Sex in troubled waters: Widespread agricultural contaminant disrupts reproductive behaviour in fish

Michael G. Bertram [a,*], Minna Saaristo [a,b], John B. Baumgartner [c], Christopher P. Johnstone [a], Mayumi Allinson [d], Graeme Allinson [d,e], Bob B.M. Wong [a]

[a] School of Biological Sciences, Monash University, Victoria, Australia
[b] Department of Biosciences, Åbo Akademi University, Turku, Finland
[c] ARC Centre of Excellence for Environmental Decisions, School of Botany, The University of Melbourne, Victoria, Australia
[d] Centre for Aquatic Pollution Identification and Management (CAPIM), The University of Melbourne, Bio21 Institute, Victoria, Australia
[e] Department of Environment and Primary Industries (DEPI), Victoria, Australia

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ABSTRACT

Chemical pollution is a pervasive and insidious agent of environmental change. One class of chemical pollutant threatening ecosystems globally is the endocrine disrupting chemicals (EDCs). The capacity of EDCs to disrupt development and reproduction is well established, but their effects on behaviour have received far less attention. Here, we investigate the impact of a widespread androgenic EDC on reproductive behaviour in the guppy, Poecilia reticulata. We found that short-term exposure of male guppies to an environmentally relevant concentration of 17β-trenbolone—a common environmental pollutant associated with livestock production—influenced the amount of male courtship and forced copulatory behaviour (sneaking) performed toward females, as well as the receptivity of females toward exposed males. Exposure to 17β-trenbolone was also associated with greater male mass. However, no effect of female exposure to 17β-trenbolone was detected on female reproductive behaviour, indicating sex-specific vulnerability at this dosage. Our study is the first to show altered male reproductive behaviour following exposure to an environmentally realistic concentration of 17β-trenbolone, demonstrating the possibility of widespread disruption of mating systems of aquatic organisms by common agricultural contaminants.

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Introduction

Chemical pollutants have accumulated in ecosystems globally, endangering wildlife, ecosystem function and human health (Schwarzenbach et al., 2006). One class of chemical pollutant, known as endocrine disrupting chemicals (EDCs), comprises environmental contaminants with the capacity to disrupt the natural hormonal functioning of organisms (Colborn et al., 1993). Endocrine disruptors are of particular concern given their extreme potency, with exposure to concentrations as low as nanograms per litre having deleterious effects, as well as the propensity of some EDCs to bioaccumulate, persist temporally and act transgenerationally (Diamanti-Kandarakis et al., 2009). Conventionally, studies in ecotoxicology have focussed on direct mortality and chronic sub-lethal effects of EDCs on development and reproduction (Melvin and Wilson, 2013). However, EDCs can also induce alarming changes in

behaviour. Indeed, the particular sensitivity of behaviour to EDCs has driven recent interest in behavioural ecotoxicology as a tool for investigating endocrine disruption at environmentally relevant pollutant concentrations (reviewed in Melvin and Wilson, 2013). Existing studies in behavioural ecotoxicology typically focus on EDCs that disrupt gonadal steroid signalling by interacting with vertebrate estrogen or androgen receptors, as chemical interference with this pathway has the potential to disrupt sexual selection (e.g., Saaristo et al., 2009). However, the vast majority of these efforts have concentrated on EDCs with estrogenic activity. This is surprising because the handful of studies that have considered androgenic EDCs suggest that they are also capable of markedly altering animal behaviour (e.g., Hoffmann and Kloas, 2012).

