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ENVIRONMENTAL ESTROGENS SUPPRESS HORMONES, BEHAVIOR, AND REPRODUCTIVE FITNESS IN MALE FATHEAD MINNOWS

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Abstract—This study explored the possibility that environmental estrogens in sewage effluent may reduce the reproductive fitness of adult male fish by suppressing their reproductive behaviors, including their ability to compete for nests and females. Male fathead minnows (*Pimephales promelas*) were exposed for three weeks to either blank control, effluent released by a sewage treatment plant (STPE), waterborne estradiol (E_2), or a synthetic androgen (methyltestosterone [MT]). Afterward, fish were placed with females and a nest, and their behavior was monitored for 5 d in either the presence or the absence of a competing (unexposed control) male. Males exposed to either the STPE or E_2 (~50 ng/L, a level chosen to mimic the estrogenic content of the STPE) had elevated levels of circulating vitellogenin ($p < 0.05$) and lower levels of 11-ketotestosterone (KT; $p < 0.05$). Nearly all STPE- and E_2-exposed males spawned successfully in the absence of a competing male, but in both cases, exposed males suffered nearly total reproductive failure when they had to compete. Conversely, males exposed to MT (~50 ng/L) outcompeted control males. Behavioral observations suggested that subtle differences in agonistic behaviors, typically associated with circulating androgens (i.e., KT), were responsible. We speculate that male fish exposed to estrogenic effluent in the field are less likely to reproduce successfully within large populations of wild fish, thereby causing abnormal and potentially detrimental patterns of gene flow within those populations.

Keywords—Endocrine disruptors Behavior Competition Fitness Fish

INTRODUCTION

Although it is well established that many sewage treatment plants (STPs) release estrogenic compounds into the environment (environmental estrogens [EEs]), the long-term consequences of exposure to these compounds on populations of wild fish are unclear [1]. Information is particularly lacking about what effects these effluents might have on the reproductive behavior and success of adult fish as they move about the environment seeking optimal temperatures, food, and spawning habitats. Likely, exposure regimes for wild, free-ranging adult fish vary greatly between individuals. Unlike developing larval fish that suffer from severe gonadal abnormalities (e.g., intersex) when exposed to EEs [2,3], adult fish typically do not experience overt developmental problems when exposed for only a few weeks [4,5]. To date, most studies concerning the effects of short-term exposure to EEs have focused on males, and although such studies have routinely described increased expression of the egg yolk protein–precursor vitellogenin (VTG) and suppressed levels of androgenic hormones (testosterone and 11-ketotestosterone [KT]), few effects on fertility have been noted [6–9]. Almost all of these studies, however, have employed relatively simple laboratory bioassays that involved exposing males to effluents or synthetic estrogens and then testing the performance of these males when given ready access to females for extended periods of time (see, e.g., [7,8]). To date, little emphasis has been placed on understanding the behavior and fitness of exposed adult male fish, especially in groups.

Outside of the laboratory, the life histories of most fish involve intense competition for mates and/or spawning substrate [10–12]. Behavior typically plays a critical role determining individual reproductive success in groups of fish, the social dynamics of which can amplify individual differences [13]. Notably, circulating androgenic hormones typically are suppressed by EEs and are known to modulate agonistic behaviors of mature males [14–16]. Especially strong positive correlations have been described between KT and the performance of agonistic behaviors during periods of social instability (e.g., establishing a new territory or responding to territorial intrusion) [17,18]. For many fish, including the fathead minnow (*Pimephales promelas*), acquisition of spawning territory is a fiercely competitive process in which more aggressive males tend to acquire and maintain high-quality spawning territories (i.e., nest sites), whereas subordinate males often fail to reproduce [12]. Additionally, both testosterone and KT appear to influence nest-site preparation before spawning in at least some fish species in which males acquire and guard nests [19,20]. In the wild, disruption of androgen-related behaviors in male fish, where exposure to EEs may vary, could alter the dynamics of spawning and gene flow within populations.

The present study evaluated whether exposure to estrogenic effluent released by a STP might impair the behavioral performance of male fish and, as a consequence, suppress their competitive reproductive fitness. We examined the behaviors of male fathead minnows exposed to effluent released by a well-studied and significant STP, both in the presence, and in the absence of competing males, and we determined both whether and how their reproductive fitness changed over time. We used the fathead minnow because this species is native to

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our region of North America and is an important and widely used ecotoxicological model [21]. We posed two questions: First, does exposure to STP effluent (STPE) alter agonistic and nest-directed behaviors of male fathead minnows, and if so, do these behavioral changes lead to a reduction in their competitive reproductive fitness? Second, might EEs in that STPE be responsible for any observed impairments of behavioral performance and reproductive fitness?

