# EFFECTS OF EXPOSURE TO LOW LEVELS OF WATER-BORNE 17b-ESTRADIOL ON NEST HOLDING ABILITY AND SPERM QUALITY IN FATHEAD MINNOWS

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## ABSTRACT

This study assessed the effects of exposure to low levels of estradiol (E2), as they have been found in some treated sewage effluents, on nest holding ability and sperm quality, two measures likely related to reproductive success in fathead minnows (*Pimephales promelas*), a species with paternal nest care. Male fathead minnows were exposed for 21 days to either 50 ng/L (E2) in 25 µl/L ethanol, an E2 concentration found in the effluent of some British and Canadian sewage treatment plants (STP), or to a 25 µl/L ethanol control (aqueous flow-through exposure). After exposure, groups of six males from one treatment were placed into an observation aquarium with females and a limited number of spawning sites and allowed to spawn for four days. Plasma vitellogenin (VTG) concentrations, the ability to acquire and defend a nest site, and sperm quality were compared between E2 exposed and control males. E2 exposure resulted in a significant increase in VTG concentrations in E2 exposed males, but did not affect nest holding ability or sperm quality. These preliminary results indicate little effect of 21-day exposure to levels of E2 that match the highest reported E2 loads in STP effluent, and parallel our findings from a previous study using goldfish. However, caution is warranted in extrapolating these results to populations of wild fish, as other factors such as the overall estrogenic potency of effluent, the duration and timing of exposure to estrogenic compounds, and the competition between fish for spawning opportunities need to be estimated.

## INTRODUCTION

The steroid 17ß-estradiol (E2) is an important hormone in endocrine pathways of fishes (Arcand-Hoy & Benson 1998) and occurs frequently in effluent discharge of municipal sewage treatment plants (STP) (Desbrow et al., 1998; Ternes et al., 1999; Rodgers-Gray et al., 2000) where it can cause endocrine disruption in fishes (Purdom et al., 1994; Harries et al., 1997; Routledge et al., 1998). Numerous studies have assessed the effects of E2 and associated xenoestrogens on wild fish (Folmar et al., 1996; Jobling et al., 1998; Lee et al., 2000; Folmar et al., 2001), fish that were caged (Purdom et al., 1994; Harries et al., 1996; Harries et al., 1997; Routledge et al., 1998; Rodgers-Gray et al., 2001), and on fish in the laboratory (Kramer et al., 1998; Panter et al., 1998; Miles-Richardson et al., 1999; Korte et al., 2000; Bjerselius et al., 2001). All of these studies found induction of the yolk protein vitellogenin (VTG) in exposed male fish, as well as other effects such as gonadal abnormalities (Gimeno et al., 1998; Jobling et al., 1998; Miles-Richardson et al., 1999; Rodgers-Gray et al., 2001), changes in behavior (Bayley et al., 1999; Bjerselius et al., 2001), and sperm production (Gimeno et al., 1998; Bjerselius et al., 2001). However, little is known about the reproductive effects of exposure to E2 at these low concentrations. In a previous study (Schoenfuss et al., in press), we found that 50 ng/L E2 exposure resulted in minor reductions in spawning behavior and sperm production in male goldfish (*Carassius auratus*), which have no parental care. Based on these results, we designed a new experiment to determine whether exposure to similar E2 concentrations would be more detrimental to fish with greater paternal involvement in reproduction. E2 exposure concentrations were chosen to match the highest E2 concentrations reported in sewage effluent in Great Britain (Desbrow et al., 1998; Rodgers-Gray et al., 2000) and Canada (Ternes et al., 1999). In this study we exposed male fathead minnows (*Pimephales promelas*), a species with paternal nest care, to low levels of E2 and assessed the effects of exposure on the fishes ability to hold nest sites and to produce viable sperm.

