



Original Article

An androgenic endocrine disruptor alters male mating behavior in the guppy (*Poecilia reticulata*)

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Hormonally active chemical pollution threatens human and wildlife populations globally. However, despite the well-established capacity of endocrine-disrupting chemicals (EDCs) to alter reproductive traits, relatively few studies have examined the impacts of EDCs on mechanisms of sexual selection. This study investigated the effects of short-term exposure to an environmentally realistic level of 17 β -trenbolone—a potent anabolic steroid used in livestock production worldwide—on male mate preference, reproductive behavior, and morphology in the guppy (*Poecilia reticulata*). Male guppies prefer to mate with larger females because such females are generally more fecund. Hence, males gain direct fitness benefits by being choosy. Here, we found no significant effect of 17 β -trenbolone exposure on male courting behavior, with both unexposed and exposed males courting larger females more often. However, exposure to 17 β -trenbolone significantly altered the amount of coercive copulatory behavior (“sneak” matings) performed. Specifically, while both unexposed and exposed males demonstrated a preference for larger females by conducting more sneaking attempts toward these females, exposed males carried out a greater number of sneaks toward large females than did unexposed males. Further, exposure resulted in increased male condition index (i.e., mass relative to length). Together, our results show for the first time that 17 β -trenbolone can alter reproductive behavior and morphology in male fish at concentrations as low as 4 ng/L, highlighting the potential for disruption of reproductive processes in wildlife exposed to this potent agricultural contaminant.

Key words: agricultural pollution, endocrine disrupting chemical, pharmaceutical, reproductive behavior, sexual selection, trenbolone.

INTRODUCTION

Human and wildlife populations worldwide are increasingly being exposed to chemicals capable of altering hormone signaling (WHO-UNEP 2012). Endocrine-disrupting chemicals (EDCs) disturb the natural homeostatic functioning of the endocrine system by interfering with the synthesis, secretion, transport, metabolism, binding, action, and/or elimination of natural hormones (Kavlock et al. 1996; Diamanti-Kandarakis et al. 2009). In this regard, agricultural activity is a leading source of endocrine-disrupting pollution (Horrigan et al. 2002; Yin et al. 2002; Diamanti-Kandarakis et al. 2009; WHO-UNEP 2012). Although many EDCs produce inadvertent effects in nontarget species as an unintended by-product of other functions (Wilson et al. 2002; Clotfelter et al. 2004),

endocrine-disrupting pollution from agriculture is concerning given the use of high-potency synthetic chemicals known as hormonal growth promotants (HGP), which have been specifically developed to act on the endocrine system. Currently, vast amounts of HGP are administered in beef-producing nations worldwide, including the United States, Canada, Australia, New Zealand, Mexico, Chile, South Africa, and Japan (Hunter 2010; Johnson 2015). For example, approximately 20 million beef cattle per annum receive growth-promoting implants in the United States, representing around two-thirds of beef livestock in the country (Johnson 2015; USDA 2016). This widespread use of HGP, however, excludes the European Union, where the use of growth hormones in domestic production and imported beef has been banned, dating back to the early 1980s (Johnson 2015).

Chemical compounds used in HGP implants commonly include androgens (e.g., trenbolone acetate), estrogens (e.g., 17 β -estradiol, zeranol) and progestins (e.g., melengestrol acetate) (Lange et al. 2001).

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Trenbolone acetate—a powerful synthetic steroid with 15–50 times the androgenic and anabolic potency of testosterone (Neumann 1976)—is the androgen most commonly administered to beef cattle (Hunter 2010). After implantation, trenbolone acetate is rapidly hydrolyzed and excreted in the form of various metabolites, the most biologically potent of which is 17 β -trenbolone (Khan et al. 2008; Parker et al. 2012). This excrement can then run off into freshwater systems, where 17 β -trenbolone is highly persistent (half-life: ~260 days; Schiffer et al. 2001) and has repeatedly been detected at levels ranging from 1 to 20 ng/L in discharge and diffuse run-off (Durhan et al. 2006) to as high as 162 ng/L in tile-drained agroecosystems (Gall et al. 2011). Rapidly taken up by fish, 17 β -trenbolone reaches a steady state at approximately 8 h in fathead minnows (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*) (Schultz et al. 2013). Further, 17 β -trenbolone can bioconcentrate in fish—with a bioconcentration factor on the order of 13 reported in fathead minnows exposed for 21 days (Ankley et al. 2003)—and, therefore, potentially also represents a threat to wild-life at higher trophic levels that feed on fish. As a known androgenic endocrine disruptor, reported impacts of 17 β -trenbolone exposure on nontarget species are wide-ranging (reviewed in Ankley et al. 2018), including altered gonad morphology (Orn et al. 2006), reduced fecundity (Ankley et al. 2003), decreased fertility (Mizukami-Murata et al. 2016), altered sexual differentiation (Olmstead et al. 2012), skewed sex ratios (Orn et al. 2006; Olmstead et al. 2012), and even complete and functional female-to-male sex reversal (Larsen and Baatrup 2010; Morthorst et al. 2010). Despite this, as is true for many EDCs, relatively little is known about the impacts of exposure to 17 β -trenbolone on ecologically important behaviors, including those under sexual selection.

