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Reproductive Consequences of Exposure to Waterborne Phytoestrogens in Male Fighting Fish *Betta splendens*

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Abstract Phytoestrogens are plant compounds that can act as endocrine disruptors in vertebrates. Biologically active levels of phytoestrogens have been found in aquatic habitats near wood pulp and paper mills, biofuel manufacturing plants, sewage-treatment plants, and agricultural fields. Phytoestrogens are known to cause hormonal and gonadal changes in male fish, but few studies have connected these effects to outcomes relevant to reproductive success. In one experiment, we exposed sexually mature male fighting fish *Betta splendens* to environmentally relevant (1 μg L^{-1}) and pharmacological concentrations (1000 μg L^{-1}) of the phytoestrogen genistein as well as to a positive control of waterborne 17β-estradiol (E2; 1 μg L^{-1}), and a negative control of untreated water. In a second experiment, we exposed male *B. splendens* to environmentally relevant concentrations (1 μg L^{-1}) of genistein and β-sitosterol singly and in combination as well as to the positive and negative controls. All exposures were 21 days in duration. We measured sex-steroid hormone levels, gonadosomatic index (GSI), sperm concentration and motility, and fertilization success in these fish. We found that exposure to genistein did not affect circulating levels of the androgen 11-ketotestosterone or the estrogen E2 relative to negative-control fish. We also found that neither of the compounds nor their mixture affected GSI, sperm concentration or motility, or fertilization success in exposed fish relative to negative-control fish. However, fish exposed to phytoestrogens showed some evidence of fewer but more motile sperm than fish exposed to the positive control E2. We conclude that sexually mature male *B. splendens* are relatively immune to reproductive impairments from short-term exposure to waterborne phytoestrogens.

Phytoestrogens are estrogenic compounds found in >300 species of plants (Dixon 2004). They occur at biologically active levels in effluent from wood pulp and paper mills, biofuel manufacturing, and municipal sewage-treatment facilities, as well as in agricultural soils (Kiparissis et al. 2001; Spengler et al. 2001; Burnison et al. 2003; Mahmood-Khan and Hall 2003, 2008; Puglisi et al. 2003; Lundgren and Novak 2009). Phytoestrogen contamination represents a potentially significant threat in many aquatic habitats. Investigations of the effects of these compounds on fishes have used a range of approaches, including collection and analysis of fish from natural populations (Howell et al. 1980), exposure of fish to effluent in semi-natural conditions (Sepúlveda et al. 2003), and exposure of fish to effluent or its constituent components in the laboratory (MacLatchy et al. 1997; Mattsson et al. 2001; Zhang et al. 2002; Wartman et al. 2009). Laboratory studies have focused primarily on genistein and β-sitosterol. These compounds are two of the most abundant and biologically active phytoestrogens, and both are known to bind to estrogen receptors in fish (Tremblay and Van Der Kraak 1998; Latonnelle et al. 2002).

In a range of fish species, phytoestrogens have a variety of hepatic, gonadal, hormonal, and gametic effects. Hepatic

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effects include the induction of vitellogenin in male fish and changes in 7-ethoxyresorufin-O-deethylase (EROD) activity. Vitellogenin is an egg yolk glycoprotein typically produced in the livers of female fish, and its induction in male fish is a reliable indicator of endocrine disruption (Sumpter and Jobling 1995). EROD activity is related to the activity of the cytochrome P4501A (CYP1A) enzyme, which is responsible for conversion of the endogenous estrogen 17β-estradiol (E2) to hydroxylated estrogen (Green and Kelly 2009). Vitellogenin and EROD induction have been reported in male fish in a range of fish species exposed to genistein (Pollack et al. 2003; Bennetau-Pelissero et al. 2001), β-sitosterol and other phytosterols (Mellanen et al. 1996; Tremblay and Van Der Kraak 1999; Christianson-Heiska et al. 2007), and whole effluent (Tremblay and Van Der Kraak 1999; van den Heuvel and Ellis 2002; Orrego et al. 2009).

Phytoestrogens can also affect the size or tissue composition of fish gonads. With respect to gonad size, evidence that phytoestrogens affect gonad size relative to body size is mixed (Sepúlveda et al. 2003; Sharpe et al. 2006); however, some studies have shown that phytoestrogens actually accelerate gonadal development (Bennetau-Pelissero et al. 2001). In an early life-stage exposure experiment, Kiparissis et al. (2003) reported that the phytoestrogen metabolite equol induced gonadal intersex, a condition in which the gonads contain both ovarian and testicular tissue, among medaka (*Oryzias latipes*). There were fewer intersex individuals among medaka exposed to genistein, and individuals exposed to both compounds displayed altered secondary sex characteristics (Kiparissis et al. 2003). Gonadal intersex has also been observed in channel catfish (*Ictalurus punctatus*) after dietary exposure to genistein (Green and Kelly 2009).

Gonadal production of the sex-steroid hormones testosterone (T), 11-ketotestosterone (11-KT), and E2, as well as levels of these hormones circulating in blood plasma, can be affected by phytoestrogens. Zhang et al. (2002, 2003) reported that exposure to genistein decreased production and circulating levels of T in medaka, and Bennetau-Pelissero et al. (2001) found that genistein-enriched diets suppressed circulating T levels in rainbow trout (*Oncorhynchus mykiss*). Similar suppressive effects on T have been found for β-sitosterol, which decreases T production by ex vivo testes in goldfish (*Carassius auratus*) (MacLatchy et al. 1997; Sharpe et al. 2006). Decrease of 11-KT and increased E2 levels have also been reported in fishes exposed to whole effluent (van den Heuvel and Ellis 2002; Sepúlveda et al. 2003).

Much less is known about the gametic effects of phytoestrogen exposure. Green and Kelly (2008) incubated testes of channel catfish and walleye (*Sander vitreus*) in genistein and found that genistein had a significant negative effect on sperm motility, adenosine triphosphate (ATP) content, and in vitro fertilization rates in both species. Similarly, sperm motility and concentration decreased in a dose-dependent manner in rainbow trout fed diets enriched with genistein (Bennetau-Pelissero et al. 2001). Both of these studies used aquaculture species to examine the effect of dietary phytoestrogens on sperm quality. Nothing is known about how environmentally relevant exposure to phytoestrogens might affect sperm in the context of natural spawning behavior.

