# Exposure to 17α-Ethinylestradiol Decreases Motility and ATP in Sperm of Male Fighting Fish *Betta splendens*

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**ABSTRACT:** The synthetic estrogen 17α-ethinylestradiol (EE2) is an endocrine-disrupting chemical released into aquatic environments from sewage treatment facilities. We tested the effects of two environmentally relevant concentrations of waterborne EE2, 10 and 100 ng $L^{-1}$, on reproductive endpoints in the teleost fish *Betta splendens*. In the first experiment, testes were removed from males and sperm were exposed to EE2 directly through the activation water. Direct exposure to EE2 had no effect on any measure of sperm swimming performance. In the second experiment, we exposed sexually mature male *B. splendens* to EE2 using a semi-static exposure protocol for 4 weeks. There were no significant treatment effects in the 10 ng $L^{-1}$ treatment group, but at the 100 ng $L^{-1}$ dose we found that fish had smaller gonads and reduced sperm swimming velocity. When allowed to interact freely with female conspecifics, males exposed to 100 ng $L^{-1}$ EE2 built smaller nests and showed a nonsignificant decrease in fertilization success. To investigate further the potential mechanism underlying the decrease in sperm quality, we repeated the chronic exposure experiment and analyzed the ATP content of sperm from fish in each treatment group. We found that males exposed to 100 ng $L^{-1}$ of EE2 had fewer moles of ATP per sperm than did fish in the other two treatment groups, suggesting that a decrease in intracellular ATP caused a reduction in sperm swimming velocity. The current study adds to the growing body of literature that indicates the risks to aquatic organisms of exposure to environmentally relevant concentrations of EE2. © 2012 Wiley Periodicals, Inc. Environ Toxicol 29: 243–252, 2014.

**Keywords:** endocrine disruption; gonadosomatic index; sperm velocity; nest building; spawning

## INTRODUCTION

The synthetic estrogen 17α-ethinylestradiol (EE2) is an active component in oral contraceptives and hormone replacement therapy (Kime, 2001). As a result, low levels of EE2 are typically detected in sewage treatment plant effluent (Larsson et al., 1999). Maximum levels of EE2 in treated effluent can be as high as 40–60 ng $L^{-1}$ (Belfroid et al., 1999; Ternes et al., 1999), but are generally measured at 1–10 ng $L^{-1}$ (Kolpin et al., 2002; Ying et al., 2002; Huggett et al., 2003). EE2 and related metabolites have also been detected in surface water, groundwater, and drinking water (Halling-Sørensen et al., 1998; Heberer, 2002; Hannah et al., 2009). For example, a study of 139 rivers across 30 states in the US found maximum and median concentrations of 831 and 73 ng $L^{-1}$ respectively, and reported that nearly 6% of rivers had EE2 concentrations greater than 5 ng $L^{-1}$ (Kolpin et al., 2002).

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EE2 is similar in structure to the endogenous hormone 17$\beta$-estradiol (E2), but binds with greater affinity to E2 receptors (Thorpe et al., 2003; Nash et al., 2004; Balch et al., 2004). EE2 is also more persistent in the environment than natural steroids; the half-life of EE2 is about 14 days in water, exhibiting much lower aerobic biodegradation than E2 (Shore et al., 1993; Jürgens et al., 2002). In addition, as a hydrophobic organic compound, EE2 also has a strong tendency to bioaccumulate (Lange et al., 2001). For example, Larsson et al. (1999) showed that juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to 50 $\mu$g $L^{-1}$ EE2 accumulated 350 $\mu$g EE2 per gram of bile after only 2 days of exposure, with no evidence of de-ethinylation or metabolism of the compound. Lai et al. (2002) determined that the concentration of EE2 in fish can be up to 332-fold higher than the surrounding environment.

Because of its high estrogenicity, environmental persistence, and accumulation, EE2 poses a significant risk to fish, even at the relatively low concentrations at which it occurs in the environment (Fent et al., 2006). The most frequently used biomarker of EE2 exposure is vitellogenin (VTG; Zhang and Hu, 2008), the egg yolk precursor glycolipoprotein produced in the liver. Exposure to EE2 induces vitellogenin production in male fish at doses as low as 1 ng $L^{-1}$ in early life stages and 4 ng $L^{-1}$ in adult stages (Van den Belt et al., 2003; Zha et al., 2007, 2008). Circulating levels of sex steroid hormones, particularly the androgens testosterone and 11-ketotestosterone, are also affected by EE2 exposure. Decreased androgen levels have been found in fish following doses of 4–65 ng $L^{-1}$ in juveniles (Labadie and Budzinski, 2006; Brown et al., 2007) and 10–250 ng $L^{-1}$ in adults (MacLatchy et al., 2003; Schultz et al., 2003; Coe et al., 2008).