Sexual selection, by influencing reproductive success, is fundamental to individual fitness, population and species viability, as well as broader evolutionary processes (Candolin and Heuschele 2008; Wong and Candolin 2015). Given that sex steroid hormones modulate the expression of a wide range of behaviors under sexual selection (Beyer et al. 1976; Munakata and Kobayashi 2010), these processes are likely to be vulnerable to disruption by hormone-like contaminants. Although endocrine disruptor exposure has been linked to breakdowns in sexual selection processes as a consequence of altered female mate choice (e.g., Saaristo et al. 2009a; Secondi et al. 2009; Partridge et al. 2010; Tomkins et al. 2016, 2018), comparatively little is currently known about the potential for these contaminants to influence male mating preferences and reproductive behavior (but see Saaristo et al. 2009b; Jayasena et al. 2011; Bertram et al. 2015). This is surprising because male mate choice, regarding not only the decision to mate but also the allocation of reproductive investment to each mate or mating, can afford both direct (i.e., material) and indirect (i.e., genetic) benefits to choosy males (Kokko et al. 2003; Edward and Chapman 2011).

The guppy (*Poecilia reticulata*) is a small live-bearing freshwater fish endemic to north-eastern South America that is now established in at least 69 countries outside of its native range due to numerous accidental and deliberate introductions (Deacon et al. 2011). This species' life history and mating behaviors have been exceptionally well studied (Houde 1997; Magurran 2005), which, in combination with a propensity to inhabit freshwater systems receiving agricultural waste (e.g., Phillip 1998; López-Rojas and Bonilla-Rivero 2000; Widianarko et al. 2000; Araújo et al. 2009), means that guppies are an ideal model to study the potential behavioral impacts of endocrine disruptor exposure. Although the guppy mating system is predominantly driven by female choice, with females

responding to elaborate male courtship displays and avoiding coercive “sneak” mating attempts (Houde 1997), male guppies can also be choosy and are known to prefer larger females as mates (Dosen and Montgomerie 2004; Herdman et al. 2004). Because female guppy fecundity (brood size) increases with body size (Herdman et al. 2004), males gain direct fitness benefits by mating with larger females. Wild guppies live in mixed-sex shoals, where males routinely encounter multiple females concurrently (Houde 1997), and females vary greatly in body size and fecundity (Reznick and Endler 1982; Kelly 1999), making the fitness benefits of male choosiness particularly pronounced.

Here, we examine the impact of short-term (21-day) exposure to an environmentally realistic concentration (average measured concentration: 4 ng/L) of 17 β -trenbolone on male guppy preference for female size, as well as male reproductive behavior (i.e., performance of courtship behavior vs. unsolicited sneaking behavior). In addition to expecting altered behavior, we hypothesized that 17 β -trenbolone's potent growth-promoting activity would lead to increased male mass.

MATERIALS AND METHODS

Ethics statement

Methods for animal housing and experimental protocols were approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2013/09) and observed all relevant State and Federal laws of Australia.

Animal housing

Guppies used in this experiment were laboratory-reared descendants of wild fish collected from Alligator Creek (19°26'79"S, 146°58'65"E), a pristine rainforest-fed stream located in Bowling Green Bay National Park, Queensland, Australia (Queensland permit: WITK07655010). Water samples drawn from this site over consecutive years indicate no contamination from EDCs (ALS Group, unpublished data). Sexually mature male and female guppies were acclimated to laboratory conditions for 2 months in large mixed-sex holding tanks (81 L; 60 cm × 45 cm × 30 cm; 25 to 27 °C; 12:12 h light:dark regime), and were presumed to be nonvirginal considering the incessant mating pressure applied by males toward females (Magurran and Seghers 1994; Houde 1997), with nonvirginal fish being used to simulate mixed-sex wild populations. Fish were fed *ad libitum* once per day (Otohime Hiramé larval diet; 580–910 μ m).