Despite these advances in our understanding of the reproductive effects of phytoestrogens on fishes, relatively few studies integrate proximate mechanisms, such as gonadal development and steroid hormone levels, with outcomes that are directly linked to fitness, such as sperm motility and fertilization success (Bennetau-Pelissero et al. 2001; Sepúlveda et al. 2003). Furthermore, in many previous studies phytoestrogens were delivered by way of the diet (Bennetau-Pelissero et al. 2001; Pollack et al. 2003; Ishibashi et al. 2004; Green and Kelly 2009) or through intraperitoneal injection (MacLatchy and Van Der Kraak 1995; Zhang et al. 2002) rather than aqueous exposure, which more closely mimics natural conditions. Thus, the objectives of our study were (1) to examine the effects of exposure to the waterborne phytoestrogens genistein and β-sitosterol on gonad size and circulating steroid hormone levels in sexually mature male fighting fish *Betta splendens* and (2) to relate these findings to observed changes in sperm concentration and motility and in vivo fertilization success. To achieve these objectives, we assayed levels of 11-KT, the most potent fish androgen (Borg 1994), as well as levels of the estrogen E2. We used computer-assisted sperm analysis (CASA) to measure sperm concentration and motility and quantified the fertilization success of free-swimming male fish. We chose to work with *B. splendens* because we have optimized steroid hormone assays and CASA protocols for this species and because previous work in our laboratory identified behavioral and neurochemical effects of phytoestrogen exposure in this species (Clotfelter and Rodriguez 2006; Clotfelter et al. 2010), suggesting that this is a promising system in which to investigate reproductive consequences of exposure to phytoestrogens. Furthermore, there is some evidence that *B. splendens* populations are vulnerable to phytoestrogen exposure in the wild (Foberg 2003).

Based on the literature reviewed previously, we hypothesized that exposure to waterborne genistein and β-sitosterol would decrease relative gonad size of sexually mature male *B. splendens*. We predicted that circulating levels of 11-KT and E2 would decrease and increase, respectively, in exposed fish. These gonadal and hormonal changes would lead to a decrease in sperm concentration and motility, which we predicted would ultimately lead to a

decrease in fertilization success in exposed male fish relative to control male fish.

Materials and Methods

Experimental Subjects

We used 221 sexually mature (approximately 1 year old) male *B. splendens* in this study. Each fish was only used once during the experiments. Fish were obtained from a commercial supplier, and body mass (±0.01 g) and standard length (snout to caudal peduncle ±0.02 mm) were recorded. Body mass and standard length ranged from 0.95 to 2.93 g and 32.82 to 43.70 mm, respectively. Fish were fed approximately 5% of their body mass in freeze-dried chironomid larvae five times per week, except during exposure treatment periods, when they were fed three times per week to limit water fouling. Fish were housed in individual, visually isolated 1-L beakers with 800 mL reverse-osmosis (RO) water reconstituted to a conductivity of 110–140 μS. Water was maintained at 27 to 28°C, and the light-to-dark cycle was 12:12. All animal care protocols used in this study were approved by the Institutional Animal Care and Use Committee of Amherst College.

Steroid Hormones

We first focused on the effects of genistein on circulating levels of the androgen 11-KT and the estrogen E2. We did not include β-sitosterol in this portion of the study. Fish were housed in water containing an environmentally relevant dose of 1 μg L^{-1} genistein (Kiparissis et al. 2001), a pharmacological dose of 1000 μg L^{-1}, a positive control treatment of 1 μg L^{-1} E2, and a negative control treatment with only the ethanol vehicle (see later text). Low doses in this and other experiments were selected for their environmental relevance and because they have previously been shown to induce behavioral effects in male *B. splendens* (Clotfelter and Rodriguez 2006). Pharmacological doses were selected so as to exceed environmental levels by 2–3 orders of magnitude. Genistein and E2 were dissolved in ethanol, and all treatment groups received 0.1 mL L^{-1} ethanol (Organization for Economic Cooperation and Development 1992). Fish were exposed to the compounds for 21 days (Haubruge et al. 2000; Christianson-Heiska et al. 2007) using a semistatic exposure protocol in which 25% of the water (200 mL) was replaced three times per week. The doses used were nominal concentrations. Water samples analyzed by high-performance liquid chromatography (HPLC) from the 1 μg L^{-1} genistein group showed that actual concentrations (4.03 ± 1.54 μg L^{-1}, $n = 5$) were within the same order of magnitude as nominal concentrations and well within the environmentally relevant range.

Hormones circulating in the bloodstream pass by way of the gills and the epidermis to the surrounding water, from which they can be retrieved by way of solid-phase extraction. This technique has been shown in *B. splendens* and other species to provide accurate assessment of plasma hormone levels (Ellis et al. 2004; Dzieweczynski et al. 2006; Earley et al. 2006). Hereafter, we refer to these released and excreted hormones as "circulating" levels. After the 21-day exposure period, each male fish was placed in a beaker with 400 mL reconstituted RO water and presented with a sexually receptive (indicated by vertical barring on flanks) female conspecific in a separate, adjacent beaker for 12 h to stimulate steroid hormone release. Immediately after this visual stimulation, the male fish was visually isolated for 2 h to allow hormones to diffuse into the beaker water (Earley et al. 2006), after which 120 mL water was collected in 40-mL aliquots. These 40-mL samples were then run through extraction columns, eluted in methanol, dried down under a stream of nitrogen gas, and resuspended in 300 μL buffer for later analysis. The concentrations of 11-KT and E2 were quantified using enzyme immunoassay (EIA) kits from Cayman Chemical (11-KT #582751; E2 #582251). For the 11-KT assays, our intra-assay coefficient of variation (CV) was 11.19 ± 5.32%, and the interassay CV was 17.94%. For the E2 assays, our intra-assay CV was 10.11 ± 2.50%, and the interassay CV was 11.13%. Hormone levels are expressed in picograms per gram fish body mass per hour.