An androgenic EDC of particular concern is 17β-trenbolone, the most bioactive metabolite of trenbolone acetate, a hormonal growth promotant used extensively in livestock production around the world (Kolodziej et al., 2013). Trenbolone acetate is a powerful steroid, with androgenic and anabolic potency 15–50 times greater than testosterone (Kolodziej et al., 2013; Neumann, 1976). Its metabolite 17β-trenbolone acts as a powerful androgen receptor agonist in the environment, is highly temporally persistent (with a half-life of approximately 260 days;

Schiffer et al., 2001) and has been repeatedly detected in aquatic environments associated with feedlot operations. Detected concentrations of 17β-trenbolone range from ≤20 ng/L in diffuse run-off (Durhan et al., 2006), to as high as 162 ng/L in fields directly receiving effluent (Gall et al., 2011). Recent studies report that exposure to 17β-trenbolone adversely impacts physiological and morphological endpoints in fish species (e.g., Morthorst et al., 2010). However, despite the potency and widespread global use of 17β-trenbolone, very little is known about its effects on behaviour. This is concerning as the ability of animals to produce and maintain behaviour appropriate to their environment is fundamental for survival and reproduction, so that disruption of these behaviours can have dire ecological and evolutionary consequences (reviewed in Candolin and Wong, 2012).

The mating system of the guppy, *Poecilia reticulata*, is ideal for investigating the effects of 17β-trenbolone on reproductive behaviour. The guppy is a small, live-bearing freshwater fish native to north-eastern South America that has a widespread global distribution, precipitated by numerous deliberate and accidental introductions (Lindholm et al., 2005). Importantly, throughout their range, guppies have the potential to be exposed to 17β-trenbolone, as they are known to inhabit water bodies receiving agricultural waste (e.g., Araújo et al., 2009; López-Rojas and Bonilla-Rivero, 2000; Widianarko et al., 2000). Male guppies employ two alternate mating strategies, either soliciting copulations from females through courtship ('sigmoid displays') or coercing copulations through unsolicited 'sneaking' behaviour (Luyten and Liley, 1991). The latter involves males surreptitiously approaching females from behind to insert their gonopodium (a modified anal fin serving as an intromittent organ) into the female's genital pore (Luyten and Liley, 1991). Female guppies are choosy and are known, for example, to prefer males possessing greater orange pigmentation (Houde, 1987). By preferentially associating with certain males over others, females are able to directly influence mating outcomes (Shohet and Watt, 2004).

Here we test the hypothesis that short-term (21-day) exposure to an environmentally relevant concentration of 17β-trenbolone (22 ng/L) alters male and female reproductive behaviour in guppies. A short-term exposure duration was employed as agricultural pollutants often enter aquatic habitats in pulses and these temporally discrete contamination events can have persistent consequences (García et al., 2011; Morthorst et al., 2010).

Materials and methods

Ethical statement

The research detailed in this paper was approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2013/09) and complies with all relevant State and Federal laws of Australia.

Animal housing

This study used laboratory-reared descendants of wild caught guppies from Alligator Creek (19° 26 18 S, 146° 57 01 E), Queensland, Australia. Sexually mature guppies reared in large mixed-sex holding tanks (90 cm × 45 cm × 45 cm) were assumed to be non-virginal given the near-constant mating pressure exerted by males in mixed-sex populations (Houde, 1997; Magurran and Seghers, 1994). Non-virgin fish were used to simulate mixed-sex wild populations, and because mate choice in virgin females can be indiscriminate (Pitcher et al., 2003). Fish were separated by sex into 81 L housing tanks (60 cm × 45 cm × 30 cm) and acclimated to laboratory conditions (25–27 °C; 12:12 h light regime) for 2 months. Fish were fed once daily (Otohime Hirame larval diet; 580–910 μm).

Exposure set-up and monitoring

A flow-through exposure design was used, as described by Saaristo et al. (2013). Fish were assigned to identical 54 L separate-sex aquaria (60 cm × 30 cm × 30 cm), which were monitored for temperature ($\bar{x}$ = 26.38 °C, SD = 0.52 °C) and flow-through rates ($\bar{x}$ = 18.88 mL/min, SD = 0.59 mL/min). In total, 308 fish were randomly assigned to one of seven 17β-trenbolone-exposure tanks, or one of seven unexposed tanks containing fresh water (22 fish per tank). Exposed and unexposed aquaria each comprised four male tanks and three female tanks, with a surplus of fish exposed to ensure adequate sample sizes for each treatment.