MATERIALS AND METHODS

Experimental design

Two experiments were conducted. In experiment 1, we exposed male fathead minnows to effluent from the Metropolitan Wastewater Treatment Plant (MWTP) located in St. Paul (MN, USA; described below) for 21 d (a duration commonly used in short-term reproduction tests recommended by the Endocrine Disruptor Screening and Testing Advisory Committee [21]). We hypothesized that exposure to effluent from this plant, the estrogenicity of which has been established, would impair the performance of androgen-related behaviors, thereby causing a reduction in individual reproductive fitness that would be particularly pronounced during competitive situations. In experiment 2, we tested whether exposure to a model estrogen at concentrations designed to mimic the estrogenicity of this effluent (as evaluated by estrogen equivalents [EEQs]) might explain any observed effects. We exposed fish either to a model estrogen or to a model androgen (the first treatment to mimic the effects of the effluent and the latter to have the opposite effect). This experiment had two components: The first component (experiment 2A) tested the effects of exposing adult males to a concentration of 17β-estradiol (E_2) designed to reproduce the estrogenic activity of MWTP effluent based on measurements of its EEQ activity in an in vitro assay. The second component (experiment 2B) tested the effects of exposing adult males to a similar concentration of methyltestosterone (MT). We used MT because this synthetic steroid exerts actions that closely resemble those of KT and is less than one-thousandth the cost of the latter [14]. Male performance was evaluated in two test scenarios during each experiment: A ''noncompetitive'' scenario, in which individual males were placed into aquaria with a nest and two mature females, and a ''competitive'' scenario, in which individual males were placed together with an unexposed (competing) male, two females, and a nest.

Experimental animals and effluent

Fathead minnows obtained from the U.S. Environmental Protection Agency (Duluth, MN) were bred and their young reared at the University of Minnesota following established protocols [22]. The fish used in the present study were sexually mature (age, approximately seven months) and had no previous spawning experience. Males were identified based on the presence of secondary sexual characteristics (SSCs; i.e., nasal tubercles and dorsal pads).

The effluent used for exposure came from the MWTP in St. Paul. This source was selected because it is a major contributor to the Mississippi River (USA), uses many modern technologies, is close to our laboratory, and has an estrogenic nature that has already been established [23,24]. Influent to the MWTP is composed mostly of commercial and residential sewage (91%) and is subjected to both primary and advanced secondary treatment before being discharged into the Mississippi River (~700 million L/d; R. Polta, MWTP, St. Paul, MN, USA, personal communication). Because the identities of the compounds responsible for the estrogenic nature of the effluent are as yet unknown and, thus, unmeasurable (D. Swackhamer, University of Minnesota, St. Paul, MN, USA, personal communication), we estimated the total estrogenic activity of the effluent using an in vitro competitive rainbow trout estrogen receptor–binding assay (rtERA) [25]. To accomplish this, 6-L grab samples ($n = 3$) were collected, extracted using sets of six C18 columns (Waters Corporation, Milford, MA, USA), eluted with 100% methanol, and concentrated under a stream of nitrogen (37°C) to a volume of 100 μl. Duplicate samples of eluate were then diluted to create five dilutions (final concentration factors, 0.01–1,000) to generate complete binding curves. Competitive binding curves and the concentration that causes 50% inhibition (IC50) were then generated using nonlinear regression and a one-site competitor equation (GraphPad Software, San Diego, CA, USA) [25]. To calculate effluent EEQs, we divided the IC50 for E_2 (ng/L) by the IC50 for the effluent.

Experiment 1: Assessing the effects of effluent

Eight groups of eight mature, male fathead minnows were removed from stock tanks and placed into 70-L glass aquaria that received 100 ml/min of well water (25°C). After a week-long acclimation period, inflow to four of these aquaria was gradually changed to either the STPE or an appropriate control.

Effluent was collected daily (December 2–24, 2002) at the discharge channel of the MWTP and transported in a 400-L polypropylene container (Nalgene, Rochester, NY, USA) to our laboratory, where it was transferred to a stock tank and heated to 25°C using 100-W glass aquarium heaters. Heated effluent was then pumped to an overhead head-tank, from which it flowed into the exposure aquaria at a constant rate (100 ml/min). As a control, well water was pumped into an identical set of stock tanks, where it was heated and delivered to another head-tank and then into the exposure aquaria (100 ml/min). All fish were fed daily, and no disease or mortality was noted in either the control or effluent-exposed fish.