Gonadosomatic Index and Sperm Motility

Our next experiment used the same semistatic exposure protocol described previously; however, fish were instead allocated to the following treatment groups: negative control (ethanol vehicle only), 1 μg L^{-1} E2 (positive control), 1 μg L^{-1} genistein, 1 μg L^{-1} β-sitosterol, or a mixture of 1 μg L^{-1} genistein and 1 μg L^{-1} β-sitosterol. The latter treatment group was included because genistein and β-sitosterol are both present in wood pulp mill effluent (Kiparissis et al. 2001; Mahmood-Khan and Hall 2008). After the 21-day exposure period, half of the fish were anesthetized with tricaine methanesulfonate (Western Chemical) and killed. We removed their gonads under a Nikon SMZ800 dissecting microscope, after which their bodies and gonads were weighed to estimate GSI [gonad mass/(gonad mass + body mass)]*100). Gonads were suspended in 100 μL sperm extender mixed according to the "catfish extender" recipe (Volckaert et al. 1994), which has been used in a variety of species (Kime et al. 2001). To

release sperm for CASA, the gonads were perforated 40–50 times with a needle. Fifty microliters of this solution was mixed with 50 μL fresh sperm extender and put on ice until analysis.

Sperm samples were mounted onto Leja 2-chamber 20-micron slides (Spectrum Technologies), viewed under a Nikon Eclipse E400 microscope, and photographed using a SPOT Insight QE Model 4.1 camera. Video sequences were recorded using the SPOT basic program. Sperm were activated by mixing freshwater with the sperm extender solution. Within 60 s of sperm activation, videos from three different parts of each slide were taken for CASA using a Java plug-in for ImageJ (Wilson-Leedy and Ingermann 2007). In addition to sperm concentration (number of sperm in each slide view), we measured the % motility (percentage 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 along a path based on the average of the traveling sperm). We chose these parameters because they are known indicators of fertilization success in a range of fish species (Fitzpatrick et al. 2009). For each sperm sample, we averaged the values of the previous parameters for each of the three video sequences recorded.

Fertilization Success

The other half of the fish from the second experiment (see previous text) were placed individually in 38-L glass aquaria filled halfway with fresh reconstituted RO water, a small terra cotta pot, and a plastic cup lid under which the male fish could build their bubble nests (Clotfelter et al. 2006). Female fish were placed in the aquaria in beakers, and the pairs were able to visually interact. After 48 h, female fish were released into the tanks. After an additional 48 h, male and female fish were returned to beakers of clean water, and all nests were removed to check for the presence of eggs. If nests contained eggs, they were kept in 2-L aquaria for 72 h to allow further development, after which the eggs were scored as fertilized or unfertilized under 10× magnification with a Nikon SMZ800 microscope. Unfertilized eggs were white and opaque, whereas fertilized eggs developed into small fry with distinct eyes, body, and tail.

Statistical Analysis

All data analysis was performed using SPSS 15.0 (SPSS, Chicago, IL). For hormone and fertilization data that were not normally distributed, we applied a $\log_{10}$ transformation to achieve normality. Data were analyzed with univariate analysis of variance with Scheffe's post-hoc tests. Covariates (body size, sperm concentration) were included where relevant. Means are presented ± SE, and differences were considered statistically significant at $p < 0.05$.

Results

Steroid Hormones

We obtained information on circulating levels of 11-KT and E2 from 70 and 64 fish, respectively. Overall analysis of variance (ANOVA) showed a significant effect of exposure to genistein on circulating 11-KT levels in male *B. splendens* ($F_{3,66} = 3.51$, $p = 0.02$), which was due primarily to a pairwise difference between the 1 and 1000 μg L^{-1} genistein treatment groups (Scheffe's post-hoc test, $p = 0.027$; Fig. 1). All other pair-wise comparisons were not significant ($p \geq 0.18$). Exposure to genistein had nonsignificant effects on E2 levels in male *B. splendens* ($F_{3,60} = 2.52$, $p = 0.066$; Fig. 2). None of the other pairwise comparisons approached statistical significance ($p \geq 0.077$).

Gonadosomatic Index and Sperm Motility

Male gonadosomatic index (GSI), which we obtained from 60 male fish, did not vary among treatment groups, indicating that there was no effect of genistein, β-sitosterol, or the genistein + β-sitosterol mixture on relative male gonad size ($F_{4,53} = 0.65$, $p = 0.63$). Of these fish, we were able to successfully apply CASA to 54 of them. Sperm concentration was significantly different among treatment

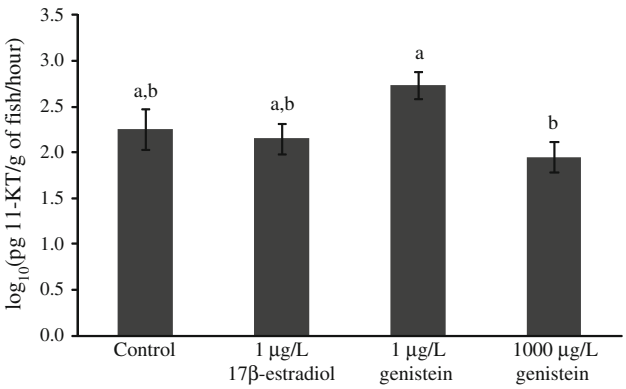

Fig. 1 Effect of exposure to waterborne E2 and genistein on circulating levels of 11-KT in male *B. splendens* ($F_{3,66} = 3.51$, $p = 0.02$). Bars sharing a letter are not significantly different from each other. Male fish exposed to the high dose of genistein released significantly lower levels of 11-KT than those exposed to the low dose of genistein (Scheffe's post-hoc test, $p = 0.027$). Sample sizes were 18, 16, 18, and 18 fish, respectively

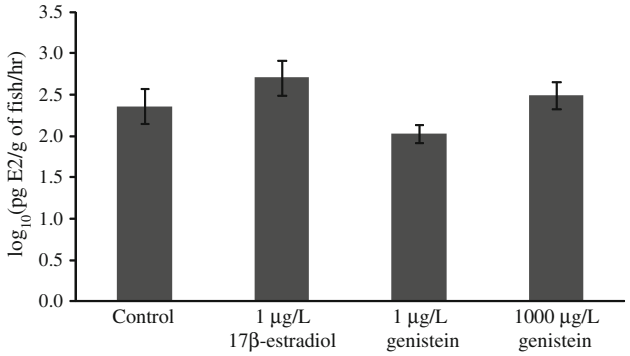

Fig. 2 Effect of exposure to waterborne E2 and genistein on circulating levels of E2 in male *B. splendens* ($F_{3,60} = 2.52$, $p = 0.066$). None of the differences were statistically significant; male fish exposed to the low dose of genistein released slightly lower levels of E2 than those exposed to E2 (Scheffe's post-hoc test, $p = 0.077$). Sample sizes were 15, 16, 16, and 17 fish, respectively