The fathead minnow is widely used in toxicological studies (Ankley et al., 1998) and reproduces in the

laboratory. Fathead minnows breed year round with the male guarding a cavernous nest site until eggs hatch. The ability to defend a nest is determined by the individual's social status in which dominant males, identifiable by their tubercles, dorsal pad, and bright banding pattern, establish successful breeding territories (Cole & Smith, 1987). Smith (1974) suggested that these morphological characteristics are associated with elevated androgen levels. The male defends the nest site continuously until hatching as eggs are quickly eaten by other fathead minnows in the absence of the nest-guarding male (Sargent, 1989). Estrogen exposure could increase circulating E2 levels in male fish and suppress androgen levels, by altering neuroendocrine feedback loops (Trudeau et al., 1993; Jobling et al., 1996; Ankley et al., 1998).

We expect nest holding ability to be a sensitive indicator of reproductive success as the opportunity to spawn is conditional upon possession of a nest site (Unger, 1983; Huntingford & Torricelli, 1993). We also expect sperm production to serve as a sensitive and relevant measure of the effects of estrogen exposure as sperm production is important in externally fertilizing fish (Petersen & Warner, 1998). A reduction in sperm quantity (which normally increases in association with interactions with ovulating females (Stacey & Sorensen, 1986; Dulka et al., 1987; DeFraipont & Sorensen, 1993)) or quality can lead to reduced fertilization rates (Nakatsuru & Kramer, 1982; Zheng et al., 1997; Suquet et al., 1998; Cosson et al., 2000). By exposing fathead minnows to E2 we addressed two questions. (1) Is sperm quality in fathead minnows compromised by E2 exposure? (2) Are E2 exposed male fathead minnows able to maintain nest sites?

## MATERIALS & METHODS

### Experimental Animals

Fish for this study were bred and reared at the University of Minnesota. Fish were held in 28°C water (flow-through, 300 ml/min) from an in-house well, at a constant photoperiod (16 h:8 h light:dark, 0800 lights on), and were fed frozen brine shrimp (San Francisco Bay Company, CA) twice daily *ad libitum.*

### Experimental Design

An experiment was conducted to assess the effects of E2 exposure on nest holding ability and sperm quality in male fathead minnows. In four trials, males were exposed for 21 days to either E2 or an ethanol control. After exposure, groups of males were placed with females and allowed to spawn. Male behavior was observed, after which plasma VTG concentrations, and sperm quality were quantified.

### Exposure

For each trial, 16 spermiating fathead minnows were netted from a stock aquarium and distributed evenly among four 20 L aquaria. Aquaria were aerated, shielded on three sides, and fish were maintained otherwise as in the stock aquaria. Males were held for 21 days in either (i) 50 ng/L E2 dissolved in 25 µl/L ethanol, or in (ii) a 25 µl/L ethanol control. Experiments with guppies (*Poecilia reticulata*) and goldfish (Bayley et al., 1999; Schoenfuss et al., in press) found no effects of ethanol exposure on spawning behavior and sperm production. Both treatments were administered via continuous flow-through delivery (280 ml/min). A peristaltic pump continuously supplied two aquaria with an E2 stock solution, while the other two aquaria received ethanol at concentrations similar to the one used to prepare the E2 stock solution. E2 was extracted weekly from E2 exposure aquaria and bi-weekly from the control aquaria. Water samples were concentrated using C18 SepPaks (Waters Corporation, MA), and than measured with an E2 ELISA test kit (Cayman Chemicals, MI). Aquarium concentrations of E2 were approximately 50 percent of the 50 ng/L nominal concentration at 24 ± 3.6 ng/L E2 (mean ± standard error). E2 concentrations in the ethanol control aquaria were below detection limit (9 ng/L).

### Spawning Assay

After 21 days, groups of six male fish from the same treatment were moved from the exposure aquaria into 160 L observation aquaria (day one). Each observation aquarium contained six mature female fathead minnows and three nest sites (3" PVC pipe sections) to ensure that only nest sites are limiting reproductive success. In preliminary studies performed with unexposed males in a similar setting, males routinely occupied all nest sites within 24 hours. Males were marked using a combination of the same three latex colors that were injected subcutaneously. A pre-experiment found no effects of latex injection on the reproductive ability of male fathead minnows.