A suite of morphological changes are also associated with exposure to EE2 in fishes. Full life-cycle exposure to concentrations $<$10 ng $L^{-1}$ can result in impaired growth, altered sexual differentiation including feminization of gonads and secondary sexual traits, and skewed sex ratios (Lange et al., 2001; Robinson et al., 2003; Nash et al., 2004; Schäfers et al., 2007; Larsen et al., 2008). Similar results occur in adult exposure experiments at higher concentrations (10–100 ng $L^{-1}$). Adult male zebrafish (*Danio rerio*) exposed to 40 ng $L^{-1}$ EE2 exhibited decreased GSI and the presence of an ovipositor (Salierno and Kane, 2009). Likewise, exposure to 64 ng $L^{-1}$ of EE2 for 21 days created intersex gonads in adult male medaka (*Oryzias latipes*) (Seki et al., 2002).

EE2 also affects both fecundity and fertility in fishes. Male exposure to EE2 has been shown to reduce fertilization success (Peters et al., 2007; Zha et al., 2008). Male and female exposure to EE2 leads to reduced hatching success and embryonic survival (Zillioux et al., 2001; Brown et al., 2007), and egg fertilization was reduced by 50–90% (Robinson et al., 2003; Schultz et al., 2003). In a multigenerational study, Nash et al. (2004) exposed breeding populations of zebrafish to 5 ng $L^{-1}$ EE2 and observed complete population failure with no fertilization in the F1 generation. In perhaps the most dramatic example, Kidd et al. (2007) conducted a whole-lake manipulation and found that prolonged exposure to 5–6 ng $L^{-1}$ of EE2 was sufficient to cause a population collapse in fathead minnows (*Pimephales promelas*) due to gonadal intersex in males and impaired oogenesis in females.

Sperm motility is a reliable indicator of fertilization success in fishes (Rurangwa et al., 2001; Fitzpatrick et al., 2009). In most teleost species fertilization is external, with males and females releasing gametes almost simultaneously into the water column (Schoenfuss et al., 2009). Fish sperm are immotile until they contact water, when the sharp drop in osmolality activates them (Takai and Morisawa, 1995). Once activated, the sperm are motile for only a few seconds or minutes (Morisawa and Suzuki, 1980; Billard et al., 1995), during which time they must enter the egg via the micropyle (Kime, 1999). The first minute after induction of motility is crucial to fertilization success (Casselman et al., 2006; Hara et al., 2007), because the sperm must swim fast enough and in the right direction to find the micropyle (Rurangwa et al., 2004). Immotile sperm may further decrease fertilization rates by blocking motile sperm from reaching or entering the micropyle (Levanduski and Cloud, 1988; Rurangwa et al., 2001).

We examined the effects of exposure to environmentally relevant concentrations of waterborne EE2 on the reproductive performance of sexually mature male fighting fish, *Betta splendens*, a species previously used to study endocrine disruption of physiology and behavior (Clotfelter and Rodriguez, 2006; Clotfelter et al., 2010; Stevenson et al., 2011). To place the current study in the context of previous studies on the effects of EE2 in fishes, we tested the following three hypotheses. First, waterborne EE2 has a direct inhibitory effect on the swimming performance and the fertilization potential of *B. splendens* sperm once they are released from the fish (Lahnsteiner et al., 1999; Abascal et al., 2007; Schoenfuss et al., 2009). Second, chronic exposure of fish to EE2 results in decreased gonad size, sperm performance, parental behavior, and/or fertilization success, as has been reported by many previous authors (MacLatchy et al., 2003; Schultz et al., 2003; Tilton et al., 2005; Brian et al., 2006; Santos et al., 2007; Coe et al., 2008; Zhang and Hu, 2008; Saaristo et al., 2010). Third and finally, the effect of EE2 exposure on sperm performance, if any, is related to a reduction in intracellular adenosine triphosphate (ATP) in *B. splendens* sperm (Green and Kelly, 2008).