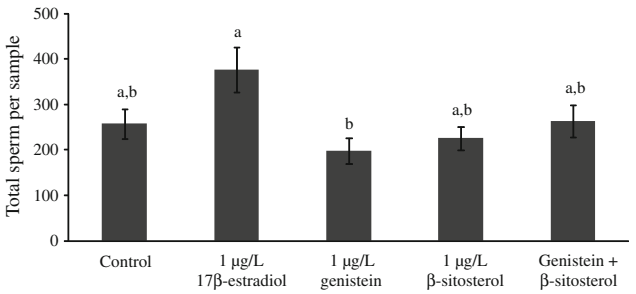

Fig. 3 Effects of exposure to waterborne E2, genistein, β-sitosterol, or the genistein plus β-sitosterol mixture on sperm concentration in male *B. splendens* ($F_{4,47} = 3.41$, $p = 0.016$). Fish exposed to E2 had significantly more sperm than fish exposed to genistein (Scheffe's post-hoc test, $p = 0.028$) and marginally more sperm than fish exposed to β-sitosterol ($p = 0.093$). *Bars* sharing a letter are not significantly different from each other. Sample sizes were 10, 9, 11, 10, and 12 fish, respectively

groups ($F_{4,47} = 3.41$, $p = 0.016$; Fig. 3); however, the only significant pairwise difference occurred between fish exposed to waterborne genistein and fish exposed to the positive control of E2 (Scheffe's post-hoc test, $p = 0.028$). The covariate of fish body mass did not affect sperm concentration ($F_{1,47} = 0.84$, $p = 0.37$).

Exposure to phytoestrogens did not significantly affect % motile sperm ($F_{4,47} = 1.07$, $p = 0.38$) or VSL of sperm ($F_{4,47} = 1.71$, $p = 0.16$). VCL of sperm was significantly different among treatment groups ($F_{4,47} = 5.07$, $p = 0.002$; Fig. 4). Fish treated with β-sitosterol (Scheffe's post-hoc test, $p = 0.004$), fish treated with the genistein + β-sitosterol mixture ($p = 0.028$), and fish in the negative-control treatment ($p = 0.034$) all had sperm with greater curvilinear velocities than the sperm of fish treated with E2. All other pairwise comparisons were not statistically significant ($p \geq 0.12$). Sperm concentration had a

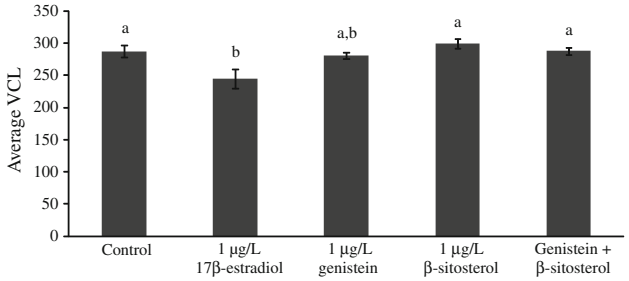

Fig. 4 VCL of sperm from male *B. splendens* exposed to E2, genistein, β-sitosterol, or the genistein plus β-sitosterol mixture ($F_{4,47} = 5.07$, $p = 0.002$). The sperm of the male fish exposed to E2 swam significantly more slowly than those of control male fish (Scheffe's post-hoc test, $p = 0.034$) or those exposed to β-sitosterol ($p = 0.004$) and the genistein plus β-sitosterol mixture ($p = 0.028$). *Bars* sharing a letter are not significantly different from each other. Sample sizes were 11, 9, 10, 10, and 12 fish, respectively

significant effect on VCL; however, its inclusion as a covariate did not eliminate the significant effect of treatment (main treatment effect $F_{4,46} = 2.90$, $p = 0.032$; covariate of sperm concentration $F_{1,46} = 14.52$, $p < 0.001$). Overall ANOVA for VAP was statistically significant ($F_{4,47} = 2.65$, $p = 0.045$); however, none of the pairwise differences among the individual treatment groups were significant ($p \geq 0.08$).

Fertilization Success

Of the fish whose gonads were not removed, 20 male fish from each of the five treatment groups (negative control, E2, genistein, β-sitosterol, and genistein plus β-sitosterol mixture) were paired with unexposed female fish, and of these 100 pairs, 66 of them (66%) successfully spawned. The proportion of unfertilized eggs was not significantly different among treatment groups ($F_{4,61} = 1.45$, $p = 0.23$). The addition of clutch size as a covariate did not change this relation (main effect of treatment $F_{4,60} = 1.73$, $p = 0.16$; covariate of clutch size $F_{1,60} = 1.70$, $p = 0.19$).

Discussion

We found that when sexually mature male *B. splendens* were exposed to two concentrations (one environmentally relevant, one pharmacological) of two waterborne phytoestrogens, the effects on reproductive end points in these fish were relatively minimal. Circulating levels of the sex steroids 11-KT and E2 were not significantly different in fish exposed to genistein compared with control fish. Relative gonad size (GSI), sperm concentration, sperm motility, and fertilization success were generally unaffected by either genistein or β-sitosterol exposure.

Phytoestrogens are naturally occurring; however, they are unnaturally concentrated as a result of human activity. Wood pulp and paper mills are important sources of phytoestrogens because woody plants contain high levels of isoflavones, such as genistein, and sterols, such as β-sitosterol (Dixon 2004), which are introduced into aquatic habitats by way of effluent release. The environmentally relevant concentrations we used in the current study are lower than those reported by several investigators. For example, Mahmood-Khan and Hall (2008) reported that biologically treated effluent from two pulp and paper mills in Canada contained an average of 200 $\mu g\ L^{-1}$ plant sterols, of which β-sitosterol was the most abundant, with levels sometimes reaching 500 $\mu g\ L^{-1}$. Kiparissis et al. (2001) found that treated effluent from another Canadian wood pulp and paper mill contained 10.5 $\mu g\ L^{-1}$ genistein. Thus, the environmentally relevant concentrations we used in the current study were 1–2 orders of magnitude lower than those found in treated effluent, suggesting that aquatic organisms downstream from such anthropogenic inputs are at significant risk of endocrine disruption or other exposure effects. The physical transport of phytoestrogens is poorly understood; however, some studies have reported undiluted plumes of pulp and paper mill effluent dozens of kilometers downstream from the point source (Servos et al. 1995).

