exposed to effluent had elevated VTG (0.025 mg/ml vs. 0.003 mg/ml for controls; $p < 0.05$, $n = 10$) as did the E2-exposed males (3.52 mg/ml vs. 0.0 mg/ml, $p < 0.05$, $n = 10$). Analysis of the effluent using rtRNA found it to contain 3.8 ± 0.04 ($n = 4$) equivalents of estrogen while E2 concentrations measured by ELISA in experiment 1B were 33 ng/l ± 8.85 ($n = 5$).

3.2. Experiment 2 and model

Experiment 2 provided no evidence that fish were either attracted to, or repelled by, effluent; equal numbers of males and females were found in both effluent and well water control arms in both tests (Fig. 2). No side bias was evident and fish were seen to move readily between arms. Evaluation of the maze studies previously conducted by Cherry et al. (1977) found that adult FHM (males were not evaluated independently of females) moved into warmer water as long as the difference was less than 9 °C and that they have an optimal temperature preference of 26 °C. Continuous temperature data for the effluent channel and river were available for 2001–2003 and were averaged by month after determining that these were relatively typical years. This analysis showed that MWTP effluent was typically 1–14 °C warmer than Mississippi River water from September through early June (Fig. 3), after which time the river was warmer. Together, these data allowed us to construct a simple model (Fig. 3) which suggested that FHM likely move from the river into the effluent during the month of May, from the effluent into the river from June to September, and then return to the effluent in September. Data from Isaac (1961) showed that FHM spawn between 16 and 18 °C, a temperature which the effluent reached slightly in advance of the river in May of 2001 and 2002, suggesting that exposed and unexposed fish likely interact while spawning. Large numbers of fish may also become trapped in effluents during the winter months.

3.3. Experiment 3

Males exposed to the high concentration of E2 (which ELISA showed to be 14.8 ± 2.3 ng/l), were only half as likely to acquire and defend nests as competing, unexposed control males ($p < 0.05$ on days 3 and 4; Fig. 4). Further, although egg production was highly variable, control males sired nearly four times as many eggs (1573 vs. 371) as did E2-exposed males (Table 2). Males exposed to the high concentration of E2 experienced VTG induction (0.26 mg/ml vs. 0.001 mg/ml for the control; $p < 0.05$), slightly (but not significantly) reduced secondary sexual characteristics, and equivalent HSIs and GSIs ($p > 0.05$, Table 2). A week after being exposed to E2, males had fully recovered and competed on par with control fish.

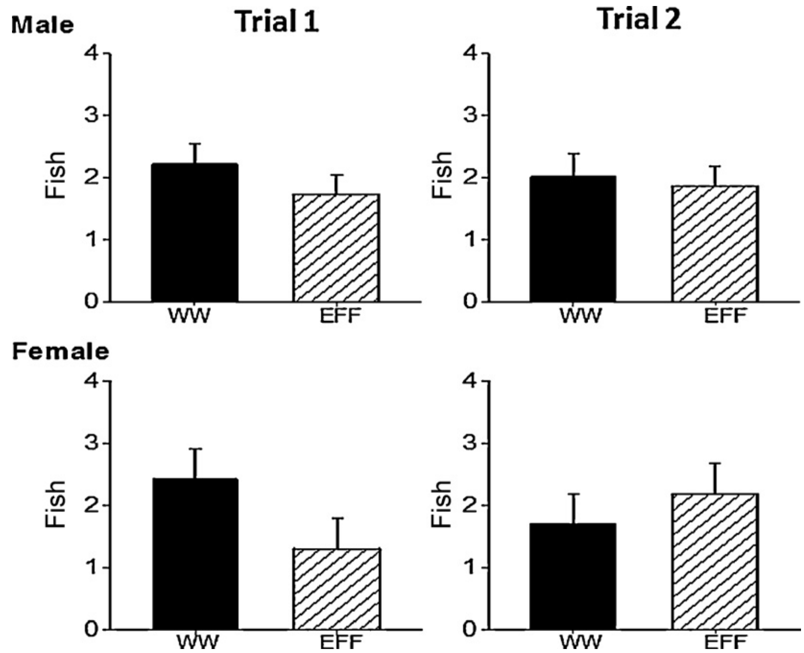

Fig. 2. Mean number of fish located in each side of the maze during each of the two test periods (Trial 1 and 2; by showing both trials we address possible side bias) in experiment 2. WW = well water control; EFF = MWTP effluent.

Male FHM given three weeks to recover also did not differ in any way from their controls (Table 2).