## MATERIALS AND METHODS

### Experimental Subjects

Sexually mature males from a domesticated strain of *Betta splendens* were purchased from a commercial supplier and

acclimated for at least 2 days in the laboratory prior to each experiment (domesticated *B. splendens* typically recover from handling stress within a few hours [EDC and ACB, personal observations; see also Verbeek et al., 2008]). Each fish was used only once. Fish were housed in individual, visually isolated 1-L beakers containing 800 mL of reverse-osmosis (RO) water reconstituted to a conductivity of 110–140 μS using R/O Right (Kent Marine). Fish were fed freeze-dried chironomid larvae five times per week, except during exposure treatment periods when they were fed three times per week to reduce fouling of holding water. Water was maintained at 27°C and the light cycle was kept at 14:10 L:D. Water quality parameters (temperature, pH, conductivity) were checked regularly to ensure consistent testing conditions. All animal care protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Amherst College.

## Direct Exposure of Sperm to EE2

This experiment was designed to mimic the effects of EE2-contaminated water during spawning on sperm activation and motility (Hara et al., 2007; Schoenfuss et al., 2009). Sixty-six *B. splendens* were anesthetized with buffered tricaine methanesulfonate (MS-222) and sacrificed. Their gonads and bodies were weighed to calculate gonadosomatic index or GSI ([gonad weight/total body weight] × 100). To preserve the sperm, the testes were suspended in 100 μL of catfish sperm extender (Volckaert et al., 1994), which has been used successfully in a variety of species (Kime et al., 2001). The testes were then punctured 40 times with a needle to release the sperm.

The activation water contained concentrations of either 10 or 100 ng $L^{-1}$ EE2 (EE2 dissolved in 1 μL ethanol/10 mL water). The control group contained the ethanol vehicle only. We mixed 4 μL of activation water with 12 μL of the sperm solution described above. Five μL of this now activated sperm sample was immediately mounted onto Leja® 20-μm slides and viewed under a Nikon Eclipse E400 microscope. Using a SPOT Insight™ QE, Model 4.1 camera, three videos (~6 s in duration, 70 frames $s^{-1}$) from different parts of each slide were recorded within 60 s of sperm activation. Each video was analyzed with computer-assisted sperm analysis (CASA) using the Java™ plug-in for ImageJ (Wilson-Leedy and Ingermann, 2007). We measured sperm number (in each slide view), which was standardized by multiplying the number of detected sperm by the dilution factor and dividing by the testes mass (g) of each fish. We also recorded percent motility (percent of sperm that were motile), curvilinear velocity (VCL; point-to-point velocity per second), straight line velocity (VSL; velocity measured along a straight line from the first point of movement to the point furthest from the origin of the sperm), and smooth path velocity (VAP; point-to-point velocity based upon the average path of the traveling sperm) for the samples obtained from each fish. These parameters are known indicators of fertilization success in a range of fish species (Fitzpatrick et al., 2009). For each sperm sample, the values of the above parameters were averaged for the three recorded video sequences to obtain a single value per parameter per fish.

## Four-Week Exposure of Fish to EE2

To examine the effects of longer-term exposure to EE2 on reproductive parameters in *B. splendens*, 145 sexually mature males were exposed to EE2 for 28 days using a semi-static exposure protocol in which 25% of the water was replaced three times per week (Clotfelter and Rodriguez, 2006; Clotfelter et al., 2010; Stevenson et al., 2011). There were three treatment groups: a control group (ethanol vehicle only) and two experimental groups with concentrations of 10 or 100 ng $L^{-1}$ EE2. EE2 was dissolved in 1 μL ethanol/10 mL treatment water (same concentration as used in the previous experiment); control fish received the same amount of ethanol. After the 28-day exposure period, 70 fish from this experiment were anesthetized with buffered MS-222 and sacrificed. GSI and sperm quality were measured using methods described in the previous section.

The remaining 75 fish were bred to test for effects of EE2 on reproductive success. Briefly, males were placed in individual 38-L glass aquaria filled with 13–15 cm of fresh water. A small terra cotta pot was placed in each aquarium to provide refuge, along with a plastic disk (10 cm diameter) under which the males could build their bubble nests (Clotfelter et al., 2006). A female was placed in each aquarium in a covered beaker, allowing the pair to visually interact. After 48 h, females were released from beakers into the aquaria. After another 48 h, the male and female were separated, and all nests were removed to check for the presence of eggs. Nests that contained eggs were kept in 2-L tanks for 3 days to allow for egg development. Nests were photographed from below and images were scored by eye as the percentage (in 5% increments) of the plastic disk that was covered by the bubble nest. Each image was scored three times by an observer (TMM) who was blind to the male's treatment group, and the values were averaged to obtain a single value per male. Fry were then counted, and any remaining eggs were scored as fertilized or unfertilized (Stevenson et al., 2011).