Exposure to these environmental sources of phytoestrogens is known to decrease circulating levels of steroid hormones, including 11-KT and E2, in fishes (Munkittrick et al. 1991; Sepúlveda et al. 2003; Martinović et al. 2007). The use of waterborne exposure, intraperitoneal injection, or subcutaneous implants has also demonstrated that β-sitosterol decreases 11-KT and T levels in goldfish (*C. auratus*) (MacLatchy and Van Der Kraak 1995; MacLatchy et al. 1997; Sharpe et al. 2006) and that genistein decreases T levels in medaka (Zhang et al. 2002). However, one study found that adult male zebrafish (*Danio rerio*) exposed to a phytosterol mixture, 80% of which was β-sitosterol, actually had increased levels of T and 11-KT (Christianson-Heiska et al. 2007). The precise mechanism of this generalized inhibition is unknown; Leusch and MacLatchy (2003) found that β-sitosterol decreased the amount of reactive cholesterol inside the mitochondria without affecting the enzyme that initiates its conversion into sex steroids. This indicates that phytoestrogens may impede the transfer of cholesterol across the mitochondrial membrane, thus decreasing androgen levels because the cholesterol precursor cannot be converted.

In our study, male *B. splendens* treated with a high dose of genistein had lower levels of circulating 11-KT than male fish treated with the low dose of genistein, which is generally consistent with the androgen suppression observed in other species. Curiously, however, none of the phytoestrogen treatment groups differed significantly from the negative-control group. With respect to circulating E2 levels, fish exposed to E2 had slightly higher circulating levels of that hormone than male fish treated with 1 $\mu g\ L^{-1}$ genistein. Again, neither group differed significantly from negative-control fish. Our results suggest that sex-steroid levels in sexually mature male *B. splendens* are relatively impervious to short-term exposure to waterborne genistein, β-sitosterol, or E2.

Our research group previously reported (Clotfelter and Rodriguez 2006) that environmentally relevant concentrations of E2, genistein, and equol, a metabolite of the isoflavone daidzein, significantly decreased aggressive behavior in male *B. splendens*. Behavior has been used as an endpoint in many studies of endocrine disruption, especially in fish (see Clotfelter et al. 2004). One cause of this decreased aggression in male fish may be depressed levels of 11-KT and E2. However, because we did not find this effect of phytoestrogens on sex steroids in this study, decreased aggression is likely not caused by hormonal changes; however, it may instead be linked to changes in monoamine neurotransmitter activity and metabolism (Clotfelter et al. 2010).

Sex-steroid inhibition by phytoestrogen exposure has the potential to affect gonadal development in male fish. Sexual determination and differentiation is a highly plastic process in fish and thus is highly sensitive to environmental perturbation. GSI is a widely used biomarker for exposure to environmental estrogens (Scholz and Klüver 2009; Björkblom et al. 2009), and several studies have shown that phytoestrogens decrease GSI (Hassanin et al. 2002; Sepúlveda et al. 2003; Sharpe et al. 2006) and absolute gonad size (Munkittrick et al. 1991). Other studies, however, have reported that exposure to effluent from paper mills accelerates gonadal maturation in male fish, thus leading to increased GSI (Couillard et al. 1999; Orrego et al. 2006). We found no effect of waterborne E2, genistein, β-sitosterol, or the genistein + β-sitosterol mixture on GSI in male *B. splendens*. Although gonadal development is clearly most susceptible to perturbation by phytoestrogens and pulp mill effluent in juvenile fish (Scholz and Gutzeit 2001; Kiparissis et al. 2003; Örn et al. 2006), reversal of sex determination by way of administration of oral estrogens has been demonstrated in adult *B. splendens* (Kavumpurath and Pandian 1993), suggesting the potential for adults of this species to be sensitive to waterborne estrogenic compounds.

Our results show that phytoestrogens and the positive control E2 do not affect sex-steroid levels in adult male *B. splendens*. This inconsistency with previous studies on other fish species may be due to differences among species or exposure duration. The relatively short duration (21 days) used in the current study may have been

insufficient to produce gonadal changes in sexually mature fish because it is generally accepted that fish are more susceptible to environmental stressors as they proceed through early life stages of development (Brucker-Davis 1998; Jobling and Tyler 2003; Sepúlveda et al. 2003). Short-term laboratory experiments can be effective but have been criticized as being less sensitive than field exposures (Munkittrick et al. 1998).

Spermatogenesis is another potential target of phytoestrogen exposure because it is regulated in male fish by gonadotropins and androgens, specifically 11-KT (Borg 1994). Exposure to other environmental estrogens (or antiandrogens) has been shown to decrease sperm counts in fish (Haubruge et al. 2000; Bayley et al. 2002; Toft et al. 2003). Unlike mammalian sperm, fish sperm are inactive until contacted by water, after which they have a relatively short active period in which to achieve fertilization (Rurangwa et al. 2004). Thus, sperm motility is a key predictor of fertilization success in fishes (Fitzpatrick et al. 2009; Schoenfuss et al. 2009). Previous studies have shown that estrogenic compounds similar to those used in our study can decrease sperm concentration and motility in adult rainbow trout, channel catfish, walleye, and Arctic grayling (*Thymallus thymallus*), an effect that in some cases appears mediated by decreased ATP content of sperm (Bennetau-Pelissero et al. 2001; Lahnsteiner et al. 2006; Green and Kelly 2008). However, we did not find any effect of genistein or β-sitosterol on % motile sperm or sperm velocity. This result is consistent with the finding that mosquitofish (*Gambusia holbrooki*) exposed to paper mill effluent had similar sperm counts compared with those exposed to a control stream (Toft et al. 2004). Our positive control E2 caused a slight increase in sperm concentration (relative to genistein), which was associated with decreased sperm motility. Inverse relations between sperm concentration and motility have been reported for other fishes (Rurangwa et al. 2004) and in our study was possibly due to the physical constraints imposed by the slides on which sperm motility was measured.