Exposure set-up

A flow-through system was used to expose male fish to 17 β -trenbolone, following previously established methods (Saaristo et al. 2013; Bertram et al. 2015; Tomkins et al. 2016, 2017, 2018), for a period of 21 days. Males were allocated to identical exposure tanks (54 L; 60 cm × 30 cm × 30 cm), which were monitored daily for temperature (mean \pm SD = 25.83 \pm 0.44 °C) and flow-through rates (mean \pm SD = 18.43 \pm 0.37 mL/min) maintained using flow meters (BES, MPB Series 1200). Males were randomly allocated to 1 of 4 17 β -trenbolone-exposure tanks, or 1 of 4 identical unexposed tanks containing fresh water only (22 fish per tank). Survivorship over the exposure period was 92.0% for unexposed fish and 94.3% for exposed fish (7 and 5 deaths, respectively). This is in line with expected natural mortality rates for adult (i.e., \geq 7 weeks; Magurran 2005) male *P. reticulata*, given that males of this

species have an average lifespan of ~24 months (Reznick et al. 2006).

Exposure dosing and GC-MS/MS analysis

The 17 β -trenbolone concentration used (mean \pm SD = 4.48 \pm 1.53 ng/L, n = 16) was attained by dissolving 17 β -trenbolone (17 β -hydroxyestra-4,9,11-trien-3-one; CAS: 10161-33-8; Novachem, Germany) in ethanol (HPLC grade, \geq 99.99%) to create a stock standard (400 mg/L). This stock standard was diluted to 4 μ g/L with deionized water, and further diluted within the flow-through system, to yield the desired exposure concentration. The exposure level of 4 ng/L was chosen as this concentration falls within the range (1–7 ng/L) of 17 β -trenbolone concentrations detected in river water (Durhan et al. 2006).

Levels of 17 β -trenbolone were monitored weekly—in exposed tanks, as well as in unexposed tanks (to ensure the absence of contamination)—using gas chromatography–tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware). The analysis was performed by Envirolab Services (MPL Laboratories, Perth; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025). No significant concentration differences were detected across 17 β -trenbolone exposure tanks within the flow-through system (ANOVA: $F_{3,12}$ = 0.074, P = 0.973). For additional details on the collection and analysis of water samples, see Tomkins et al. (2018).

Behavior trials

To examine the impact of exposure to 17 β -trenbolone on male guppy mate preference and reproductive behavior, fish were tested in 4 treatments: 1) unexposed male paired with large female (hereafter UL; n = 19), 2) unexposed male paired with small female (US; n = 19), 3) exposed male paired with large female (EL; n = 25), and 4) exposed male paired with small female (ES; n = 24). A pair of 1 male and 1 female fish were tested to disentangle impacts of 17 β -trenbolone exposure on male mating preferences and reproductive behavior (if any) from potentially interacting factors, such as male–male competition (Jirotkul 1999) and/or audience effects (Makowicz et al. 2010). Further, stimulus females were unexposed, to ensure that potential 17 β -trenbolone-induced changes in these stimulus fish did not influence the behavior of males, a technique employed in previous ecotoxicological research (e.g., Tomkins et al. 2017, 2018; Bertram et al. 2018a). Stimulus females comprised large (standard length minimum and maximum cutoffs of 19.00 and 20.00 mm, respectively; mean \pm SD = 19.58 \pm 0.34 mm, range: 19.05–19.98 mm) and small (standard length minimum and maximum cutoffs of 15.00 and 16.00 mm, respectively; mean \pm SD = 15.45 \pm 0.33 mm, range: 15.02–15.99 mm) sexually mature fish. Across these size classes, large and small stimulus females differed significantly in standard length (Mann–Whitney U = 1892, P < 0.001).

Behavioral trials involved males being drawn randomly from flow-through exposure tanks and allocated to 1 of 12 observation tanks (54 L; 60 cm \times 30 cm \times 30 cm) filled with aged carbon-filtered fresh water (mean temperature \pm SD = 25.89 \pm 0.56 $^{\circ}$ C) to 20 cm depth, with stimulus females being drawn randomly from 1 of 2 holding aquaria (54 L; 60 cm \times 30 cm \times 30 cm). Free-swimming behavioral trials (n = 87) were preceded by a 5-min period of acclimation, after which the male and female were released from their respective holding containers and allowed to interact, with their behavior being video-recorded (Canon PowerShot S120).

Behavior was recorded for 15 min following Herdman et al. (2004), where the same trial duration was used to demonstrate a preference in male guppies for greater female size. Observation tanks were drained and refilled with aged water upon completion of each trial.