After 21 d of exposure, a subsample of 10 fish from each treatment ($n = 2$–3 from each aquarium) was killed by overanesthetizing them with 0.1% phenoxy-ethanol (Sigma Chemical, St. Louis, MO, USA), and a sample of their blood was collected from the caudal vasculature for KT and VTG analyses (see below). Each of the remaining males was then anesthetized, and 2 μl of blue latex (Fisher Scientific, Pittsburgh, PA, USA) were injected below their dorsal fin on either their right or left side so that we could identify individuals when they were paired for mating and observation. A pilot experiment showed that such injections did not affect survival or fish behavior (D. Martinovic and P. Sorensen, unpublished data). The day after latex injection (to permit recovery), individual fish were transferred for behavioral testing into 40-L glass test aquaria, each of which was supplied with a 50:50 mixture of effluent and well water (100 ml/min) so that both control and effluent-exposed males had to make similar adjustments to new water conditions. Two test scenarios were used ($N = 10$ for each scenario). In the first (noncompetitive scenario), single males (either control or exposed) were placed with two unexposed mature females and a nest (an 8-cm cross-section of polyvinyl chloride pipe [diameter, 0 cm]). In the second (competitive scenario), a control male was added along with the effluent-exposed male to aquaria containing two un-

exposed females and a nest. Male fish were selected so that their total lengths were within 3 mm of each other; one fish had a right-hand mark and the other a left-hand mark (marking pattern was not treatment specific).

Behavioral evaluation proceeded in two steps. On the first day of the experiment (day 0), we noted a variety of behaviors exhibited by males as they established territories (details below). Thenceforth (days 1–5), we noted only the number of males that had nests (i.e., ''nest holders'') and whether those nests had eggs. We followed this regime because we knew from previous studies (D. Martinovic and P. Sorensen, unpublished data) that once pairs of male fathead minnows have established territories in our test aquaria, they rarely lose them (≤10% of fish will change), that only nest holders sire young (a result confirmed by DNA fingerprinting), and that behaviors exhibited during the initial 24 h of competition are particularly intense and determine which fish become dominant. Experiments lasted 5 d because eggs hatch after approximately one week, and we wanted to collect the eggs before that time so that we could monitor reproductive success. All behavioral observations and nest-holder identification was performed blind; that is, the observer did not know the treatment group of the fish being observed.

Detailed behavioral observations were made just 10 min after the fish were placed into test aquaria (day 0), when we knew from previous experience that fish would start to compete for nest sites. For consistency, this period was always between 0800 to 1400 h. For these observations, individual males were first located and then observed for 10 min by an observer who was behind a blind and did not know the identity of the treatment group. The frequencies of six key reproductive behaviors described by McMillan and Smith [26] were noted: Butting/biting, an agonistic behavior in which males push or bite each other; tailbeating, another agonistic behavior in which males position themselves alongside another and then undulate their body and/or caudal fin to push the other; charging/chasing, a related agonistic behavior in which males swim toward and/or chase other fish; touching (the nest), a behavior in which males contact the nest with their bodies; rubbing/circling (the nest), a nest tending/cleaning behavior in which males rub the nest with their dorsal pad; and nibbling (the nest), a nest tending/cleaning behavior in which males contact and nibble the ceiling of their nest.

Identification of nest holders commenced after 24 h had elapsed from the time the fish were introduced into test aquaria (i.e., day 1). We identified nest holders in aquaria and whether their nests had eggs. To accomplish this, each aquarium was observed each day between 1000 and 1400 h for 5 min, and those fish that spent the majority of their time in a nest while also exhibiting any of the behaviors quantified on day 0 were categorized as nest-holders. We observed fish for 5 min because we were interested in establishing the identities of nest holders, not in quantifying all aspects of their behavior. After 5 d of performing such observations, fish were killed, and nests containing eggs were transferred to hatching jars, which were examined after another 7 d to count the number of hatched larvae. We assumed that nest holders sired the young hatched from the eggs collected from their nests based on previous experiments that employed DNA fingerprinting and confirmed, first, that only nest holders sired young and, second, that no successful ''sneaking behavior'' occurred under our experimental conditions [27]. Male-specific SSCs (nuptial tubercles and size of the dorsal pad) were scored postmortem according to established methods [12] in the males used during the competitive scenario. Briefly, we scored development of both their tubercles and dorsal pads using a scale that ranged from 0 (i.e., no tubercles or pad) to 3 (i.e., sharp, prominent tubercles and a wide, thick dorsal pad forming a sharp nape behind head). We summed tubercle and dorsal pad scores to obtain a SSC score (maximum possible score, 6).