Likewise, phytoestrogens have been shown to decrease fertilization success in several fish species (Lehtinen et al. 1999; Bennetau-Pelissero et al. 2001; Karels et al. 2003). This decreased fertilization may be due to decreased sperm motility (see previous text) because lower fertilization rates were found when exposed adult male sheepshead minnows (*Cyprinodon variegatus*) were mated with unexposed female minnows (Karels et al. 2003). However, another study reported that decreased fertilization success is attributable to abnormalities in maturing female fish from phytoestrogen contamination because higher frequencies of deformed or diseased larvae and lower fertilization rates were found when control male fish were mated with exposed female fish (Lehtinen et al. 1999). We found that fertilization success, estimated here by the proportion of unfertilized eggs, was unaffected by E2 or the two phytoestrogens we tested. We did not expose female fish to phytoestrogens in the current study; thus the potential contribution of female exposure to fertilization success in *B. splendens* remains unexplored.

One factor that could have affected our fertilization data is that male–female pairs of *B. splendens* were placed in aquaria without conspecific competitors. Other studies have examined the effect of male–male competition for access to female fish on fertilization success of male fish contaminated with environmental estrogens. Martinović et al. (2007) found that the fertilization success of male fathead minnows (*Pimephales promelas*) was unaffected by exposure to sewage-treatment effluent in the absence of a competing male fish. However, exposed fish experienced almost complete reproductive failure when placed in proximity to control male fish because they were outcompeted for both nests and female fish. Kristensen et al. (2005) and Bistodeau et al. (2006) reported similar results in competitive fertilization tests with male guppies (*Poecilia reticulata*) and fathead minnows, respectively, exposed to environmental contaminants. We previously documented that similar exposure to genistein and β-sitosterol affects male–male aggressive behavior (Clotfelter and Rodriguez 2006) and neurotransmitter activity in *B. splendens* (Clotfelter et al. 2010), suggesting that social interactions may significantly interact with phytoestrogen exposure to affect fertilization success in this species.

In summary, we found that sexually mature male *B. splendens* were not susceptible to the endocrine-disrupting effects of short-term exposure to genistein and β-sitosterol. Future studies on the effects of waterborne phytoestrogens on reproductive end points in fishes should include direct comparisons of fish in juvenile and mature life stages as well as a range of exposure durations, which will help determine windows of vulnerability to phytoestrogen exposure. Phytoestrogen exposure of adult fish has been demonstrated to affect phytoestrogen levels in offspring (Mattsson et al. 2001), suggesting that future studies focused on sexually mature adult fish should also consider the potential for transgenerational effects. Furthermore, additional exposure concentrations between the extremes used here (1–1000 $\mu g\ L^{-1}$) would help refine our understanding of phytoestrogen effects on reproductive end points. Finally, our study was exclusively focused on male fish, although there is ample evidence that phytoestrogens can affect reproductive competence in female fish (Bennetau-Pelissero et al. 2001; Sepúlveda et al. 2003). Our research group is currently investigating what effects, if any, genistein and β-sitosterol have on sex-steroid hormone levels and ovarian development in female *B. splendens*.

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References

Bayley M, Junge M, Baatrup E (2002) Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. Aquat Toxicol 56:227–239

Bennetau-Pelissero C, Breton B, Bennetau B, Corraze G, Le Menn F, Davail-Cuisset B et al (2001) Effect of genistein-enriched diets on the endocrine process of gametogenesis and on reproduction efficiency of the rainbow trout *Oncorhynchus mykiss*. Gen Comp Endocrinol 121:173–187

Bistodeau TJ, Barber LB, Bartell SE, Cediel RA, Grove KJ, Klaustermeier J et al (2006) Larval exposure to environmentally relevant mixtures of alkylphenolethoxylates reduces reproductive competence in male fathead minnows. Aquat Toxicol 79:268–277

Björkblom C, Högfors E, Salste L, Bergelin E, Olsson P, Katsiadaki I et al (2009) Estrogenic and androgenic effects of municipal wastewater effluent on reproductive endpoint biomarkers in three-spined stickleback (*Gasterosteus aculeatus*). Environ Toxicol Chem 28:1063–1071

Borg B (1994) Androgens in teleost fishes. Comp Biochem Physiol C 109:219–245

Brucker-Davis F (1998) Effects of environmental synthetic chemicals on thyroid function. Thyroid 8:827–856

Burnison BK, Hartmann A, Lister A, Servos MR, Ternes T, Van Der Kraak G (2003) A toxicity identification evaluation approach to studying estrogenic substances in hog manure and agricultural runoff. Environ Toxicol Chem 22:2243–2250

Christianson-Heiska I, Smeds P, Granholm N, Bergelin E, Isomaa B (2007) Endocrine modulating actions of a phytosterol mixture and its oxidation products in zebrafish (*Danio rerio*). Comp Biochem Physiol C 145:518–527

Clotfelter ED, Rodriguez AC (2006) Behavioral changes in fish exposed to phytoestrogens. Environ Pollut 144:833–839

Clotfelter ED, Bell AM, Levering KR (2004) The role of animal behaviour in the study of endocrine-disrupting chemicals. Anim Behav 68:465–476

Clotfelter ED, Curren LJ, Murphy CE (2006) Mate choice and spawning success in the fighting fish *Betta splendens*: the importance of body size, display behavior and nest size. Ethology 112:1170–1178

Clotfelter ED, McNitt MM, Carpenter RE, Summers CH (2010) Modulation of monoamine neurotransmitters in fighting fish *Betta splendens* exposed to waterborne phytoestrogens. Fish Physiol Biochem

Couillard CM, Williams PJ, Courtenay SC, Rawn GP (1999) Histopathological evaluation of Atlantic tomcod (*Microgadus tomcod*) collected at estuarine sites receiving pulp and paper mill effluent. Aquat Toxicol 44:263–278