## Analysis of Sperm ATP

A separate group of male *B. splendens* ($n = 45$) were exposed to EE2 or control conditions for 28 days following the same protocol described above, after which they were anesthetized in buffered MS-222 and sacrificed. Their testes

were extracted in sperm extender as described above and immediately frozen at −20°C for later analysis of ATP content. These sperm solution samples were later thawed and lysed with 5% trichloroacetic acid with pH indicator dye for 30 min, after which they were neutralized with 2.5 mL tris-acetate-EDTA buffer (pH = 7.75) and kept on ice. We plated 10 μL of this solution into each well of a white 96-well plate according to the manufacturer's specifications (ENLITEN® ATP assay system; Promega). Plates were read on a SpectraMax M5 microplate reader running Softmax Pro 5.3 software. ATP is reported as the moles of ATP per sperm.

## Analysis of Waterborne EE2

We validated our concentrations of waterborne EE2 by collecting 50 mL of holding water from randomly selected beakers. These samples were collected between water changes, and thus represent the likely lower limits of EE2 concentration experienced by the fish. Water samples were analyzed using a Waters 600 (Waters Corp.) high performance liquid chromatography (HPLC) system. Briefly, water samples and 17α-estradiol analytical standards were dried under nitrogen gas and resuspended in 600 μL mobile phase. Samples were manually injected into a 100 μL loop and were run on a 3.9 × 150 $mm^2$ column (Waters Corp. no. 86344) using 55:45 water:acetonitrile as the mobile phase. Detection was set for UV at 220 nm and flow rate was 1 mL per minute. The median EE2 concentrations in water samples from the control, 10 ng $L^{-1}$ EE2 and 100 ng $L^{-1}$ EE2 treatment groups were 0.0 ng $L^{-1}$ ($n = 7$), 5.9 ng $L^{-1}$ ($n = 7$), and 70.0 ng $L^{-1}$ ($n = 8$), respectively.

## Statistical Analysis

All data analysis was performed using SPSS 15.0. For variables that were not normally distributed, we applied a log, arcsine, or squared transformation to achieve normality. Most data were analyzed with univariate analysis of variance with Scheffe's post hoc tests. Sperm count was included as a covariate in all analyses of sperm parameters. When transformations were unable to achieve normality, we used Kruskal–Wallis or Mann–Whitney nonparametric tests. Means are presented ± SE and differences were considered statistically significant if $P < 0.05$.

# RESULTS

## Direct Exposure of Sperm to EE2

When EE2 was added directly to sperm activation water, there were no significant differences among treatment groups for any of the sperm parameters analyzed ($P \geq 0.23$ for all groups). Sperm count was significantly correlated with percent motile sperm ($P < 0.001$) and sperm straight line velocity (VSL; $P = 0.021$).

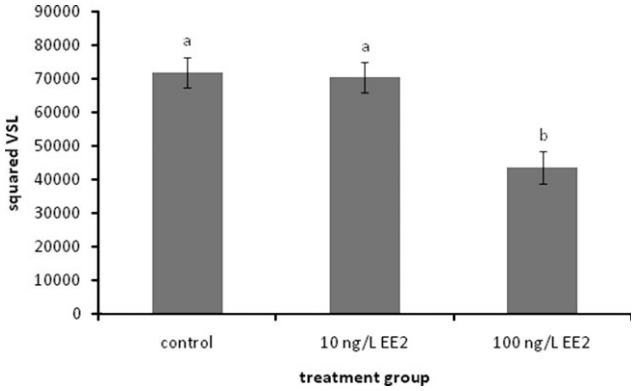


**Fig. 1.** Effect of semi-static exposure to EE2 on straight line velocity (VSL) of sperm in male *B. splendens*. Males were exposed to control conditions, 10 or 100 ng $L^{-1}$ of EE2 for 4 weeks ($n = 22$, 24, and 23, respectively). Males exposed to 100 ng $L^{-1}$ EE2 had significantly slower sperm (lower VSL) than the other treatment groups (ANOVA, $P = 0.01$). Data were square-transformed for statistical analysis. Bars sharing a letter are not significantly different from each other.