Experiment 2: Assessing the effects of E_2 and MT

To determine if the effects observed during experiment 1 could have been caused by EEs, we exposed males to either waterborne E_2 (experiment 2A; described here) or waterborne androgen (experiment 2B; see below) or to a well-water control for 21 d. Protocols closely followed those of experiment 1. For both experiments, we selected 50 ng/L of steroid as the target concentration based on our rtERA analysis of the effluent (see above). For experiment 2A, mature males ($n = 80$) were evenly distributed between eight 70-L aquaria, each of which was supplied with both well water (400 ml/min, a faster flow than used in experiment 1 to promote dilution of the concentrated steroid stock) and either a continuous inflow (1 ml/min) of E_2 stock solution (20 ng/ml of E_2 and containing 5 μl of ethanol/L) or solvent control (5 μl/L of ethanol). As measured by enzyme-linked immunosorbent assay (ELISA; Cayman Chemicals, Ann Arbor, MI, USA), the concentration (mean ± standard error [SE]) of E_2 in exposure aquaria was 31.01 ± 1.25 ng/L ($n = 4$) versus 0.23 ± 0.04 ($n = 4$) for the control. Fish were exposed for 21 d, after which 20 fish were killed for blood samples and 48 were distributed into thirty-six 40-L test aquaria, where they were allowed to reproduce in either competitive ($n = 12$) or noncompetitive ($n = 12$) scenarios as described in experiment 1.

Experiment 2B was identical to experiment 2A except that we exposed males to MT instead of E_2. Sample size also was slightly larger ($n = 12$ for the noncompetitive scenario and $n = 19$ for the competitive scenario). Lacking an assay for MT, exposure conditions were based on nominal concentrations only.

Blood processing and analyses

Blood was placed on ice immediately after collection and centrifuged at 10,000 rpm for 1 min. Half the plasma was then transferred into aprotinine-coated microcentrifuge tubes to prevent degradation and stored at −20°C for VTG analyses. Plasma VTG concentrations were measured using an ELISA that employed a fathead minnow antibody [28]. The other half of the plasma was stored at −20°C, and KT was extracted and measured later using a radioimmunoassay following established protocols [29].

Statistical analyses

Differences in the KT and VTG levels of control and exposed males were assessed for each experiment using *t* tests (STATISTICA; StatSoft, Tulsa, OK, USA). Differences in SSCs also were evaluated by *t* tests. Agonistic and nest-directed behaviors were analyzed using nonparametric Mann–Whitney *U* tests, because these data were not normally distributed (STATISTICA). To determine if the ability of fish to obtain and hold nests was associated with endocrine-disrupting chemicals, we compared the number of control and exposed nest holders in each scenario each day using Fisher's exact tests (STATISTICA). Finally, differences in the cumulative number of hatched larvae sired by control versus exposed

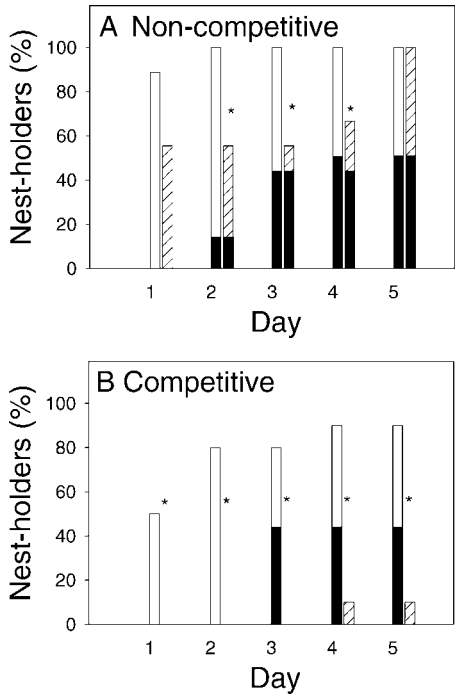

Fig. 1. Relative number (percentage) of control males (clear bars) and effluent-exposed males (hatched bars) that held nests in the (**A**) non-competitive and (**B**) competitive assays in experiment 1. The relative number (percentage) of the males that had eggs in their nests is blackened. $^{*}p < 0.05$, $^{+}p < 0.1$.

males were evaluated using *t* tests (STATISTICA). All tests were two-tailed, and findings were considered to be significantly different at $p < 0.05$.

RESULTS

Experiment 1: Assessing the effects of effluent

The rtERA analysis revealed that MWTP effluent had an EEQ activity (mean ± SE) of 44 ± 0.9 ng/L. Exposure to this effluent induced VTG production (5.67 ± 1.93 vs 0.001 ± 0.0002 mg/ml in control males; $p < 0.01$). Circulating levels of KT also were suppressed after exposure to this STPE (8.42 ± 2.251 vs 34.07 ± 3.87 ng/ml in control males; $p < 0.05$). In the absence of competition, nearly 90% of all unexposed control males held nests within 1 d and all had a nest within 2 d, whereas it took 5 d for all STPE-exposed males to hold nests (Fig. 1). Nevertheless, approximately half of all males in both treatment groups had fertilized eggs in their nests after 5 d, resulting in similar levels of overall reproductive fitness (i.e., mean numbers of hatched larvae) (Fig. 2).