Strikingly different results were evident for male FHM exposed to the low dose of E2 which was measured as 2.16 ± 0.5 ng/l by ELISA. Following three weeks of exposure to this dose, males consistently outcompeted control males by a ratio of 2:1 across all 5 days ($p < 0.05$; Fig. 4) and produced nearly 7 times more eggs (Table 3). These exposed male FHMs had both elevated VTG (0.04 mg/ml vs. 0.001 mg/ml for the control; $p < 0.05$) and larger GSIs ($p < 0.05$). Male fish that had been exposed to this dose and permitted to recover in estrogen-less water for either one or three weeks did not differ in any way from unexposed males (Fig. 4, Table 3).

4. Discussion

This study describes laboratory evidence that wild free-ranging fishes in the Mississippi River are likely attracted into estrogenic wastewater plumes in the spring where they will compete with exposed males for reproductive opportunities with varying degrees of success which may override other processes to the long term determent of the population. Effects on recruitment cannot be ruled out and are likely complex and location/situation-specific as the effects of effluent exposure are influenced by dose and recovery is rapid. These effects may be especially significant in more extreme environments where fish likely are attracted into warm water effluents for long periods of time during the winter. The later situation particularly warrants direct study as does study of other fish species in the field. The details of how fish behavior likely drives exposure regime also merits examination.

The present study maybe one of the first to examine the effects of realistic levels of EEs on adult female fish and suggests that females may not be seriously affected by EE exposure. This result is not especially surprising because female cyprinid fishes already have high levels of circulating E2 and their sexual behavior is not medi-

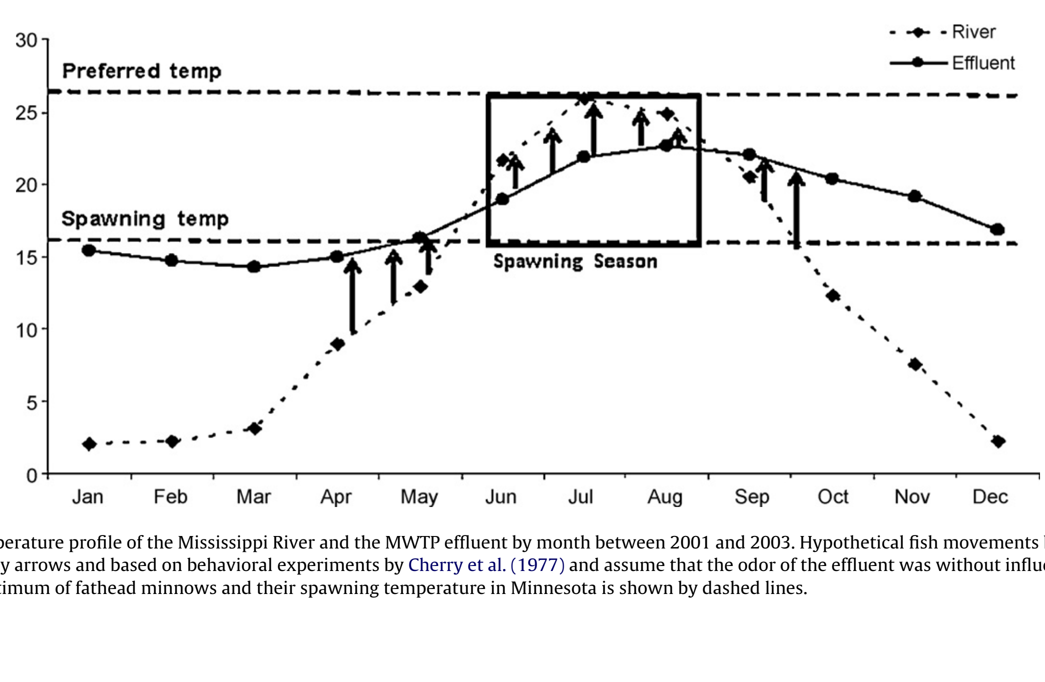

Fig. 3. Average temperature profile of the Mississippi River and the MWTP effluent by month between 2001 and 2003. Hypothetical fish movements between the river and effluent are shown by arrows and based on behavioral experiments by Cherry et al. (1977) and assume that the odor of the effluent was without influence (experiment 2A). The temperature optimum of fathead minnows and their spawning temperature in Minnesota is shown by dashed lines.