## Four-Week Exposure of Fish to EE2: Gonadosomatic Index

Exposure to EE2 significantly affected the GSI of male *B. splendens* ($F_{2,65} = 10.8$, $P < 0.001$). Males exposed to 100 ng $L^{-1}$ EE2 for 4 weeks ($n = 23$) had significantly smaller GSI ([gonad weight/total body weight] × 100) than males exposed to control conditions ($n = 22$) or 10 ng $L^{-1}$ EE2 ($n = 24$) (ANOVA, $P < 0.001$). The GSI of the 10 ng/L EE2 group did not significantly differ from the control group ($P = 0.94$).

## Four-Week Exposure of Fish to EE2: Sperm Motility

Four-week exposure to EE2 significantly affected sperm count and VSL ($P \leq 0.01$ for all). Males exposed to 100 ng $L^{-1}$ EE2 for 4 weeks ($n = 23$) had significantly lower sperm counts than males exposed to control conditions ($n = 22$) or 10 ng $L^{-1}$ EE2 ($n = 24$) (ANOVA, $P < 0.001$). The 100 ng $L^{-1}$ EE2 group had a significantly lower VSL than both the control group ($P = 0.005$) and the 10 ng $L^{-1}$ EE2 group ($P = 0.011$) (Fig. 1). Four-week exposure to EE2 did not significantly affect percent motility ($F_{2,65} = 1.41$, $P = 0.25$), VCL ($F_{2,65} = 2.63$, $P = 0.08$), or VAP ($F_{2,65} = 2.96$, $P = 0.06$), though the trends were in the same direction: fish in the 100 ng $L^{-1}$ treatment group had nonsignificantly reduced average sperm motility and sperm VCL and VAP.

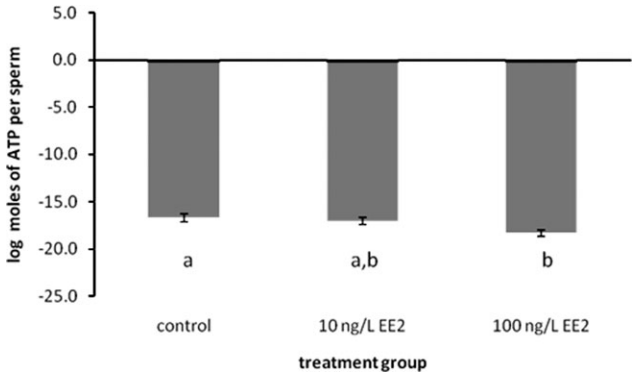


**Fig. 2.** Effect of semi-static exposure to EE2 on ATP content of male *B. splendens* sperm. ATP content of sperm from male *B. splendens* was significantly different among treatment groups ($F_{2,42} = 4.56$, $P = 0.016$). Fish exposed to 100 ng $L^{-1}$ of EE2 had less intracellular ATP per sperm than did control fish (Scheffe's post hoc test, $P = 0.02$) but only marginally less than fish exposed to 10 ng $L^{-1}$ EE2 ($P = 0.09$). Bars sharing a letter are not significantly different from each other.

## Four-Week Exposure of Fish to EE2: Sperm ATP

ATP content of *B. splendens* sperm was analyzed from 45 individuals, of which 16 fish had been exposed to control conditions, 15 fish were exposed to 10 ng $L^{-1}$ EE2, and 14 fish were exposed to 100 ng $L^{-1}$ EE2. We found significant differences in the moles of ATP per sperm in male *B. splendens* from the different treatment groups ($F_{2,42} = 4.56$, $P = 0.016$; Fig. 2). Specifically, fish exposed to 100 ng $L^{-1}$ of EE2 had less ATP per sperm than did control fish (Scheffe's post hoc test, $P = 0.02$) but only marginally less than fish exposed to 10 ng $L^{-1}$ EE2 ($P = 0.09$). Moles of ATP per sperm was weakly, though significantly, correlated with two measures of sperm velocity, VCL and VAP ($r = 0.34$, $P = 0.02$, $n = 45$ for both).