Dixon RA (2004) Phytoestrogens. Annu Rev Plant Biol 55:225–261

Dzieweczynski TL, Eklund AC, Rowland WJ (2006) Male 11-ketotestosterone levels change as a result of being watched in Siamese fighting fish, *Betta splendens*. Gen Comp Endocrinol 147:184–189

Earley RL, Edwards JT, Aseem O, Felton K, Blumer LS, Karom M et al (2006) Social interactions tune aggression and stress responsiveness in a territorial cichlid fish (*Archocentrus nigrofasciatus*). Physiol Behav 88:353–363

Ellis T, James JD, Stewart C, Scott AP (2004) A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout. J Fish Biol 65:1233–1252

Fitzpatrick JL, Craig PM, Bucking C, Balshine S, Wood CM, McClelland GB (2009) Sperm performance under hypoxic conditions in the intertidal fish *Porichthys notatus*. Can J Zool 87:464–469

Foberg A (2003) The Siamese fighting fish (*Betta splendens*): an alternative fish species to use in evaluating the impact of endocrine disrupting chemicals with focus on aggressive performance. Master's thesis, Uppsala, Sweden, Uppsala University

Green C, Kelly A (2008) Effect of the exogenous soyabean phytooestrogen genistein on sperm quality, ATP content and fertilization rates in channel catfish *Ictalurus punctatus* (Rafinesque) and walleye *Sander vitreus* (Mitchill). J Fish Biol 72:2485–2499

Green C, Kelly A (2009) Effects of the estrogen mimic genistein as a dietary component on sex differentiation and ethoxyresorufin-O-deethylase (EROD) activity in channel catfish (*Ictalurus punctatus*). Fish Physiol Biochem 35:377–384

Hassanin A, Kuwahara S, Nurhidayat, Tsukamoto Y, Ogawa K, Hiramatsu K et al (2002) Gonadosomatic index and testis morphology of common carp (*Cyprinus carpio*) in rivers contaminated with estrogenic chemicals. J Vet Med Sci 64:921–926

Haubruge E, Petit F, Gage MJ (2000) Reduced sperm counts in guppies (*Poecilia reticulata*) following exposure to low levels of tributyltin and bisphenol A. Proc R Soc Lond B 267:2333–2337

Howell WM, Black D, Bortone S (1980) Abnormal expression of secondary sex characters in a population of mosquitofish, *Gambusia affinis holbrooki*: evidence for environmentally-induced masculinization. Copeia 4:676–681

Ishibashi H, Tachibana K, Tsuchimoto M, Soyano K, Tatarazako N, Matsumura N et al (2004) Effects of nonylphenol and phytoestrogen-enriched diet on plasma vitellogenin, steroid hormone, hepatic cytochrome P450 1A, and glutathione-S-transferase values in goldfish (*Carassius auratus*). Comp Med 54:54–62

Jobling S, Tyler CR (2003) Endocrine disruption in wild freshwater fish. Pure Appl Chem 75:2219–2234

Karels AA, Manning S, Brouwer TH, Brouwer M (2003) Reproductive effects of estrogenic and antiestrogenic chemicals on sheepshead minnows (*Cyprinodon variegatus*). Environ Toxicol Chem 22:855–865

Kavumpurath S, Pandian TJ (1993) Determination of labile period and critical dose for sex reversal by oral administration of estrogens in *Betta splendens* (Regan). Indian J Exp Biol 31:16–20

Kime DE, Van Look KJW, McAllister BG, Huyskens G, Rurangwa E, Ollevier F (2001) Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp Biochem Physiol C 130:425–433

Kiparissis Y, Hughes R, Metcalfe C (2001) Identification of the isoflavonoid genistein in bleached kraft mill effluent. Environ Sci Technol 35:2423–2427

Kiparissis Y, Balch G, Metcalfe T, Metcalfe C (2003) Effects of the isoflavones genistein and equol on the gonadal development of

Japanese medaka (*Oryzias latipes*). Environ Health Perspect 111:1158–1163

Kristensen T, Baatrup E, Bayley M (2005) 17α-ethinylestradiol reduces the competitive reproductive fitness of the male guppy (*Poecilia reticulata*). Biol Reprod 72:150–156

Lahnsteiner F, Berger B, Kletzl M, Weismann T (2006) Effect of 17β-estradiol on gamete quality and maturation in two salmonid species. Aquat Toxicol 79:124–131

Latonnelle K, Fostier A, Le Menn F, Bennetau-Pelissero C (2002) Binding affinities of hepatic nuclear estrogen receptors for phytoestrogens in rainbow trout (*Oncorhynchus mykiss*) and Siberian sturgeon (*Acipenser baeri*). Gen Comp Endocrinol 129:69–79

Lehtinen KJ, Mattsson K, Tana J, Engström C, Lerche O, Hemming J (1999) Effects of wood-related sterols on the reproduction, egg survival, and offspring of brown trout (*Salmo trutta lacustris* L.). Ecotoxicol Environ Saf 42:40–49

Leusch FD, MacLatchy D (2003) In vivo implants of β-sitosterol cause reductions of reactive cholesterol pools in mitochondria isolated from gonads of male goldfish (*Carassius auratus*). Gen Comp Endocrinol 134:255–263

Lundgren MS, Novak PJ (2009) Quantification of phytoestrogens in industrial waste streams. Environ Toxicol Chem 28:2318–2323

MacLatchy D, Van Der Kraak G (1995) The phytoestrogen β-sitosterol alters the reproductive endocrine status of goldfish. Toxicol Appl Pharmacol 134:305–312

MacLatchy D, Peters L, Nickle J, Van Der Kraak G (1997) Exposure to β-sitosterol alters the endocrine status of goldfish differently than 17β-estradiol. Environ Toxicol Chem 16:1895–1904

Mahmood-Khan Z, Hall E (2003) Occurrence and removal of plant sterols in pulp and paper mill effluents. J Environ Eng Sci 2:17–26

Mahmood-Khan Z, Hall E (2008) Quantification of plant sterols in pulp and paper mill effluents. Water Qual Res J Can 43:173–181

Martinović D, Hogarth WT, Jones RE, Sorensen PW (2007) Environmental estrogens suppress hormones, behavior, and reproductive fitness in male fathead minnows. Environ Toxicol Chem 26:271–278