Male fathead minnows were observed twice each morning (days two through five) for five-minute intervals. During the observation period the nest holding male in each of the three nest cavities was identified. Each nest defended by a male fathead minnow was scored as a "nest holding event" for the treatment. Data from all four replicates were pooled

resulting in a total of 48 "nest holding events" per aquarium and treatment (three nests x four observation days x four replicates).

On the fifth day, fish were anesthetized with 0.1% phenoxyethanol (Sigma, St. Louis, MO), placed upside-down into a grooved sponge, and then milt (sperm and seminal fluid) was drawn into a capillary tube by applying light abdominal pressure following established protocols (Stacey & Sorensen, 1986). Care was taken not to contaminate the sample with feces or urine. For storage, 1 µl of milt was transferred into 100 µL of isoosmotic sperm extender following published protocols (Chao et al., 1987). For the sperm analysis, 10 µl of sperm extender solution was suspended in 100 µl distilled water (1:2000 final dilution) and vortexed. The drop in osmotic pressure activated the sperm sample (Billard et al., 1995), which was then analyzed using a Hamilton-Thorn motility analyzer (Danvers, MA). Mean Progressive Velocity (MPrV), a measure of sperm velocity along its path was used to characterize sperm quality (Kime et al., 1996). Each sperm sample was analyzed three times and values were averaged to determine the MPrV for each fish. MPrV was calculated only for those fish with expressible milt.

VTG Assay

After completion of sperm analysis, blood was collected from the caudal vasculature, stored on ice, and centrifuged at 10,000 rpm for one minute. Plasma was transferred into aprotinine coated microcentrifuge tubes and stored at –20°C for later analysis. Plasma VTG concentrations were determined using a fathead minnow-specific ELISA following published protocols (Parks et al., 1999). For the statistical analysis VTG concentrations below detection limit were reported as zero.

Statistical Analysis

Sperm quality and plasma vitellogenin concentrations were analyzed using a nonparametric Mann-Whitney U test as some of the data were not normally distributed. The proportional nest holding data were analyzed using a Fisher's Exact test. All calculations were performed with the Prism 2.0 statistical package for the Macintosh (Graphpad Software Inc., CA).

**RESULTS**

Plasma VTG concentrations in E2 exposed male fathead minnows were significantly higher than in control males ($p<0.01$, Mann-Whitney U test; Table 1). Despite VTG induction in exposed males, sperm quality did not differ between E2 exposed and control males and the MPrV was virtually identical ($p>0.05$, Mann Whitney U test, Table 1). The ability to hold nest sites also did not differ between E2 exposed and control fish. The number of nest holding events was similar for E2 exposed and control male fathead minnows ($p>0.05$, Fisher's Exact test; Table 1). E2 exposed male fathead minnows were holding nest sites during all 48 observations, while control males held nests during 44 out of 48 observations. As males were color-coded it was also possible to identify changes in the identity of the nest holder from day to day. However, very few incidents in which the nest holding male changed from one day to the next were observed in either treatment.

**DISCUSSION**

Our preliminary results indicate that three-week exposure to 50 ng/L E2, a concentration exceeding E2 loads in the effluent of at least two of the few STP effluents that have been analyzed for E2 loads, consistently induced VTG production in mature male fathead minnows. The reproductive consequences associated with this physiological effect appear minor under the experimental conditions presented in this study. These results match our earlier findings in goldfish, for which ten week exposure to E2 at similar concentrations results in VTG induction but had relatively minor effects on spawning behavior and sperm production (Schoenfuss et al., in press). Ours is the first study to evaluate the effects of aqueous exposure to E2 at concentrations that simulate E2 loads in some STP effluents on nest holding ability and sperm quality in fathead minnows.