## Four-Week Exposure of Fish to EE2: Nest Building

The size of the bubble nests built by males varied significantly among treatment groups (Kruskal–Wallis $H = 17.2$, $P < 0.001$). The mean area of the nest substrate covered by the bubble nest was 53.2% ± 5.6% for the control group, 48.5% ± 5.0% for the 10 ng $L^{-1}$ EE2 group, and 23.3% ± 4.4% for the 100 ng $L^{-1}$ EE2 group (Fig. 3). The 100 ng $L^{-1}$ EE2 group built significantly smaller nests than either the control group (Mann–Whitney $U = 124$, $Z = -3.66$, $P < 0.001$) or the 10 ng $L^{-1}$ EE2 group ($U = 135$, $Z = -3.46$, $P = 0.001$). The nest size of the control group and the 10 ng $L^{-1}$ EE2 group was not significantly different ($U = 288$, $Z = -0.49$, $P = 0.63$). Nests with eggs were no different in size than nests without eggs ($U = 323$, $Z = -1.42$, $P = 0.16$).

## Four-Week Exposure of Fish to EE2: Fertilization Success

Males in the 100 ng $L^{-1}$ EE2 group had more unfertilized eggs in their nests (35.9% ± 6.7%) than males in either the control group (21.5% ± 4.5%) or the 10 ng $L^{-1}$ EE2 group (24.2% ± 4.7%), but these differences were not statistically significant ($F_{2,58} = 1.78$, $P = 0.18$). Furthermore, the total number of eggs in each nest did not differ among treatment groups ($F_{2,58} = 0.54$, $P = 0.59$), nor did clutch size affect the significance of fertilization results when included as a covariate ($F_{2,57} = 1.87$, $P = 0.16$).

## DISCUSSION

In this study, we exposed sexually mature male fighting fish (*Betta splendens*) and their sperm to two environmentally relevant concentrations of waterborne 17α-ethinylestradiol (EE2), a widespread endocrine disruptor and water contaminant. Consistent with other published studies, the current study suggests that EE2 could have negative effects on reproduction in free-living fish. Exposure of sexually mature males to 100 ng $L^{-1}$ of EE2 for 4 weeks caused a significant reduction in gonadosomatic index (GSI), sperm count, sperm straight line velocity (VSL), and sperm adenosine triphosphate (ATP) content. These reductions were found only in the 4-week semi-static exposure experiment; no significant changes in sperm motility were found for sperm directly exposed to EE2 in activation water. We also examined the nest building behavior of exposed fish and found that male *B. splendens* exposed to 100 ng $L^{-1}$ EE2 built significantly smaller bubble nests than males in the other

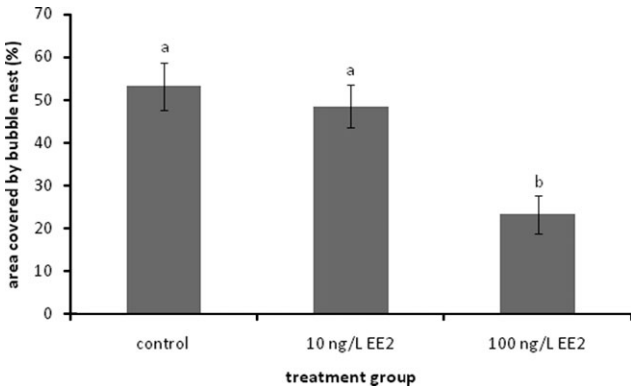


**Fig. 3.** Effect of semi-static exposure to EE2 on bubble nest size produced by male *B. splendens*. Males ($n = 25$ for each group) were exposed to environmentally relevant levels of EE2 or to control conditions for 4 weeks. Males exposed to 100 ng $L^{-1}$ EE2 built significantly smaller bubble nests than the other treatment groups (Kruskal–Wallis, $P < 0.001$). Bars sharing a letter are not significantly different from each other.

treatment groups. Overall, the reductions in sperm quality and GSI and the suppression of behavior at 100 ng $L^{-1}$ EE2 would be expected to negatively affect fertilization, but we found no evidence that the reduced sperm quality translated into a significant decline in fertilization success.