Especially dramatic differences were evident between control and effluent-exposed males when they were tested together in the competitive scenario. Thus, whereas nearly half the unexposed control males had acquired nests by day 1 and 80% by day 2, no STPE-exposed males acquired nests until day 4 in this testing scenario (Fig. 1). Only one STPE-exposed fish had reproduced successfully by the last day of the experiment, whereas five of the competing control animals had reproduced. None of the eggs found in the STPE-exposed fish's nest hatched, whereas the control fish sired nearly 100 young. We observed that control males exhibited much higher levels of agonistic and nest-related behaviors than did STPE-exposed fish ($p < 0.05$ for butting/biting, charging/chasing, nibbling, and rubbing/circling behaviors) on day 1 (Table 1). Finally, effluent-exposed males had less developed, sexually dimorphic features than did control males (average SSC scores, 2.3 ± 0.50 vs 4.1 ± 0.59; $p < 0.05$).

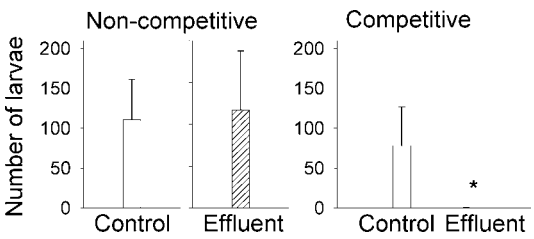

Fig. 2. Cumulative number (mean ± standard error of the mean) of offspring sired over 5 d by control (clear bars) and effluent-exposed (hatched bars) males tested in both competitive and noncompetitive scenarios in experiment 1. $^{*}p < 0.05$.

Table 1. Frequency of agonistic and nest-directed behaviors (no. of behaviors/10 min) of males exposed to either well water (control) or sewage treatment plant effluent (effluent) on the first day of testing (day 0) in a competitive scenario (median no. of behaviors/10 min [25th and 75th percentiles])[a]

	Control (n = 10)	Effluent (n = 10)
Agonistic behaviors		
Butt/bite	3.5 (2, 13)	1.5 (1, 3)*
Charge/chase	4.0 (2, 7)	0.0 (0, 2)*
Tailbeat	0.5 (0, 2)	0.0 (0, 2)
Nest-directed behaviors		
Touch	9.0 (3, 17)	0.0 (0, 3)
Nibble	3.0 (2, 7)	0.0 (0, 0)*
Rub/circle	6.0 (1, 12)	0.0 (0, 0)*

[a] Mann–Whitney *U* test (*$p < 0.05$).

Experiment 2A: Assessing the effects of E_2

Exposure to E_2 induced VTG production in males (18.01 ± 1.4 vs 0.009 ± 0.006 mg/ml in control males; $p < 0.05$). As with effluent-exposed fish, circulating levels of KT also were suppressed in E_2-exposed males (2.31 ± 2.18 vs 11.01 ± 2.18 ng/ml in controls; $p < 0.05$). Although a trend was observed toward decreased SSC scores in E_2-exposed males (3.9 ± 0.23 vs 4.4 ± 0.16 for controls), the difference was not significant ($p = 0.09$). When tested in the noncompetitive scenario, both E_2-exposed and control males acquired nests at similar rates (Fig. 3) and produced equivalent numbers of fertilized eggs, which then hatched to produce similar numbers of young (Fig. 4). A notable decrease in the number of males holding nests (but lacking eggs) was observed for the E_2-exposed fish on the last day (Fig. 3). In the competitive assay, E_2-exposed males performed agonistic and nest-directed behaviors less frequently ($p < 0.05$ for butting/biting, charging/chasing, touching, and rubbing/circling) (Table 2). As a result, as with effluent-exposed males, E_2-exposed males were less able ($p < 0.05$) to acquire nests (Fig. 3), to fertilize eggs, and to produce young in the presence of competing control males (Fig. 4).