Mattsson K, Tana J, Engstrom C, Hemming J, Lehtinen KJ (2001) Effects of wood-related sterols on the offspring of the viviparous blenny, *Zoarces viviparus* L. Ecotoxicol Environ Saf 49:122–130

Mellanen P, Petänen T, Lehtimäki J, Mäkelä S, Bylund G, Holmbom B et al (1996) Wood-derived estrogens: studies in vitro with breast cancer cell lines and in vivo in trout. Toxicol Appl Pharmacol 136:381–388

Munkittrick KR, Portt CB, Van der Kraak G, Smith IR, Rokosh DA (1991) Impact of bleached kraft mill effluent on population characteristics, liver MFO activity, and serum steroid-levels of a Lake Superior white sucker (*Catostomus commersonii*) population. Can J Fish Aquat Sci 48:1371–1380

Munkittrick KR, McMaster ME, McCarthy LH, Servos MR, Van Der Kraak GJ (1998) An overview of recent studies on the potential of pulp-mill effluents to alter reproductive parameters in fish. J Toxicol Environ Health 1:347–371

Organization for Economic Cooperation and Development (1992) Fish prolonged toxicity test: 14 day study. Guideline 204. Paris, France

Örn S, Svenson A, Viktor T, Holbech H, Norrgren L (2006) Male-biased sex ratios and vitellogenin induction in zebrafish exposed to effluent water from a Swedish pulp mill. Arch Environ Contam Toxicol 51:445–451

Orrego R, Burgos A, Moraga-Cid G, Inzunza B, Gonzalez M, Valenzuela A et al (2006) Effects of pulp and paper mill discharges on caged rainbow trout (*Oncorhynchus mykiss*): biomarker responses along a pollution gradient in the Biobio River, Chile. Environ Toxicol Chem 25:2280–2287

Orrego R, Guchardi J, Hernandez V, Krause R, Roti L, Armour J et al (2009) Pulp and paper mill effluent treatments have differential endocrine-disrupting effects on rainbow trout. Environ Toxicol Chem 28:181–188

Pollack SJ, Ottinger MA, Sullivan CV, Woods LC (2003) The effects of the soy isoflavone genistein on the reproductive development of striped bass. North Am J Aquacult 65:226–234

Puglisi E, Nicelli M, Capri E, Trevisan M, Del Re AAM, Trevisan M (2003) Cholesterol, β-sitosterol, ergosterol, and coprostanol in agricultural soils. J Environ Qual 32:466–471

Rurangwa E, Kime DE, Ollevier F, Nash JP (2004) The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234:1–28

Schoenfuss HL, Levitt JT, Rai R, Julius ML, Martinovic D (2009) Treated wastewater effluent reduces sperm motility along an osmolality gradient. Arch Environ Contam Toxicol 56:397–407

Scholz S, Gutzeit H (2001) Lasting effects of xeno- and phytoestrogens on sex differentiation and reproduction of fish. Environ Sci 8:57–73

Scholz S, Klüver N (2009) Effects of endocrine disrupters on sexual, gonadal development in fish. Sex Dev 3:136–151

Sepúlveda MS, Quinn BP, Denslow ND, Holm SE, Gross TS (2003) Effects of pulp and paper mill effluents on reproductive success of largemouth bass. Environ Toxicol Chem 22:205–213

Servos MR, Munkittrick K, Carey J, Van Der Kraak G (1995) Environmental fate and effects of pulp and paper mill effluents. St. Lucie Press, Boca Raton, FL

Sharpe R, Drolet M, MacLatchy D (2006) Investigation of de novo cholesterol synthetic capacity in the gonads of goldfish (*Carassius auratus*) exposed to the phytosterol beta-sitosterol. Reprod Biol Endocrinol 4:60

Spengler P, Korner W, Metzger JW (2001) Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 1. Chemical analysis. Environ Toxicol Chem 20:2133–2141

Sumpter JP, Jobling S (1995) Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ Health Perspect 103:173–178

Toft G, Edwards TM, Baatrup E, Guillette LJ (2003) Disturbed sexual characteristics in male mosquitofish (*Gambusia holbrooki*) from a lake contaminated with endocrine disruptors. Environ Health Perspect 111:695–701

Toft G, Baatrup E, Guillette LJ (2004) Altered social behavior and sexual characteristics in mosquitofish (*Gambusia holbrooki*) living downstream of a paper mill. Aquat Toxicol 70:213–222

Tremblay L, Van Der Kraak G (1998) Use of a series of homologous in vitro and in vivo assays to evaluate the endocrine modulating actions of β-sitosterol in rainbow trout. Aquat Toxicol 43:149–162

Tremblay L, Van Der Kraak G (1999) Comparison between the effects of the phytosterol β-sitosterol and pulp and paper mill effluents on sexually immature rainbow trout. Environ Toxicol Chem 18:329–336

van den Heuvel M, Ellis R (2002) Timing of exposure to a pulp and paper effluent influences the manifestation of reproductive effects in rainbow trout. Environ Toxicol Chem 21:2338–2347

Volckaert FA, Galbusera PH, Hellemans BA, Van den Haute C, Vanstaen D, Ollevier F (1994) Gynogenesis in the African catfish (*Clarias gariepinus*). I. Induction of meiogynogenesis with thermal and pressure shocks. Aquaculture 128:221–233

Wartman CA, Hogan NS, Hewitt LM, McMaster ME, Landman MJ, Taylor S et al (2009) Androgenic effects of a Canadian bleached kraft pulp and paper effluent as assessed using three-spine stickleback (*Gasterosteus aculeatus*). Aquat Toxicol 92:131–139

Wilson-Leedy JG, Ingermann RL (2007) Development of a novel CASA system based on open source software for

characterization of zebrafish sperm motility parameters. Theriogenology 67:661–672

Zhang L, Khan IA, Foran CM (2002) Characterization of the estrogenic response to genistein in Japanese medaka (*Oryzias latipes*). Comp Biochem Physiol C 132:203–211

Zhang L, Khan IA, Willett KL, Foran CM (2003) In vivo effects of black cohosh and genistein on estrogenic activity and lipid peroxidation in Japanese medaka (*Oryzias latipes*). J Herb Pharmacother 3:33–50