Consistent with most other studies (Tilton et al., 2005; Hoffmann et al., 2006; Ma et al., 2007; Zhang and Hu, 2008; but see Peters et al., 2007), we found that exposure to EE2 decreased the gonadosomatic index (GSI) of male *Betta splendens*. GSI is commonly used as an index to assess the effects of endocrine disruptors (Hassanin et al., 2002; Ma et al., 2007), and several studies have examined gonadal histology as well as GSI and found that exposure to EE2 inhibited spermatogenesis, causing a decrease in both testis size and gamete maturity. For example, fathead minnows exposed to 20–40 ng $L^{-1}$ EE2 for 21 days have reduced gonadal development and reduced GSI (Salierno and Kane, 2009), and the majority of testis cells in control fish were mature spermatozoa, while the majority of cells in exposed males were spermatogonia. A similarly dramatic reduction of both GSI and number of mature spermatozoa was found in short-term EE2 exposures of zebrafish (Van den Belt et al., 2002) and eelpout (*Zoarces viviparus*) (Velasco-Santamaría et al., 2010). The significant decrease in GSI in males exposed to 100 ng $L^{-1}$ EE2 suggests that the compound may also alter testis structure and interfere with spermatogenesis in sexually mature *B. splendens*.

In the current study, fish exposed to 100 ng $L^{-1}$ EE2 had fewer sperm than fish in the other treatment groups, similar to results reported in full life-cycle exposure studies in guppies (*Poecilia reticulata*) (Kristensen et al., 2005; Nielsen and Baatrup, 2006). We found similar reductions in the sperm count of sexually mature fish in our short-term study. This suggests that EE2 interferes with spermatogenesis itself, and not just in the testis differentiation that occurs during early development (Vizziano et al., 2008). In addition to decreased sperm count, we also found a significant decrease in sperm motility at 100 ng $L^{-1}$ EE2, as measured by a decrease in straight line velocity (VSL), which is consistent with previous studies in adult zebrafish (Santos et al., 2007). Our finding is at odds with work by Hashimoto et al. (2009), however, who found that EE2 exposure caused a significant increase in curvilinear velocity and percentage of motile sperm in adult male medaka.

The reduction in sperm velocity that we observed due to EE2 exposure may be due to reduced intracellular ATP. Sperm motility is powered by the hydrolysis of ATP (Burness et al., 2005; Ingermann, 2008), and the flagellar beating of fish sperm is dependent both on ATP stored prior to release (Christen et al., 1987; Ingermann et al., 2003) and on ATP synthesized while moving (Lahnsteiner et al., 1999). During motility, ATP hydrolysis cannot be sustained at the high rates necessary for ATP synthesis, resulting in rapidly declining ATP levels. This causes an associated decline in flagellar beat frequency and sperm swimming speed (Christen et al., 1987; Perchec et al., 1995; Dreanno et al., 1999; Cosson et al., 2008). Although there is considerable interspecific variation, higher stored ATP levels are generally correlated with a faster initial swimming velocity but a shorter motile period for sperm (Burness et al., 2004). The decreased sperm velocity and reduced intracellular ATP as a result of EE2 exposure in the current study are similar to the effects of the phytoestrogen genistein in directly exposed sperm of catfish (*Ictalurus punctatus*) and walleye (*Sander vitreus*) reported by Green and Kelly (2008). The reduction in intracellular ATP in the current study may be due to an initial, transient increase in sperm velocity that depletes ATP reserves (Hashimoto et al., 2009) followed by a rapid decrease in sperm velocity. Future work should examine the time course of EE2's effects on fish sperm swimming performance.

Several previous studies have reported that contaminants can directly alter fish gametes once they are released into the water. However, we found no evidence that direct exposure of sperm to waterborne EE2 affected sperm motility. In a sperm motility study using rainbow trout and Danube bleak (*Chalcalburnus chalcoides*), Lahnsteiner et al. (1999) showed that fish gametes were very sensitive to environmental influences immediately after release and that external substances, including the environmental pollutants dinitrophenol and potassium cyanide, easily penetrated the sperm membrane. Abascal et al. (2007) found that mercury had adverse effects on sea bass (*Dicentrarchus labrax*) sperm motility, and Schoenfuss et al. (2009) reported similar findings for goldfish (*Carassius auratus*) sperm in wastewater treatment plant effluent. The absence of a direct effect of EE2 on *B. splendens* sperm in the current study again supports the hypothesis that EE2 exposure disrupts gonad development and spermatogenesis, as has been demonstrated in several other species (Kime and Nash, 1999; Arukwe, 2001; Schulz et al., 2010).