Experiment 2B: Assessing the effects of MT

Exposure to MT did not induce production of VTG (0.007 ± 0.006 vs 0.004 ± 0.003 mg/ml in control males; $p > 0.10$). A trend was observed toward increased SSC scores in MT-exposed males, but the difference was not significant (2.83 ± 0.42 vs 1.94 ± 0.37; $p = 0.12$). In the absence of competition,

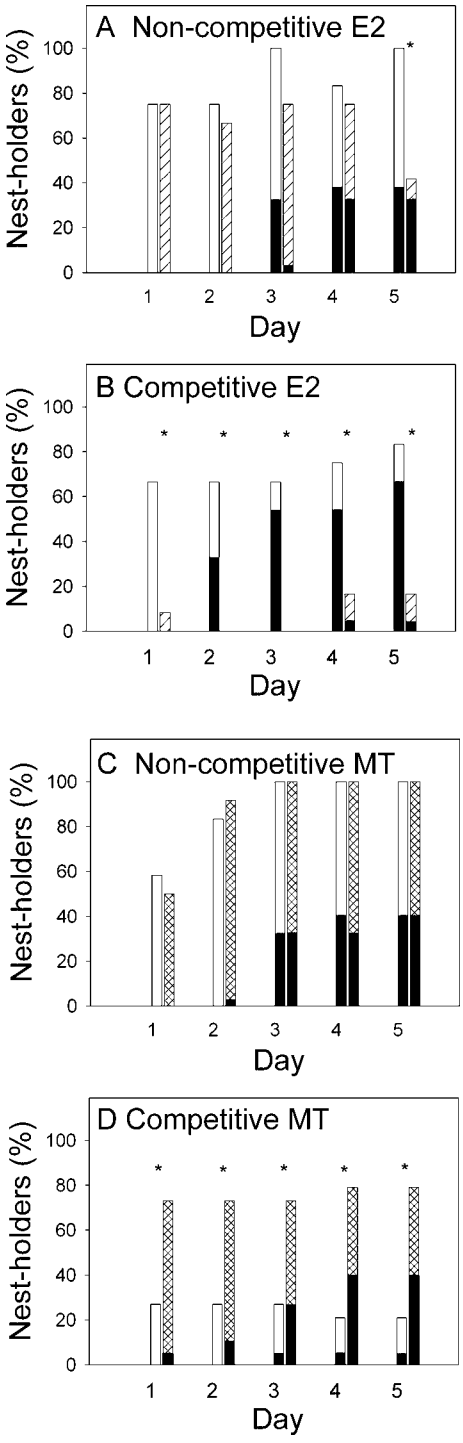

Fig. 3. Relative number (percentage) of control males (clear bars) and 17β-estradiol (E_2)–exposed males (hatched bars) that held nests in the noncompetitive (**A**) and competitive (**B**) scenarios in experiment 2A. Nest-holding control (clear bars) and 17α-methyltestosterone (MT)–exposed (cross-hatched bars) males also are shown for the noncompetitive (**C**) and competitive scenarios (**D**) for experiment 2B. The relative number (percentage) of males that had eggs in the nests is blackened in each instance. *$p < 0.05$.

MT-exposed and control males acquired nests at similar rates, and all males had nests by day 3 (Fig. 3). Each group also sired similar numbers of young (Fig. 4). In the competitive assay, MT-exposed males were more aggressive than control males ($p < 0.05$ for butting) (Table 2) and acquired more nests in the presence of competitors, which they maintained throughout the experiment (Fig. 3). As a result, MT-exposed males also produced many more eggs and larvae than did control males (Fig. 4). Notably, however, the rate at which MT-exposed males acquired nests and sired young in the noncompetitive scenario was comparable to that of the control males.

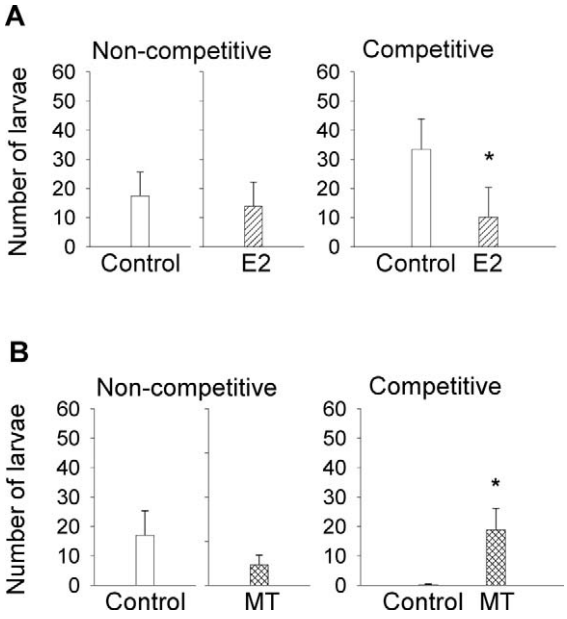

Fig. 4. Mean cumulative number of offspring sired over 5 d by control (clear bars) and 17β-estradiol (E_2)–exposed males (hatched bars) in experiment 2A (**A**) and control (clear bars) and 17α-methyltestosterone (MT)–exposed (cross-hatched bars) males tested in experiment 2B (**B**) Results from both the competitive and noncompetitive tests are shown in each instance. *$p < 0.05$.