The concentration of 17β-trenbolone used ($\bar{x}$ = 22 ng/L, SD = 14.55 ng/L, n = 28) was monitored following Saaristo et al. (2013), with some modifications, using a commercial enzyme-linked immunosorbent assay (ELISA). Weekly water samples were drawn according to the protocol detailed by Saaristo et al. (2013).

Behaviour trials

To investigate the impact of 17β-trenbolone on the reproductive behaviour of guppies, four treatments were employed: (1) unexposed male paired with unexposed female (control; hereafter UU; n = 18), (2) unexposed male with exposed female (UE; n = 19), (3) exposed male with unexposed female (EU; n = 18), and (4) exposed male with exposed female (EE; n = 20). Fish were taken at random and equally from each exposure tank and allocated to behavioural trials (n = 75), which took place in 54 L observation tanks (60 cm × 30 cm × 30 cm) containing fresh water (i.e. water free from 17β-trenbolone). Trials involved a 5-minute period of acclimation, before both fish were released from holding containers and allowed to freely interact, while their behaviour was video-recorded for 15 min. Fish were euthanized immediately after trials using an overdose (40 mg/L) of anaesthetic clove oil, following which morphological and colouration analyses were conducted.

Reproductive behaviours (see Supplementary materials Table S1 for detailed descriptions) were quantified from video recordings using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007). Briefly, for males, we counted the number of courtship bouts performed (i.e., male orienting in front of the female and performing courtship displays), as well as the number of sneaking attempts (i.e., male surreptitiously approaching the female from behind for forced copulation). For females, we counted the number of times that a female actively associated with the male, a frequently used measure of mating intent in poeciliid fishes (e.g., Kahn et al., 2010), including guppies (e.g., Shohet and Watt, 2004).

Morphological analysis

After behavioural trials, we measured the length of males and females (±0.01 mm). Males were also weighed (±0.0001 g), and an index of male condition was derived from a regression of the mass (g) of all males against their standard length (mm). This male Condition Index was calculated as the residuals from the least squares regression line (i.e., weight = −0.164 + 0.016 × length).

Colouration analysis

Because female guppies typically prefer males with greater orange pigmentation (e.g., Houde, 1987)—including in the population from which fish were sourced for the present study (Brooks and Endler, 2001; Gamble et al., 2003)—the percentage of males' body surface containing orange pigments was assessed using photographic colouration analysis, performed immediately after behavioural trials. After euthanasia, fish were photographed on the right side in a

standardised fashion (Nikon D90, shutter speed = 1/250, Nikon AF Micro-Nikkor 60 mm f/2.8D).

Colouration analysis involved using Photoshop (CS5 Version 12.0 Extended) to isolate the fishes' body surface, from snout to caudal peduncle (i.e., excluding fins). Eight reference specimens were randomly selected (4 exposed, 4 unexposed). Photoshop's Colour Range tool was used to sample the orange pigmentation of the reference fish to create an orange pigmentation colour standard, which was applied to all photographs. For each fish, the extent of orange pigmentation was calculated as the number of orange pixels (i.e., pixels with colours belonging to the orange pigmentation colour standard) as a proportion of the total body area (i.e., the number of pixels forming the body surface).

Statistical analysis

Data were analysed in R version 3.0.2 (R Core Team, 2013). Where appropriate, data were tested for normality (Shapiro–Wilk test, *shapiro.test* function; Royston, 1995) and homogeneity of variance (Fligner–Killeen test, *fligner.test* function; Conover et al., 1981). To assess whether exposure to 17β-trenbolone impacted male Condition Index, a two-sample *t*-test was used. Relationships were examined between behavioural responses and a small suite of predictors by fitting Poisson Generalised Linear Models (GLMs) (detailed in Supplementary materials Tables S2–S5). Vuong tests (*vuong* function, *pscl* package; Jackman, 2012; Vuong, 1989) indicated zero-inflation of count data used for the latter, which was accommodated by fitting Poisson models with the *zeroinfl* function (*pscl* package; Zeileis et al., 2008). Continuous predictors were centred and standardised to have zero mean and unit variance to enable a direct comparison of their coefficients. Post-hoc evaluation of the differences in the mean response across factor levels (holding other predictors at their means) was performed through General Linear Hypothesis Testing (GLHT) (*glht* function, *multcomp* package; Hothorn et al., 2008). Partial Wald tests were used to assess whether coefficients were equal to zero.