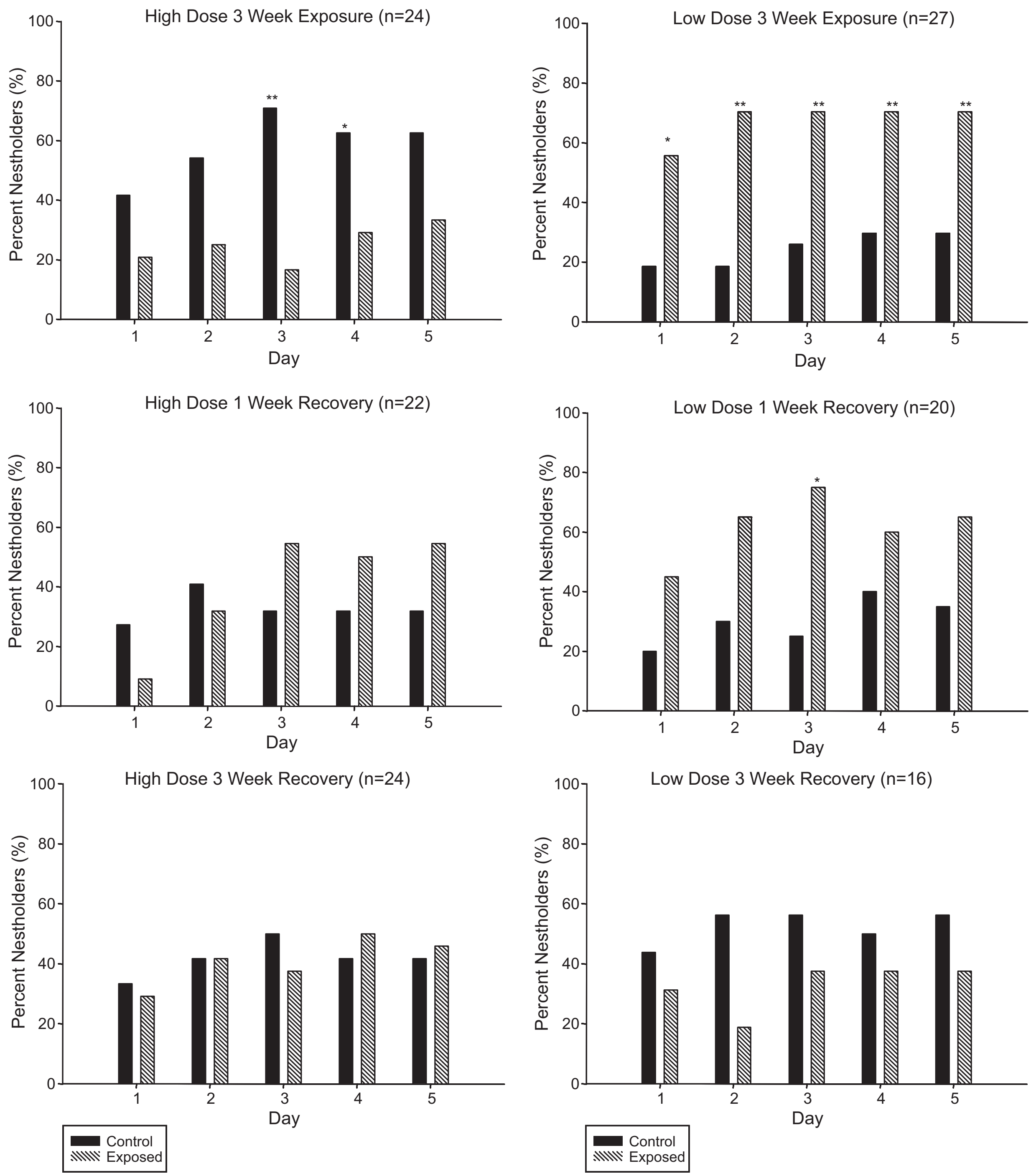

Fig. 4. Relative numbers (%) of control and E2 exposed males which held nests each day in the six competitive assays conducted for experiments 3A and 3B (n = 16–27 for each assay). The results of males exposed to the high dose of E2 (44 ng/l) and then placed immediately with unexposed males is shown in the top left with the results of similarly exposed males that were permitted to recover for one week and then three weeks are shown below that. The results of the low exposure (4 ng/l E2) shown to the immediate right. Egg production is presented in Tables 2 and 3. $^{*}p < 0.05$, $^{**}p < 0.01$.

ated by steroid hormones but rather by prostaglandin F2α (Stacey and Sorensen, 2009). In contrast, the behavioral endocrinology of internal fertilizing fishes such as the medaka (*Oryzias latipe*) is different (Liley, 1972; Balch et al., 2004) and warrants additional work. Notably, the present study does address the possibility that sexually immature females may be more sensitive to the effects of EEs or that other aspects of their behavior could be impacted (McGee et al., 2009).