DISCUSSION

The present study demonstrates that exposure to EEs suppresses androgenic hormones and reduces levels of agonistic behavior in male fathead minnows, leading to significant reductions in reproductive fitness when spawning is competitive. Both STPE and a concentration of E_2 designed to mimic the estrogenicity of the STPE exerted nearly the same behavioral and physiological effects on adult male fathead minnows, suggesting that the effects of STPE exposure likely were associated with the presence of estrogenic compounds. Reproductive impairment was most notable in the presence of a competitor, suggesting that short-term exposure to EEs may have

Table 2. Frequency of agonistic and nest-directed behaviors (no. of behaviors/10 min) of males exposed to either 17β-estradiol (E_2) or 17α-methyltestosterone (MT) during day 0 of testing in a competitive scenario (median [25th and 75th percentiles])[a]

	Experiment 2A		Experiment 2B	
	Control (n = 12)	Estradiol (n = 12)	Control (n = 19)	MT (n = 19)
Agonistic behaviors				
Butt/bite	6.0 (2, 24)	0.0 (0, 2)*	0.0 (0, 6.5)	4.0 (0, 7)*
Charge/chase	1.0 (0, 3)	0.0 (0, 0)*	0.0 (0, 2)	0.0 (0, 3)
Tailbeat	0.0 (0, 2)	0.0 (0, 1)	0.0 (0, 0)	0.0 (0, 1)
Nest-directed behaviors				
Touch	0.5 (4, 12)	0.0 (0, 3)*	0.0 (0, 1)	0.0 (0, 1)
Nibble	1.5 (0, 4)	0.0 (0, 0)	0.0 (0, 0)	0.0 (0, 1)
Rub/circle	1.0 (0, 21)	0.0 (0, 0)*	0.0 (0, 2)	0.0 (0, 1)

[a] Mann–Whitney U test (*$p < 0.05$).

important effects on male competitive reproductive fitness, a possibility that has received little attention to date. Because competition for mating opportunities is common among male fish in the wild [10–12], we believe that our findings are especially significant for populations of wild fish that are exposed to estrogenic effluents, including those from STPs.

The present study shows that subtle behavioral impairments alone can be sufficient to reduce the reproductive fitness of fish that must compete for reproductive opportunities. We also found evidence that such behavioral deficits are accentuated with time and social interaction (i.e., competition). Thus, the reduced speed with which EE-exposed males acquired nests in the absence of competitors apparently translated into a decreased ability of these fish to acquire nesting territories and, hence, into a greatly reduced ability of these fish to reproduce. We believe that this set of behavioral deficits was caused by exposure to EEs, because STPE- and E_2-exposed male fish experienced nearly identical changes in their circulating levels of KT and VTG as well as in their SSC expression, competitive behavior, and reproductive success. Even more convincingly, we found that males exposed to an androgen exhibited the opposite trends in all measured parameters. We are confident that the levels of E_2 used in the present study approximated the total estrogenicity of MWTP, because the rtERA assay that we employed has been validated by other bioassays (VTG mRNA and VTG protein) and for another STPE [27]. Furthermore, the estrogenic activity of MWTP effluent is similar to the values reported for other North American and European STPEs [30,31]. This suggests that the reduced competitive fitness we observed may not be limited to MWTP effluent and that even plants with modern processing technologies may by significant sources of EEs, which influence the competitive reproductive success of exposed fish.

Although to our knowledge the present study is the first to examine competitive reproductive fitness in externally fertilizing adult male fish exposed to endocrine-disrupting compounds that include EEs, the changes that we observed are similar to those noted by other investigators and, thus, may be broadly relevant. In perhaps the most detailed behavioral study conducted on EE-exposed fish, Bell et al. [32] noted that male three-spined sticklebacks (*Gasterosteus aucleatus*) exposed to EEs are less aggressive toward other males and are less interested in females (reproductive success was not examined). One study of the fathead minnow also provided evidence of decreased agonistic behavior after exposure to EEs (again without examining reproductive success) [33]. In an earlier study, we noted various subtle effects in the behavioral performance of the scramble-spawning goldfish (*Carassius auratus*) after exposure to MWTP effluent [7]. Finally, Kristensen et al. [34] found that juvenile guppies (*Poecilia reticulata*) exposed to high levels of 17α-ethinylestradiol for their entire life history also reproduce less successfully in the presence of a male competitor; however, these fish also experienced a variety of problems, including reduced sperm counts, reduced expression of SSCs, coloration, and reduced courtship behavior, making it difficult to deduce if competitive behavior per se was a key factor. Furthermore, no effects on reproductive fitness were observed at lower, more realistic doses of 17α-ethinylestradiol in guppies. In the present study, it appeared that EE-exposed males did not experience reduced physiological fertility, because in the absence of competition, they sired as many young as the unexposed fish did.