Results

Male behaviour

The number of courting events performed by males was associated with treatment, male orange pigmentation, male Condition Index and female length. Relative to males in the control treatment (i.e., unexposed males paired with unexposed females), both unexposed and exposed males performed fewer courting events when paired with exposed females (partial Wald test: $z = -2.219$, $p = 0.027$ and $z = -2.409$, $p = 0.016$, respectively; Fig. 1A). Increased male orange pigmentation was associated with an increase in courting event occurrence, with an average of 7.6% ($\pm$SE: 5.4–9.8%) more courting attempts for each one standard deviation (4.73%) increase in percent orange pigmentation (partial Wald test: $z = 3.633$, $p < 0.001$; Fig. 2). Male Condition Index was also positively associated with number of male courting events (partial Wald test: $z = 7.509$, $p < 0.001$), with a one standard deviation increase in male Condition Index (i.e., 0.012) yielding an 18.8% ($\pm$SE: 16.1–21.6%) increase in the number of courting events. Males performed fewer courting events when paired with longer females (partial Wald test: $z = -2.447$, $p = 0.014$), with a 4.9% ($\pm$SE: 2.9–6.9%) reduction in the number of courting events for every one standard deviation (i.e., 5.6 mm) increase in female length.

The number of sneaking attempts performed by males varied significantly with treatment, male Condition Index and female length. The number of sneaking attempts performed was significantly different between all pairs of treatments (Fig. 1B). Males in the control treatment performed fewer sneaking attempts than males in any other treatment group (partial Wald test: all $z \geq 3.932$, all $p < 0.001$; Fig. 1B). Unexposed males paired with exposed females snuck more than exposed males
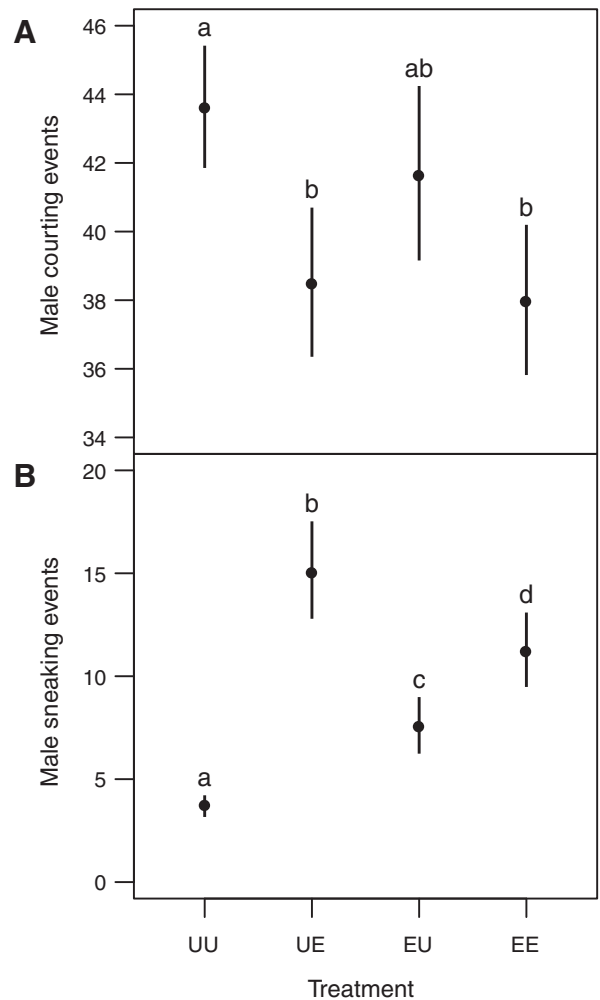