The event-recording software JWatcher V1.0 (Blumstein and Daniel 2007) was used to quantify male reproductive behaviors (as described in Houde 1997) from video recordings. Briefly, the number of courtship bouts performed by males was recorded, involving the male orienting toward the female and performing sigmoid displays (courtship) before moving behind the female for an attempted copulation. The frequency of male sneaking behavior, involving the male surreptitiously approaching a nonreceptive female from behind for a forced copulation attempt, was also quantified. Lastly, male following behavior was recorded as the number of times a male actively pursued a female (within 5 cm).

Experimenters were blind to exposure treatment both during data collection and scoring of video-footage, with all footage being scored by one observer to ensure consistency.

Morphological analysis

Immediately after each trial, fish were euthanized with anesthetic clove oil (40 mg/L). Male guppies were dabbed dry and weighed (\pm 0.0001 g), as well as being measured for standard length (\pm 0.01 mm). An index of male condition was calculated, reflecting the mass of a male relative to that expected for its standard length. Specifically, this male condition index was quantified as the residuals from a linear regression of male mass (g) on standard length (mm) (i.e., weight = $-0.211 + 0.019 \times$ length). These measures were also recorded for stimulus females after behavioral trials.

Coloration analysis

Female guppies prefer to mate with males bearing greater orange coloration (i.e., area and chroma; Houde 1997), as has been demonstrated in the source population of fish used in the present study (Brooks and Endler 2001; Gamble et al. 2003). A positive relationship also exists in the laboratory-reared descendants of this population between the area of male orange pigmentation and the number of courting bouts performed toward a female (Bertram et al. 2015). To account for these known relationships in statistical analysis, we quantified the percentage of each male's body area containing orange pigment immediately subsequent to behavioral trials. Briefly, this involved males being photographed on their right side in a standardized fashion (Nikon D90, shutter speed = 1/250, Nikon AF Micro-Nikkor 60 mm f/2.8D), before Photoshop's (CS5 Version 12.0 Extended) Color Range tool was used to sample orange pigmentation from 8 randomly selected reference fish. This orange pigmentation color standard was then used to calculate the area of each male's body surface containing orange pixels (i.e., pixels with colors belonging to the orange pigmentation color standard) as a proportion of the total body area (i.e., the number of pixels forming the body surface). For further details on photographic coloration analysis, see Bertram et al. (2015).

Statistical analysis

Data were analyzed in R version 3.2.3 (R Core Team 2015). Tests of normality (Shapiro–Wilk test; Royston 1995) and homogeneity of variance (Fligner–Killeen test; Conover et al. 1981) were performed, where appropriate. Poisson generalized linear models (GLMs) were used to investigate relationships between counts of recorded behaviors. Vuong tests (*vuong* function, *pscl*

Table 1
Summary of statistical models

Behavioral response	Model	Predictor variables
Number of male courting events	Zero-inflated negative binomial GLM	Treatment Group-means-corrected female standard length Male area of orange pigmentation (%)
Number of male sneaking events	Zero-inflated negative binomial GLM	Treatment Group-means-corrected female standard length Male condition index Male area of orange pigmentation (%)
Number of male following events	Zero-inflated negative binomial GLM	Treatment Group-means-corrected female standard length Male condition index Male area of orange pigmentation (%)

package; Vuong 1989; Jackman 2012) indicated zero-inflation of each behavioral response, with 65.5% of males performing courtship (unexposed = 63.2%, exposed = 67.3%), 35.6% conducting sneak attempts (unexposed = 36.8%, exposed = 34.7%), and 94.3% carrying out following behavior (unexposed = 94.7%, exposed = 93.9%). This zero-inflation was addressed by fitting zero-inflated Poisson (ZIP) GLMs (*zeroinfl* function, *pscl* package; Zeileis et al. 2008). To test for overdispersion of each behavioral response, zero-inflated negative binomial (ZINB) GLMs (*zeroinfl* function) were also fitted and compared with their respective ZIP GLM alternatives using likelihood-ratio tests (*lrtest* function, *lmtest* package; Zeileis and Hothorn 2002). In each case, ZINB GLMs were favored, due to overdispersion of the response variable (Zuur et al. 2009). For all models, predictors were selected based on their biological meaning (Table 1). To represent female standard length within each size class, we calculated group-means-corrected female standard length by subtracting the female groups' means (i.e., the mean of small females, and that of large females) from the lengths of females belonging to the respective groups, and dividing by the groups' standard deviations (SDs). Further, all continuous predictors were centered and standardized to have zero mean and unit variance. General linear hypothesis tests (*glht* function, *multcomp* package; Hothorn et al. 2008) were used for post hoc assessment of differences in the mean response across treatment levels. Assessment of whether coefficients of continuous predictors were significantly different from zero (at $\alpha = 0.05$) was performed using partial Wald tests. Mann–Whitney *U* tests (Mann and Whitney 1947) were used to evaluate whether exposure to 17 β -trenbolone altered male condition index, weight, and/or standard length.