Although the primary cause of reduced competitive reproductive fitness of EE-exposed male fathead minnows likely was their reduced aggressiveness, subtle changes in nest-oriented behaviors also may have played a role. The seemingly low motivation of EE-exposed fish to inspect, prepare, and maintain nests may have been responsible for the lag in nest acquisition by effluent-exposed males. Abandonment of nests by E_2-exposed males in the noncompetitive scenario of experiment 2A may have had similar causes, but no such effect was noted in effluent-exposed fish. We observed no differences in the performance of nest-directed behaviors in the MT-exposed males. This may have been caused by the fact that androgens have little influence on nest tending, but control males in that experiment also performed little nest-directed behavior. Although we are unsure why these males performed so few behaviors, it does not alter our conclusions, because comparisons were made between a matched set of control and exposed fish. Only a handful of other studies have examined the effects of estrogens on nest building and nest guarding in fish, and they too have described delayed or reduced nest-directed behavior among males exposed to EEs [35,36]. For fish in the wild, where it is important for males to acquire a nest quickly, delays in nest acquisition can lead to marked reductions in reproductive fitness [11,12]. Whether courtship behavior of fathead minnow males exposed to EEs also might have been suppressed was not determined. This seems likely, however, because it also has been commonly observed in other EE-exposed male fish [13].

Our observation that STPE- and E_2-exposed male fathead minnows had reduced levels of circulating KT has been noted in both EE- and STPE-exposed males [6,21] and could have been a root cause of the effects that we observed. A correlation between KT levels and agonistic behaviors has been noted previously [16,17,32], but speculation about a link between the two must be made cautiously. It is difficult to know whether hormones affected behavior, or vice versa, or both [15]. Estrogens also affect behavior through a variety of other mechanisms, including sensory impairments [13]. It is even more difficult to draw direct connections between nest-directed behaviors and titers of androgenic hormones; however, Sikkel et al. [19] documented a positive correlation between KT levels and algal fanning (i.e., a nest-directed behavior performed exclusively during premating) in the garibaldi (*Hypsypops rubicundus*). It will be important to determine the specific links between hormones and behavior to ascertain how endocrine disruptors exert their effects and, thus, how the threats they pose might eventually be reduced.

It is both interesting and important to consider how reductions in individual competitive drive and reproductive fitness might translate to wild populations of fish. It is possible that some populations could be continuously exposed to constant levels of EEs, thus leading to outright reproductive failure and population collapse [37], but this seems likely to be uncommon. Instead, the complex, discontinuous (temporal and spatial) distribution of effluent plumes coupled with the idiosyncrasies of natural patterns of fish movement make it probable that individual exposure regimes will vary greatly. This would be especially true in rivers such as the Mississippi River. Thus, we hypothesize that EEs may be causing reductions in the number of adults contributing to the gene pool, thereby reducing overall genetic variation. Emigration and immigration would amplify these effects, as would any physiological attributes that might make specific genotypes more susceptible to certain endocrine disruptors [38]. All of these effects could

combine to alter dramatically the patterns of individual reproductive success and, thus, lead to a loss of heterozygosity, which normally enhances the ability of a population to adapt to environmental changes [39]. Indeed, several instances of disrupted genetic structure and gene flow have been noted in populations of wild fish at contaminated sites [40], and although we are not aware that EEs per se have been suggested as a possible explanation, the present study indicates that they should be considered.

In conclusion, the present study indicates that EE-induced alterations in behavior can lead to reduced individual reproductive fitness and, thus, that competitive behavior should be considered as an end point when extrapolating or modeling effects of EEs on populations of fish. Therefore, levels of EEs that have failed to show effects on sperm production or reproduction among paired laboratory fish should not necessarily be considered as having no long-term effects on populations of wild fish. Clearly, assays that employ simple scenarios may not be adequate to fully assess the effects that fish may be experiencing in more complex ecological contexts. Use of this competitive behavioral assay offers biologically-relevant, sensitive data that can provide new opportunities for evaluating environmental risk. Even more realistic competitive assays also might be developed to investigate scenarios that involve multiple competitors, varying exposure times and concentrations, and multiple generations.

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