When whole fish were exposed to EE2, we found that 100 ng $L^{-1}$ EE2 caused male *B. splendens* to change their reproductive behavior. EE2 has been linked to many behavioral changes in exposed fish, including the suppression of reproductive behavior (copulations) (Balch et al., 2004), reduced courtship behavior (Kristensen et al., 2005), disruption of reproductive hierarchies (Coe et al., 2008), reduced aggression (Colman et al., 2009), and reduced ability to compete for mates or spawning sites (Saaristo et al., 2009; Salierno and Kane, 2009). The current study found that exposed fish built significantly smaller nests than control fish, which could limit the number of eggs each nest can accommodate. Other studies have examined the effect of EE2 on nest building in sand gobies (*Pomatoschistus minutus*) and threespine sticklebacks (*Gasterosteus aculeatus*), with which the current study is consistent. Saaristo et al. (2010) found that male sand gobies exposed to 20 ng $L^{-1}$ EE2 were significantly slower to build nests than control males (12 h in control; 3–4 days in exposed males).

Brian et al. (2006) observed a delay in nest building in threespine sticklebacks after exposure to 10 ng $L^{-1}$ EE2 for 1–6 days. Such delays could affect a male's reproductive success, as females' mate choice decisions can be influenced by nest size and quality (Saaristo et al., 2009). In addition, when Maunder et al. (2007) exposed sticklebacks to 1.75 ng $L^{-1}$ EE2 for the first 4 weeks of life, exposed males, once mature, built significantly fewer nests than control males.

Fertilization success is the most integrative estimator of sperm quality and is the culmination of nearly all other reproductive endpoints (Bobe and Labbé, 2010), but the current study did not show significantly decreased fertilization success due to exposure to high levels of EE2. In contrast to our findings, exposure to EE2 significantly decreased the fertilization success of male fish in a variety of other species, in short-term exposures of adult fish at doses ranging from 5 to 100 ng $L^{-1}$ EE2 (Van den Belt et al., 2001; Schultz et al., 2003; Santos et al., 2007) and in early life stage exposures of 1.1–10 ng $L^{-1}$ (Maack and Segner, 2004; Schäfers et al., 2007). Not surprisingly, full life-cycle exposures have decreased fertilization at doses as low as $<$1–5 ng $L^{-1}$ (Nash et al., 2004; Parrott and Blunt, 2005). Decreased fertilization success may be present in *B. splendens* at exposures of longer duration or increased EE2 concentration. Fertilization success is also influenced by the presence and behavior of others, and the absence of competing, conspecific males in our experiment could have affected its outcome and statistical significance. Exposure to EE2 has been shown to decrease the ability of males to compete with other males for spawning sites, as well as reducing male-male competitive behaviors (Saaristo et al., 2009; Salierno and Kane, 2009). EE2 also decreases the time exposed males spend in active courtship, so that females prefer to mate with control males (Saaristo et al., 2009, 2010). Furthermore, Kristensen et al. (2005) directly correlated a reduction in courtship behavior with a highly significant reduction in fertilization success in guppies. This means that our observed nonsignificant effect of 100 ng $L^{-1}$ EE2 on male fertilization success might underestimate the effect of EE2 exposure under more competitive settings.

We combined different reproductive endpoints in *B. splendens* with different exposure protocols to examine the physiological mechanisms by which EE2 interferes with normal reproduction in male fish. Four-week exposure to environmentally relevant levels of EE2 decreased gonadosomatic index and two important measures of sperm quality: sperm count and sperm velocity. The likely explanation for the reduction in sperm velocity in the 100 ng $L^{-1}$ EE2 group was a reduction in sperm ATP, as shown by our observed decrease in intracellular ATP. Direct exposure of sperm to EE2, however, did not alter sperm quality, suggesting that the changes observed in the 4-week exposure occurred during spermatogenesis. Male behavior, as measured by nest building, was altered by EE2 exposure, as males in the 100 ng $L^{-1}$ treatment group built significantly smaller nests than control males. Finally, fertilization success was nonsignificantly decreased, implying that exposure to EE2 may negatively affect reproduction in *Betta splendens* over longer time periods or higher dosages. However, reproduction was not impaired as measured in viable embryos and total numbers of eggs. Despite several nonsignificant results in the current study, our findings support the growing body of literature that documents the potentially serious effects of environmental exposure to EE2 on the reproductive processes and population dynamics of fish populations (Kidd et al., 2007; Palace et al., 2009).

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