Although our maze study was relatively simple and without great statistical power, the data offered no evidence that this wastewater effluent exerted strong effects on fish behavior. Not only did minnows spend almost exactly the same amount of time in effluent as control, but also they occupied nests in both waters (data not shown). This finding was not expected because the olfactory and taste systems of cyprinid fishes are acutely sensitive to many chemicals (Sorensen and Caprio, 1998) but perhaps could be explained by the great array of compounds that would have been present and would have lacked biological relevance. The only other study we know to examine the effects of effluent on fish behavior also described a similar result with barbs (Fava and Tsai, 1976).

Table 2
The effects of exposing adult male fathead minnows to 44 ng/l E2 (nominal) or control (ethanol carrier only) for 3 weeks on their gonadosomatic index (GSI), hepatosomatic index (HSI), secondary sexual characteristics (SSC), ability to acquire access to eggs, and nest behaviors (number with nests, eggs per nest) in the presence of an unexposed control male, immediately after a 3 week exposure, and after one or three weeks of recovery in well water. Data are expressed as mean ± SEM except where noted.

	3 Week exposure		1 Week recovery		3 Week recovery	
	Control	44 ng/l E2	Control	44 ng/l E2	Control	44 ng/l E2
VTG (mg/ml)	(n = 49) 0.001 ± 0.003	(n = 25) 0.26 ± 0.06*	NA	NA	NA	NA
GSI (%)	(n = 20) 1.2 ± 0.1	(n = 20) 1.4 ± 0.1	(n = 20) 1.2 ± 0.1	(n = 19) 1.3 ± 0.2	(n = 13) 1.3 ± 0.1	(n = 13) 1.4 ± 0.2
HSI (%)	(n = 20) 1.6 ± 0.1	(n = 20) 1.6 ± 0.1	(n = 20) 1.5 ± 0.1	(n = 20) 1.8 ± 0.2	(n = 13) 2.1 ± 0.2	(n = 13) 1.9 ± 0.2
SSC	(n = 17) 3.9 ± 0.4	(n = 17) 2.7 ± 0.4	(n = 22) 2.4 ± 0.3	(n = 21) 2.7 ± 0.4	(n = 23) 3.6 ± 0.3	(n = 24) 2.9 ± 0.4
Eggs/male[a]	0 (0, 127)	0 (0, 0)	0 (0, 0)	0 (0, 8)	0 (0, 0)	0 (0, 20)
Nests (day 5)	13/24	6/24	8/22	13/22	12/24	11/24
%NH[b] w/eggs	60	30	60	50	30	60
Total eggs	1573	371	726	462	646	555
Eggs/nest	121 ± 36	62 ± 42	91 ± 37	36 ± 19	54 ± 27	51 ± 21

NA = not available.
[a] Median(Q1, Q3).
[b] NH = nestholder.
* $p < 0.05$.

More study of the chemosensory properties of other effluents is probably warranted.

Our review of extant data suggested that like many other fish (Hazel, 1993), FHMs are strongly attracted to the relatively warmer temperatures which characterize MWTP effluent in spring (and fall) and which they likely enter in the wild. Thus, unexposed and exposed male FHM might be expected to routinely mix and compete for females and nest sites, with the result that normal competitive processes between males may be altered and no longer be solely driven by the forces of natural selection. Because even brief, pulsed exposure to EEs, such as might occur at the edge of wastewater plume, can disrupt male competitive behavior (Martinović et al., 2008), these effects could be significant but are as yet unstudied. Although our prediction that wild fish actively move through the effluent plume has yet to be validated by field sampling, large mixed-species aggregations of fish are observed within the MWTP effluent channel during the winter (MWTP personnel, St. Paul, MN; C. Lavelle and L. Levitt, personal observations, University of Minnesota) and high levels of VTG induction is routinely noted in its vicinity (Folmar et al., 1996, 2001). Because many fish species are known to be routinely attracted to temperature gradients (Hazel, 1993; Cooke and McKinley, 1999; Cooke et al., 2004), this phenomenon likely represents a significant driver of fish exposure and warrants closer study as does the effects of possible long-term exposure to EDCs during the winter.

This study is also one of the first to examine the effects of multiple levels of EEs on male fish reproductive behavior. It confirms previous studies showing that relatively high (but realistic) levels of EE exposure at several locations suppress male reproductive performance (Martinović et al., 2007, 2008) while demonstrating that exposure to the lower range of estrogens measured in wastewater effluent has the opposite effect. Although the causes of this complex dose–response relationship are unknown, evidence for it has been observed by others (Schoenfuss et al., 2008). It is interesting and significant that the effects of estrogenic exposure are relatively short-lived and may suggest a role for male hormone levels (Martinović et al., 2007). Together, these observations paint a complex picture of the effects of natural wastewater plumes on male behavior and reproductive success, especially because EE levels likely are low and variable at peripheral regions of effluent plumes.