Fig. 1. Mean ($\pm$SE) of the (A) number of male courting events (orienting and sigmoid behaviours) and (B) number of male sneaking attempts across treatment groups ($n = 18, 19, 18$ and 20, for UU, UE, EU and EE, respectively). Treatments indicate unexposed (U) and exposed (E) fish, with male treatment followed by female treatment. Treatments without lower case letters in common are significantly different.

paired with exposed females (partial Wald test: $z = 2.704$, $p = 0.007$), with males from both of these treatments performing more sneaking attempts than exposed males paired with unexposed females (partial Wald test: $z = 5.127$, $p < 0.001$ and $z = 2.777$, $p = 0.005$, respectively; Fig. 1B). Male Condition Index was negatively associated with the number of sneaking attempts performed by males (partial Wald test: $z = -5.261$, $p < 0.001$), with a one standard deviation increase in Condition Index (i.e., 0.012) associated with 20.0% ($\pm$SE: 16.5–23.3%) fewer sneaking attempts per trial. Female total length also related negatively with the number of male sneaking attempts performed (partial Wald test: $z = -6.159$, $p < 0.001$), with a one standard deviation increase in female length (i.e., 5.6 mm) yielding 28.9% ($\pm$SE: 24.8–32.7%) fewer sneaking attempts.

Female behaviour

Female association behaviour varied with male treatment. On average, unexposed females associated approximately 18.9% ($\pm$SE: 10.1–28.5%) more frequently with exposed males than with unexposed males (partial Wald test: $z = 2.239$, $p = 0.025$; Fig. 3). There was a significant positive effect of male orange pigmentation (partial Wald test: $z = 7.967$, $p < 0.001$) and a negative effect of female length (partial Wald test: $z = -13.296$, $p < 0.001$) on female association behaviour,
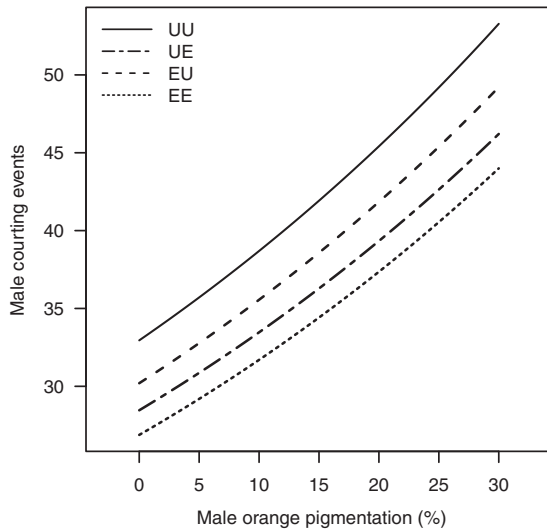
Fig. 2. Expected number of male courting events given male orange pigmentation (% of body area) and treatment (n = 18, 19, 18 and 20, for UU, UE, EU and EE, respectively), holding female total length and male Condition Index at their means. Treatments indicate unexposed (U) and exposed (E) fish, with male treatment followed by female treatment.
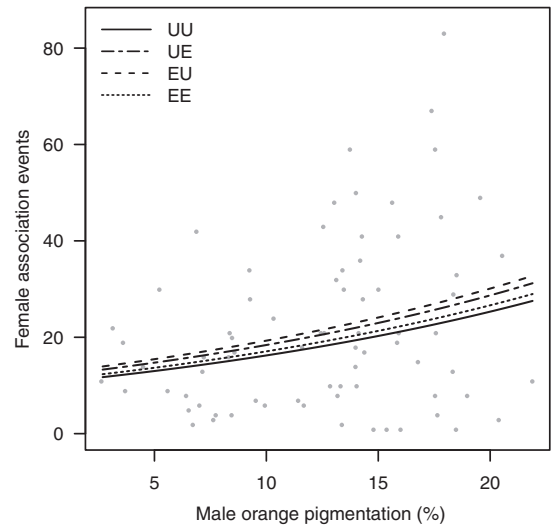