RESULTS

Mating behavior

The number of courting bouts performed by males varied significantly with treatment. Specifically, both unexposed and exposed males performed significantly more courting events when paired with large females than with small females (partial Wald test: unexposed males: $z = 2.54$, $P = 0.011$; exposed males: $z = 2.42$, $P = 0.016$; Figure 1a). However, no difference was detected between the number of courting events performed by unexposed versus exposed males towards large (partial Wald test: $z = 0.24$, $P = 0.810$) or small females (partial Wald test: $z = 0.56$, $P = 0.577$) (Figure 1a). The number of courting events performed by males was not significantly impacted by group-means-corrected female standard

length (partial Wald test: $z = -1.00$, $P = 0.318$), nor by male area of orange pigmentation (partial Wald test: $z = 1.58$, $P = 0.113$).

The frequency of male sneaking was associated with treatment, group-means-corrected female standard length and male condition index. Firstly, regardless of exposure treatment, males performed more frequent sneaking behavior toward larger females than smaller females (partial Wald test: unexposed males: $z = 2.35$, $P = 0.019$; exposed males: $z = 2.63$, $P = 0.008$; Figure 1b). However, when paired with large females, exposed males carried out significantly more sneaking attempts than did unexposed males (partial Wald test: $z = 2.62$, $P = 0.009$; Figure 1b). Specifically, when paired with large females, exposed males were expected to perform 3.51 [2.18, 5.68] (where values in brackets indicate the mean minus 1 standard error (SE), and the mean plus 1 SE, respectively) times as many copulations as unexposed males. Further, when paired with small females, there was a tendency for 17 β -trenbolone-exposed males to perform more frequent sneaking behavior relative to controls, although this result was marginally nonsignificant (partial Wald test: $z = 1.77$, $P = 0.077$; Figure 1b). More generally, there were significant negative relationships between the amount of sneaking behavior performed by males and both group-means-corrected female standard length (partial Wald test: $z = -4.15$, $P < 0.001$) and male condition index (partial Wald test: $z = -3.29$, $P = 0.001$). A 1 SD decrease in female standard length (i.e., 0.33 and 0.34 mm for small and large females, respectively) resulted in 2.59 [2.06, 3.27] times as many male sneaking events. A decrease in male condition index of 1 SD (i.e., 0.012) was predicted to increase the frequency of sneaking behavior by 1.87 [1.55, 2.26] times (Figure 2). Further, a marginally nonsignificant positive trend was observed between the number of sneaking attempts performed and male area of orange pigmentation (partial Wald test: $z = 1.78$, $P = 0.074$).

The number of events of following behavior performed by males did not differ significantly between treatment groups [$\chi^2(3) = 3.33$, $P = 0.342$; Figure 1c]. Further, the number of following events performed by males was not significantly affected by group-means-corrected female standard length (partial Wald test: $z = -1.06$, $P = 0.290$), male condition index (partial Wald test: $z = -0.80$, $P = 0.421$) or male area of orange pigmentation (partial Wald test: $z = 0.92$, $P = 0.356$).

Morphology

Exposure did not affect male weight (Mann–Whitney *U* test: $U = 890$, $P = 0.729$) or standard length (Mann–Whitney *U* test: $U = 1009.5$, $P = 0.504$). However, males exposed to 17 β -trenbolone