Table 1. Plasma vitellogenin (VTG) concentrations, sperm quality, and nest holding ability in male fathead minnows exposed to E2 or a control.

| | | **Control** | | **E2** | | |
|---|---|---|---|---|---|---|
| | | N | Mean ± Stand. Err. | N | Mean ± Stand. Err. | |
| VTG (mg/ml) | | 23 | 0.01±0.006 | 24 | **29.12 ± 0.62 | p<0.01 |
| MPrV (µm/sec) | | 11 | 45.45 ± 2.12 | 12 | 45.41 ± 0.95 | ns |
| Nest Holding | (days) | | 44 | | 48 | ns |
| | (%) | | 92% | | 100% | ns |

The E2 exposure protocol used in this study is notable because the absolute exposure concentration in this study (24 ng/L) is below the highest E2 loads reported for STP effluent. Although the E2 concentration in this study was higher than effluent E2 concentration reported for U.S. STP effluents, some studies have reported E2 concentrations in STP effluent as high as 88 ng/L in Great Britain (Desbrow et al., 1998, Rodgers-Gray et al., 2000) and 64 ng/L in Canada (Ternes et al., 1999). Thus, our exposure protocol represents a high, but still realistic exposure scenario. Furthermore, E2 is but one of many estrogenic compounds that have been found in STP effluent, and the overall estrogenic potency of the effluent could be much greater than simulated in this experiment.

The plasma VTG concentrations found in E2 exposed male fathead minnows in this study match those reported by Parks et al., (1999), in fathead minnows exposed to E2 via injection. Despite VTG induction, the fish appeared healthy and active and in each replicate spawning behavior could be observed within 24 h in both treatments. Nest holding ability of E2 exposed fathead minnows was also not affected by E2 exposure, with E2 exposed males holding all nest sites in all replicates of the experiment. The ability to defend a nest site is crucial for the reproductive success of male fathead minnows as eggs in any unguarded nest are quickly eaten by other fathead minnows (Sargent, 1989). Our results are similar to those reported in two studies on guppies that did not find reproductive impairment at 30 ng/L E2 exposure (Toft & Baatrup, 2001), and saw no behavioral impairment at 100 ng/L E2 exposure (Bayley et al., 1999). In contrast, a previous study conducted in our laboratory (Schoenfuss et al. in press) found a significant decline in reproductive behavior in male goldfish exposed to 50 ng/L E2 for ten weeks. Bjerselius et al., (2001), also found behavioral impairment at E2 exposure concentrations exceeding 1 µg/L E2, but that E2 exposure level is far above any recorded for STP effluents.

MPrV, a measure of sperm quality did not vary between E2 exposed and control fish. Sperm quality has been suggested previously (Kime et al., 1996; Kime 1998; Kime & Nash, 1999) as a tool to analyze the effects of EDCs on the reproductive fitness of fish. However, despite the strong physiological response (VTG induction) in male fish exposed to E2, sperm quality appeared unaffected. In a previous study using goldfish (Schoenfuss et al., in press), the absolute number of motile sperm per fish, a product of sperm quality and sperm quantity, declined with 50 ng/L E2 exposure after ten weeks. In contrast, Toft and Baatrup (2001) saw an increase in sperm numbers in guppies exposed to 30 ng/L E2. In this experiment we were unable to measure sperm quantity as the small size of the fathead minnow renders it impossible to strip consistently all milt while avoiding contamination by urine.

Although we did not find any impairment of nest holding ability and sperm quality in male fathead minnows exposed to E2, we remain cautious about extrapolating these laboratory results to populations of wild fish. Indeed, ongoing studies in our laboratory suggest that intra-species interactions might be an important component in evaluating the effects of E2 exposure. Furthermore, STP effluent consists of complex mixtures of chemicals, many of which have been shown to be estrogenic to fish and their combined action may exceed our exposure concentrations. Our understanding of the effects of STP effluent on fish populations is still rudimentary and further studies will be necessary to determine the effects of estrogen and xenoestrogen exposure on all life stages of fish. Answering these questions will allow us to determine the effects of effluent exposure on wild fish populations.

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