Fig. 4. Expected number of female association events given male orange pigmentation (% of body area) for each treatment (n = 18, 19, 18 and 20, for UU, UE, EU and EE, respectively), holding female total length and male Condition Index at their means. Points indicate observed data.

but no effect of male Condition Index (partial Wald test: $z = -1.898$, $p = 0.058$). An increase in male orange pigmentation of one standard deviation (i.e., 4.73%) corresponded with a 23.4% ($\pm$ SE: 20.2–26.7%) increase in the number of associations (Fig. 4). A one standard deviation increase in total female length (i.e., 5.6 mm) corresponded to a decrease in the number of female association events of 30.7% ($\pm$ SE: 28.7–32.6%).

Morphology

Exposed males had, on average, a significantly higher Condition Index than unexposed males (two-sample t-test: $t = 2.454$, df = 70.174, $p = 0.017$; Fig. 5). This was due to exposed males being heavier (two-sample t-test: $t = 2.296$, df = 72.985, $p = 0.025$), while male length was unaffected by exposure (two-sample t-test: $t = 1.231$, df = 72.926, $p = 0.222$).
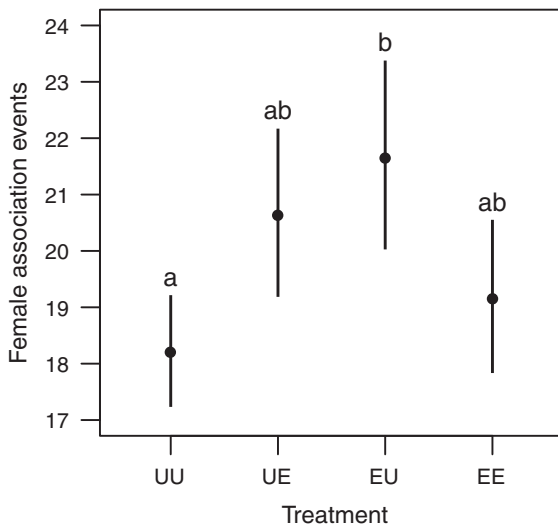
Discussion

This research is the first to document altered male reproductive behaviour following exposure to 17β-trenbolone at an environmentally relevant concentration. Males paired with exposed females performed fewer courtship bouts than did males in the control treatment. Male exposure to 17β-trenbolone led to an increase in sneaking behaviour when paired with unexposed females. However, this finding was reversed when males were paired with exposed females, with unexposed males sneaking more than exposed males. In addition, regardless of male exposure status, males performed more sneaking behaviour when paired with exposed females. Exposed males paired with unexposed females also attracted more female association behaviour than males in the control treatment. More generally, males possessing greater areas of orange pigmentation performed more courting bouts toward females than less-colourful males. This correlation was anticipated, as orange pigmentation and display rate are both honest signals of male condition (Kodric-Brown and Nicoletto, 2001; Nicoletto, 1993).
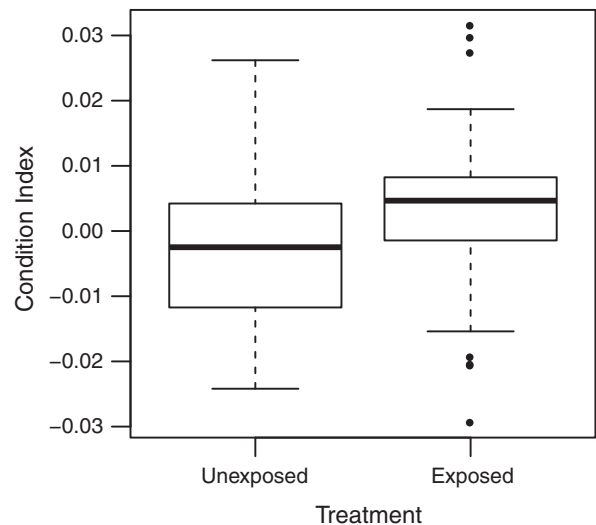