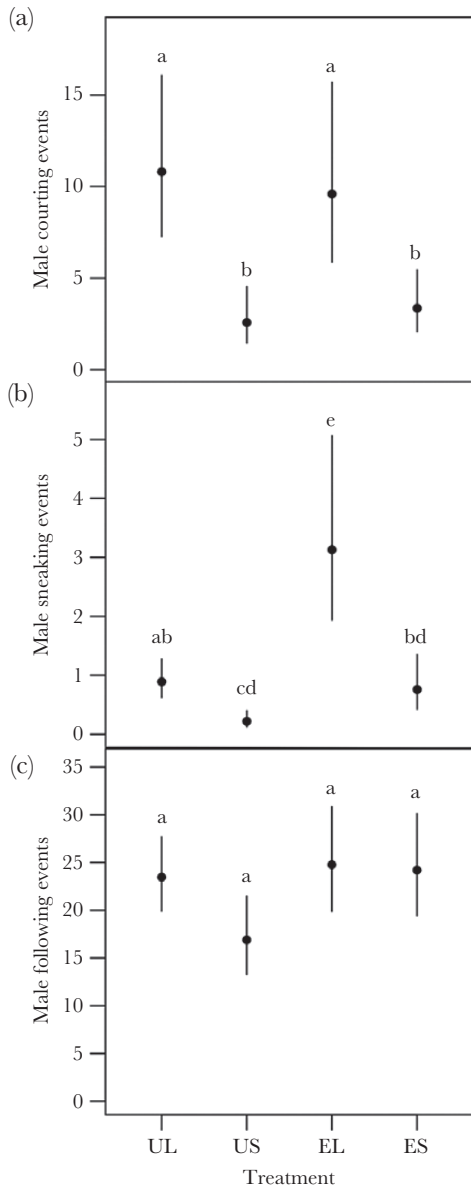


Figure 1 Mean (\pm SE) number of (a) courting events, (b) sneaking events, and (c) following events performed by males across treatments (UL: $n = 19$; US: $n = 19$; EL: $n = 25$; ES: $n = 24$) when continuous predictors were held at their means. Treatments indicate unexposed (U) or 17 β -trenbolone-exposed (E) males, paired with large (L) or small (S) stimulus females. Treatments that do not share lower case letters are significantly different.

had a significantly higher average condition index than unexposed males (Mann–Whitney U test: $U = 677$, $P = 0.030$; Figure 3).

DISCUSSION

We have demonstrated altered male reproductive behavior and morphology resulting from exposure to an environmentally realistic concentration of the androgenic endocrine disruptor 17 β -trenbolone, although male preference for greater female size was maintained at the dosage tested. To date, this is the lowest reported concentration (4 ng/L) of 17 β -trenbolone shown to influence reproductive behavior and morphology in male fish.

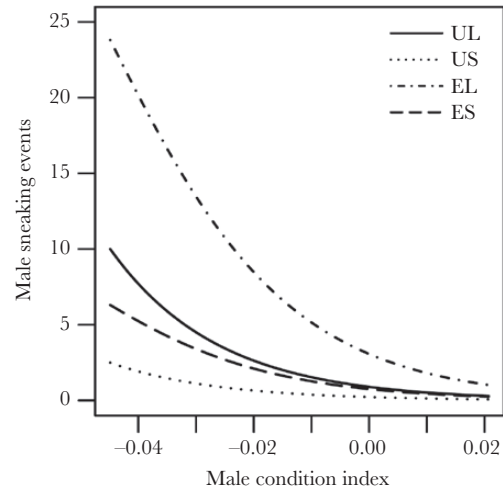


Figure 2 Expected number of male sneaking events given male condition index and treatment (UL: $n = 19$; US: $n = 19$; EL: $n = 25$; ES: $n = 24$), holding group-means-corrected female standard length and male orange pigmentation (% of body area) at their means. Treatments indicate unexposed (U) and exposed (E) males paired with large (L) or small (S) stimulus females.

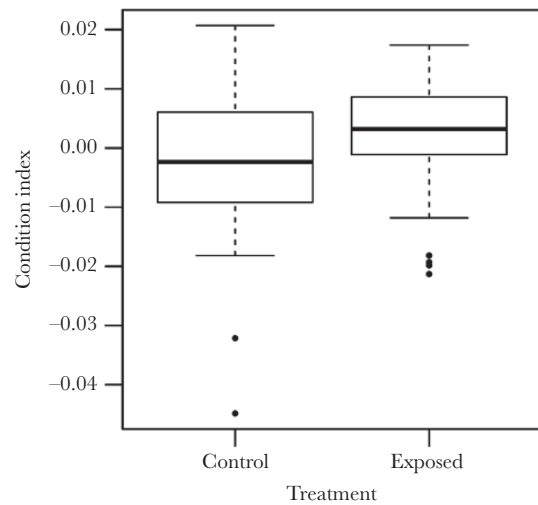


Figure 3 Condition index of unexposed males ($n = 38$) and those exposed to 17 β -trenbolone ($n = 49$).

Although the number of courting bouts carried out by males was not impacted by 17 β -trenbolone, with both unexposed and exposed males demonstrating a preference for greater female size by courting larger females more often, exposure did significantly affect the amount of coercive copulatory behavior (sneak matings) performed. Specifically, despite both unexposed and exposed males again exhibiting mate preference by performing more frequent sneaking behavior toward larger females, this effect was stronger in exposed males, which performed significantly more sneaking events toward large females than did unexposed males. Males exposed to 17 β -trenbolone also showed a significant increase in condition index (i.e., mass relative to length). More generally, male condition index associated negatively with the number of sneaking events performed. This was expected, given that female guppy preference for high-quality males is likely to make courtship behavior more

profitable for those males, while coercive sneaking behavior may be an effective alternative for lower-quality males (Houde 1997).