Although our study focused on possible effects of one estrogenic effluent on a nest guarding minnow (the fathead) living in a northern temperate river (the Mississippi River), the findings are almost certainly applicable in to other fish species and locales. Most male fishes compete actively for reproductive opportunities and have evolved traits that favor such characteristics whose expression would be expected to be effected by EDCs in similar manners because of similarities in their hormone and pheromone systems (Stacey and Sorensen, 2009). Indeed, recent work from the scramble spawning zebrafish, *Danio rerio*, seems to describe similar effects in this species (Colman et al., 2009). Fishes also commonly seek optional temperature regimes while responding to chemical cues, both of which differ in effluents which are also frequently estro-

Table 3
The effects of exposing adult male fathead minnows to 4 ng/l E2 (nominal) or control (ethanol carrier only) for 3 weeks on their gonadosomatic index (GSI), hepatosomatic index (HSI), secondary sexual characteristics (SSC), ability to acquire access to eggs, and nest behaviors (number with nests, eggs per nest) in the presence of an unexposed control male, immediately after a 3 week exposure, and after one or three weeks of recovery in well water. Data expressed as mean ± SEM except where noted.

	3 Week exposure		1 Week recovery		3 Week recovery	
	Control	4 ng/l E2	Control	4 ng/l E2	Control	4 ng/l E2
VTG (mg/ml)	(n = 49) 0.001 ± 0.003	(n = 27) 0.04 ± 0.02*	NA	NA	NA	NA
GSI (%)	(n = 14) 1.1 ± 0.11	(n = 13) 1.6 ± 0.13*	(n = 7) 1.1 ± 0.38	(n = 7) 1.4 ± 0.15	(n = 11) 1.1 ± 0.10	(n = 11) 1.3 ± 0.18
HSI (%)	(n = 14) 1.8 ± 0.19	(n = 14) 2.1 ± 0.17	(n = 8) 2.1 ± 0.22	(n = 7) 2.3 ± 0.19	(n = 11) 1.7 ± 0.15	(n = 11) 1.5 ± 0.18
SSC	(n = 27) 3.1 ± 0.31	(n = 27) 4.0 ± 0.28	(n = 20) 2.7 ± 0.49	(n = 20) 3.6 ± 0.37	(n = 16) 3.3 ± 0.37	(n = 16) 2.5 ± 0.42
Eggs/male[a]	0 (0, 0)	0 (0, 94)	0 (0,63)	0 (0, 134)	0 (0, 39.5)	0 (0, 0)
Nests (day 5)	9/27	18/27	8/20	12/20	8/16	6/16
%NH[b] w/eggs	56	50	75	67	88	33
Total eggs	206	1473	931	1053	530	295
Eggs/nest	23 ± 9	82 ± 30	116 ± 37	88 ± 25	66 ± 32	49 ± 32

NA = not available.
[a] Median(Q1, Q3).
[b] NH = nestholder.
* $p < 0.05$.

genic and/or androgenic. It is reasonable to consider the possibility that fish reproductive behavior and natural selection are commonly affected by EDCs and that long term effects on wild population may be significant but not always immediately evident.

In conclusion, the present study describes how behavioral mechanisms that are both driven, and influenced by estrogenic effluents may determine the reproductive success of male fishes in ways that could influence population health and well being over the long term. Effects are strong in the laboratory but appear relatively short-lived and dramatically influenced by dose in ways that are difficult to predict in the field. Detailed field studies are now required to determine whether our laboratory observations can be validated in the field and what their true significance on populations of wild fish might be. Efforts that track the distribution and reproduction success of wild fish might be especially insightful.

Acknowledgements

This work is the result of research sponsored by the Minnesota Sea Grant College Program supported by the NOAA Office of Sea Grant, United States Department of Commerce, under grant No. NA03OAR4170048 to PWS. The U.S. Government is authorized to reproduce and distribute reprints for government purposes, not withstanding any copyright notation that may appear hereon. This paper is journal reprint no. 592 of the Minnesota Sea Grant College Program. We thank Dalma Martinović for her advice and both Jeff Denny and Kathy Jensen from the Mid Continent Ecology Division of the EPA, Duluth, MN for assistance. Jon Hess, David Goldberg and Keith Philippe assisted with behavioral observations, data entry and husbandry. Mario Travaline helped with effluent transport and tank set-up while Haude Levesque kindly drew Fig. 2. Deb Swackhamer kindly assisted with editing.

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