Fig. 3. Mean ($\pm$ SE) number of female association events across treatment groups (n = 18, 19, 18 and 20, for UU, UE, EU and EE, respectively). Treatments indicate unexposed (U) and exposed (E) fish, with male treatment followed by female treatment. Treatments without lower case letters in common are significantly different.

Fig. 5. Boxplots of male Condition Index for unexposed males (n = 37) and those exposed to 17β-trenbolone (n = 38).

Vertebrate male sexual behaviours are reliant on androgens for their production and maintenance (Cunningham et al., 2012). As a potent non-aromatizable androgen receptor agonist (Rogozkin, 1991), 17β-trenbolone has the capacity to disturb gonadal steroid signalling pathways by disrupting the hypothalamic–pituitary–gonadal (HPG) axis. Although the varied mechanisms behind the masculinising effects of 17β-trenbolone are not yet wholly understood (Larsen and Baatrup, 2010), it is known to bind with high affinity to available androgen receptors, mimicking the effects of testosterone (Wilson et al., 2002), and is hypothesised to indirectly inhibit the production of 17β-estradiol by limiting the production of testosterone, and thereby limiting the aromatisation of testosterone to 17β-estradiol (Zhang et al., 2008). Given that a central role of androgens is the modulation of male sexual and aggressive behaviours (Cunningham et al., 2012), the anomalous presence of androgenic EDCs may result in the 'hyper-masculinisation' of these traits in males. This phenomenon has been documented previously. For example, exposure of African clawed frogs (*Xenopus laevis*) to androgenic endocrine disruptors intensified androgen-dependent male mate calling (Hoffmann and Kloas, 2012), and increased the intensity of male sexual behaviours in various cyprinid fish species (Belanger et al., 2010). The present results demonstrate that, in guppies, the relative use of alternate reproductive strategies by males can be altered by exposure to 17β-trenbolone. Specifically, despite 17β-trenbolone having no significant effect on mate solicitation by males (i.e., courtship), the increased number of sneaking attempts performed by exposed males toward unexposed females (relative to the control group) suggests that exposed males may favour this coercive reproductive strategy. Interestingly, this finding was reversed given female exposure, with unexposed males sneaking upon exposed females more than exposed males, possibly indicating a greater capacity of unexposed males to take advantage of female exposure (although the mechanisms underlying this possible phenomenon are not presently considered).

Disruption of the relative usage of alternative male reproductive strategies has implications for male reproductive success, as sneaking behaviour is associated with reduced insemination efficiency relative to copulations preceded by courtship (Pilastro and Bisazza, 1999). Although sneaking behaviour is a viable sperm transfer method, sperm transfer rates are approximately three times higher when delivered after courtship (Pilastro and Bisazza, 1999). Further, postcopulatory female choice may hamper the average reproductive success of males engaging in sneaking behaviour. Such directional postcopulatory sexual selection has been documented in female guppies, which have been shown to bias fertilisation in favour of more colourful males (Pilastro et al., 2004).