Hormones control the production and maintenance of sexual behavior in fish (Borg 1994; Munakata and Kobayashi 2010), as in other vertebrates (Rubinow and Schmidt 1996; Cunningham et al. 2012). In most fish species, male sexual behaviors are mediated by testicular androgens (Borg 1994; Munakata and Kobayashi 2010), which bind to androgen receptors (ARs), of which 2 isoforms have been characterized in teleost fish (AR α and AR β ; Harbott et al. 2007). Exogenous androgens such as 17 β -trenbolone—which is a high-affinity ligand for the fish AR (Ankley et al. 2003)—can also activate the AR, and can mimic the effect of endogenous androgens (Wilson et al. 2002; Larsen and Baatrup 2010). Further, it has been hypothesized that 17 β -trenbolone, which is nonaromatizable (Rogozkin 1991), may also indirectly reduce endogenous 17 β -estradiol production by decreasing the production of endogenous androgens (including testosterone), thereby limiting the aromatization of testosterone into 17 β -estradiol (Zhang et al. 2008). Given that a vital role of androgens is the development and regulation of male sexual behaviors (Zuloaga et al. 2008; Cunningham et al. 2012), exposure to exogenous androgens may alter these behaviors. Alteration of androgen-dependent reproductive behaviors resulting from exposure to exogenous androgen agonists has, for example, been reported in American kestrels (*Falco sparverius*) exposed to a brominated flame retardant (Martinson et al. 2015), as well as in methylidihydrotestosterone-exposed African clawed frogs (*Xenopus laevis*) (Hoffmann and Kloas 2012) and cyprinid fish species (Belanger et al. 2010).

Consistent with previous studies on guppies, exposure of males to 17 β -trenbolone did not significantly impact the number of courting events performed (4 ng/L, Tomkins et al. 2016; 22 ng/L, Bertram et al. 2015). Instead, both unexposed and exposed males demonstrated a preference for greater female size by courting large females more frequently, probably because fecundity increases with female size (Houde 1997; Herdman et al. 2004). Interestingly, recent research has demonstrated a reduced frequency of courting behavior in male guppies after exposure to 17 β -trenbolone at 8 ng/L, when under male–male competition (i.e., with rival males being allowed to freely interact and compete over an unexposed female) (Tomkins et al. 2017). This indicates that 17 β -trenbolone-induced reductions in courting behavior in adult guppy males may be context dependent, manifesting only in a competitive setting. This is suggested to result from exposed males exhibiting significantly increased levels of aggression (i.e., chases and fin-nips) toward rival males, thereby limiting the amount of time available for these males to court females (Tomkins et al. 2017).

We show that exposure to 17 β -trenbolone at concentrations as low as 4 ng/L can influence the amount of coercive copulatory behavior (sneak mating events) carried out by male guppies toward females. Although unexposed and exposed males both engaged in more frequent sneaking behavior toward larger females, again suggesting that male preference for female size was not impacted by 17 β -trenbolone at this exposure concentration, males exposed to 17 β -trenbolone performed more sneaking behavior toward large females than did unexposed males. More broadly—that is, independent of male preference for female size—a shift in male reproductive strategy towards coercive mating (sneak behavior) is consistent with previous work on guppies (Bertram et al. 2015; Tomkins et al. 2017) but contrasts with findings reported in another poeciliid, the eastern mosquitofish (*Gambusia holbrooki*), where exposure to 17 β -trenbolone at 6 ng/L did not impact the number of

gonopodial thrusts performed by males toward females (Saaristo et al. 2013). It is important to point out, however, that Saaristo et al. (2013) paired males and females from the same treatment group (i.e., unexposed or exposed), meaning that their result may have been influenced by the exposure status of the female. Moreover, mosquitofish have a coercive mating system in which males do not court females but, instead, engage exclusively in sneak copulations. Taken together, these results in guppies suggest that, in species that employ both courtship and coercive mating behavior, exposure to 17 β -trenbolone can shift relative investment in these key strategies.

The presently observed intensification of sneaking behavior in exposed males has implications for their reproductive fitness. Compared with copulations preceded by courtship, sneak copulations have a lower probability of insemination success (Matthews and Magurran 2000; Russell et al. 2006) and deliver approximately one-third as many sperm into the female's gonoduct (Pilastro and Bisazza 1999). Further, cryptic female choice during or after copulation—via, for example, sperm dumping (Cheng 2004) or biased sperm use (Eberhard 1994; Pizzari and Birkhead 2000)—may disadvantage sneaking males. To our knowledge, cryptic female choice for courting males over sneaking males has not been tested directly in guppies, yet female guppies do show cryptic preference for more colorful males (Pilastro et al. 2004), and cryptic preference for courting males has been documented in various other species (e.g., Edvardsson and Arnqvist 2000; Pizzari and Birkhead 2000). Further, male sneaking is costly to females as it circumvents female mate choice (Pilastro and Bisazza 1999), can physically damage the female's genital pore (Constantz et al. 1989) and may impart costs associated with unnecessary multiple mating, including an increased risk of disease transmission, increased predation risk and reduced foraging efficiency (Bisazza et al. 2001).