Female exposure to 17β-trenbolone led to a decrease in the frequency of male courtship behaviour, and an increase in male sneaking behaviour (relative to the control group). Male guppies typically have very high levels of sexual activity, meaning that females receive continual mating attempts that are mostly unwanted (Houde, 1997; Magurran and Seghers, 1994). Typically, females actively avoid these incessant mating attempts by swimming away from pursuing males (Houde, 1997). Despite the males in the control group showing differential courtship behaviour relative to unexposed males paired with exposed females, the present findings suggest that the impact of female exposure to 17β-trenbolone on male reproductive behaviour was primarily male-driven, as female association behaviour (a reproductive behaviour indicative of receptivity) was not influenced by female exposure. In addition, other metrics of female behaviour (i.e. swimming away from a pursuing male, non-reproductive behaviour, and stressed behaviour) also did not differ significantly between exposed and unexposed females (unpublished data). Unexposed females were, however, more likely to associate with exposed males than unexposed males. Alternatively, it is possible that 17β-trenbolone-exposure impacted females in a manner not presently investigated, driving the observed changes in male behaviour. Regarding courtship-initiated mating, females are able to exercise mating preferences—via both precopulatory and postcopulatory

mechanisms—by choosing which males to mate with, and which sperm to use for fertilisation (Pilastro et al., 2004; Shohet and Watt, 2004). Sneaking behaviour, however, circumvents female precopulatory mate choice, thus exposure to 17β-trenbolone may directly interfere with sexual selection in guppies.

No significant effect of female exposure to 17β-trenbolone was detected on female reproductive behaviour. This result was unexpected as androgen receptors are not sex-specific and endogenous androgens, as well as having an essential role in the development and maintenance of male traits, serve important functions in female vertebrates (Staub and De Beer, 1997). Androgens are involved in the regulation of female sexual and aggressive behaviours (Staub and De Beer, 1997), meaning that the mechanisms controlling these behaviours are particularly vulnerable to endocrine disruption. Female exposure to androgenic EDCs has previously been linked with physical and behavioural masculinisation, including the expression of male reproductive behaviour (Howell et al., 1980). Exposure of fathead minnow (*Pimephales promelas*) to 17β-trenbolone has been shown to severely alter female reproductive biology and suppress the production of endogenous sex steroid hormones, indicating masculinisation (Ankley et al., 2003). As such, 17β-trenbolone was expected to reduce female receptivity in the present study, but this was not observed. The resilience of the metrics of female reproductive behaviour presently considered to the concentration of 17β-trenbolone employed suggests a differential vulnerability to this EDC between sexes, a phenomenon previously documented in response to other EDCs (e.g., Kundakovic et al., 2012). Consistent with prior research, females exhibited a strong preference for males possessing greater orange pigmentation (e.g., Pilastro et al., 2004).

This study found that exposure to 17β-trenbolone was associated with an increase in male Condition Index, due to exposed males being heavier, despite there being no significant difference in length between exposed and unexposed males. This weight gain was anticipated, given the potent growth-promoting activity of 17β-trenbolone. A similar finding was reported in a study that exposed juvenile guppies to trenbolone acetate for 60 days (Zamora et al., 2008). In that study, however, fish were exposed to 300 mg/kg of trenbolone acetate, a level far higher than those having been reported in the environment. The present research, however, indicates that environmentally realistic levels of contamination are sufficient to cause weight gain, even with short-term exposure. For various taxa, including fish, heavier males have greater reproductive success in competitive breeding scenarios (e.g., Jacob et al., 2009). Although the present study did not test for the effect of 17β-trenbolone on male competitive ability, the increased weights of males exposed to 17β-trenbolone may confer an advantage in jockeying for contested fertilisations. This potential scenario holds ecological relevance as EDC concentrations are typically spatially and temporally variable (e.g., Grover et al., 2011; Lee et al., 2014), and guppies have the capacity to move considerable distances between habitats (Croft et al., 2003), making interactions between exposed and unexposed individuals likely.

Conclusion

This study reports that short-term (21-day) exposure to an environmentally relevant concentration (22 ng/L) of the androgenic endocrine disruptor 17β-trenbolone can alter reproductive behaviour and morphology in the guppy. This is the first study to show altered reproductive behaviours in male animals resulting from an environmentally realistic exposure to 17β-trenbolone. Given the prevalence and potent biological activity of 17β-trenbolone, the ongoing multidisciplinary scrutiny of this EDC is necessary to reveal the consequences of its presence in the environment.

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Funding statement

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2015.03.002.

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