A marginally nonsignificant positive association was detected between 17 β -trenbolone exposure and frequency of sneaking events performed by males towards small females. In fact, the rate of sneaking by exposed males paired with small females was similar to—that is, not significantly different from—that of unexposed males paired with large females. This suggests that exposure to 17 β -trenbolone may be causing males to become somewhat more likely to direct sneak copulations towards small females, despite theory suggesting that male reproductive fitness should be maximized by mating with larger, more fecund females (Houde 1997; Herdman et al. 2004). Ejaculate production in guppies is rate limited (Pilastro and Bisazza 1999) and costly (Wedell et al. 2002), meaning that an increase in ejaculate expenditure toward smaller and less fecund females may have negative implications for male fitness.

Exposure to 17 β -trenbolone was associated with a significant increase in male condition index. This was unsurprising given 17 β -trenbolone's strong anabolic activity (Neumann 1976) and is consistent with previous research reporting increased condition index in male guppies exposed for the same period to 17 β -trenbolone at 22 ng/L (Bertram et al. 2015). The increase in condition index observed in the current study was subtle, however, having not been sufficiently explained by either changed body length or weight alone—with neither of these traits being significantly altered by exposure. Therefore, the detected increase in relative mass was a consequence of exposed males having, on average, slightly increased weights as well as slightly smaller standard lengths. The slight difference in standard length between treatment groups is likely due to natural variation since morphogenesis of skeletal elements is complete in adults and is not, therefore, expected to be vulnerable to chemical exposure (Pandey 1969; Baatrup and Junge 2001). That

exposure did not significantly impact male weight is likely a result of the exposure concentration used (4 ng/L), because studies that have measured weight increases have reported significant differences after exposure at 22 ng/L (Bertram et al. 2015) but not at lower concentrations (4 ng/L, Tomkins et al. 2016; 8 ng/L, Tomkins et al. 2017). Weight gain in guppies has also been reported after exposure at much higher concentrations, with juvenile guppies exhibiting an increased rate of growth after a 60-day dietary exposure to trenbolone acetate—the parent compound of 17 β -trenbolone—at a dose of 300 mg/kg in feed (Zamora et al. 2008). Further, exposure of fathead minnows to 17 β -trenbolone has been associated with a concentration-dependent increase in female weight (at 0.5, 5, and 50 μ g/L), although no significant increase in female body weight was seen at lower concentrations (5 and 50 ng/L) and no effect of exposure was seen on the average weight of males (Ankley et al. 2003). Given these results, including the sex- and species-specific effects reported, additional research is needed to determine the susceptibility of fish to morphological alteration via exposure to 17 β -trenbolone at environmentally realistic levels.

CONCLUSION

We report that short-term (21-day) exposure to the pervasive endocrine disruptor 17 β -trenbolone at an environmentally realistic level (4 ng/L) altered male reproductive behavior and morphology in the guppy, although male preference for female size was maintained at this concentration. Given that the ability to appropriately perform reproductive behaviors is fundamentally important to the ecology and evolution of wildlife, the presence of hormonally active chemical pollutants in the environment that are capable of disrupting these behaviors is a major concern. More research is clearly needed to reveal the extent to which these contaminants may interfere with behavioral processes, including mating dynamics, in exposed populations (reviewed in Saaristo et al. 2018). In this regard, although our work demonstrates altered mating strategy in male fish when presented with a stimulus (i.e., unexposed) female in a one-on-one context, there is a need to examine potential impacts of 17 β -trenbolone—as well as other emerging contaminants—on increasingly complex behavioral interactions, to more closely approximate natural systems. For example, important will be to investigate potential contaminant impacts on complex mating interactions in mixed-sex shoals, including with all shoal members being similarly exposed (i.e., either unexposed or exposed). Moreover, partnering this work with interacting natural stressors—e.g., predation pressure, which could itself be impacted by contamination—will be important in further extending these findings to wild fish populations. Therefore, as behavioral analyses are increasingly being integrated into environmental toxicology research, we emphasize the need to incorporate existing knowledge in behavioral ecology to uncover hitherto unknown impacts of chemical pollution on wildlife.

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