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

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Abstract

Chemical pollution is a pervasive and insidious agent of environmental change. One class of chemical pollutant threatening ecosystems globally are 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 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. Exposure to 17β-trenbolone was also associated with greater male mass. However, no effect of exposure was detected in females, 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.

Keywords

Endocrine disrupting chemical, EDC, hormonal growth promotant, 17β-trenbolone, trenbolone acetate, guppy, *Poecilia reticulata*, behavioural ecotoxicology, sexual selection, reproductive behaviour.

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 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 are known to inhabit water bodies receiving agricultural waste (e.g., 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).

In this study we investigate the impacts of short-term (21-day) exposure to an environmentally relevant concentration of 17β-trenbolone (22 ng/L) on 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°26'17.94" S, 146°57'1.09" E), Queensland, Australia. Guppies 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 ($\overline{x}$ = 26.38°C, SD = 0.52°C) and flow-through rates ($\overline{x}$ = 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).

The concentration of 17β-trenbolone used ($\overline{x}$ = 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). Behavioural trials (*n* = 75) took place in 54 L observation tanks (60 cm × 30 cm × 30 cm) containing fresh water. Trials involved a five-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 minutes. Fish were euthanized immediately after trials using an overdose (40

mg/L) of anaesthetic clove oil, before morphological and colouration analyses were conducted.

Reproductive behaviours were quantified from video recordings using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007). For males, we quantified the number and total duration 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). Subsequently, we quantified the total time spent by females actively associating 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 = $-0.164 + 0.016 \times$ length).

Colouration analysis

Because female guppies typically prefer males with greater orange pigmentation (e.g., Houde, 1987), including in the source population of fish used in 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

175 photographed on the right side in a standardised fashion (Nikon D90, shutter speed =
176 1/250, Nikon AF Micro-Nikkor 60 mm f/2.8D).

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

186

187 *Statistical analysis*

188 Data were analysed in R version 3.0.2 (R Core Team, 2013). Where appropriate, data
189 were tested for normality (Shapiro-Wilk test, *shapiro.test* function; Royston, 1995)
190 and homogeneity of variance (Fligner-Killeen test, *fligner.test* function; Conover et
191 al., 1981). To assess whether exposure to 17β-trenbolone impacted male Condition
192 Index, a two-sample *t*-test was used. Relationships were examined between
193 behavioural responses and a small suite of predictors through Multiple Linear
194 Regression (MLR) and by fitting Poisson Generalized Linear Models (GLMs)
195 (detailed in Supplementary materials). Vuong tests (*vuong* function, *pscl* package;
196 Jackman, 2012; Vuong, 1989) indicated zero-inflation of count data used for the
197 latter, which was accommodated by fitting Poisson models with the *zeroinfl* function
198 (*pscl* package; Zeileis et al., 2008). Continuous predictors were centred and
199 standardised to have zero mean and unit variance to enable direct comparison of their

coefficients. First-order interactions between predictors were pruned iteratively through reverse stepwise elimination, leaving only those that were statistically significant. *Post-hoc* evaluation of the differences in 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). This process used partial Wald tests to assess differences between factor levels for zero-inflated Poisson GLMs, and two-tailed *t*-tests to achieve the same for MLR models.

Results

Male behaviour

Total male courting time varied significantly with both treatment and male Condition Index. Relative to males in the control treatment (UU), both unexposed and exposed males spent less time courting exposed females (*t*-test: $t = -2.177$, df = 65, $p = 0.033$ and $t = -2.190$, df = 65, $p = 0.032$, respectively; Fig. 1A). The effect of male Condition Index on time spent courting depended on treatment, with a one standard deviation increase in Condition Index (i.e., 0.012) relating to an increase in time spent courting of 80.0 ± 28.8 seconds (*t*-test: $t = 2.782$, df = 65, $p = 0.007$) for males in the control treatment. No relationship was detected between Condition Index and time spent courting in any other treatment (*t*-test: all $p > 0.05$).

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, 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. 1B). Increased

male orange pigmentation was associated with an increase in courting event occurrence (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 a 0.672 ± 0.435 increase in the log of the number of courting events (on average, an additional 95.82% courting events per trial).

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. 1C). 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. 1C). Unexposed males paired with exposed females snuck more than did exposed males 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. 1C). 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 0.223 ± 0.050 fewer sneaking attempts per trial (on the log scale; corresponding to a 19.99% decrease in sneaking attempts). 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 (5.55 mm) yielding 0.340 ± 0.055 fewer sneaking attempts (on the log scale; equivalent to 28.85% fewer sneaking attempts).

Female behaviour

Female association time was not affected by treatment (*t*-test: all $p > 0.05$). A reduced model, excluding treatment, revealed significant effects of male orange pigmentation (*t*-test: $t = 2.912$, df = 71, $p = 0.005$) and female length (*t*-test: $t = -6.298$, df = 71, $p < 0.001$) on female association time, but no effect of male Condition Index (*t*-test: $t = -0.186$, df = 71, $p = 0.853$). An increase in male orange pigmentation of one standard deviation (i.e., 4.73%) corresponded with 33.3 ± 11.4 additional seconds spent by females associating (Fig. 3). Longer females also spent less time associating with males, with a one standard deviation increase in total female length (i.e., 5.6 mm) relating with a decrease in female association time of 73.8 ± 11.7 seconds.

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. 4). 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: $t = -1.103$, df = 69.617, $p = 0.274$).

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 spent less time courting, and performed fewer courtship bouts, than did males in the control treatment. Further, 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

275 females, with unexposed males sneaking more than exposed males. In addition,
276 regardless of male exposure status, males performed more sneaking behaviour when
277 paired with exposed females. More generally, males possessing greater areas of
278 orange pigmentation performed more courting bouts toward females than less-
279 colourful males, regardless of contamination with 17β-trenbolone. This correlation
280 was anticipated, as orange pigmentation and display rate are both honest signals of
281 male condition (Kodric-Brown and Nicoletto, 2001; Nicoletto, 1993).

282 Vertebrate male sexual behaviours are reliant on androgens for their
283 production and maintenance (Cunningham et al., 2012). As a potent androgen agonist,
284 17β-trenbolone has the capacity to disturb gonadal steroid signalling pathways by
285 increasing androgen synthesis. Given that a central role of androgens is the
286 modulation of male sexual and aggressive behaviours (Cunningham et al., 2012), the
287 anomalous presence of androgenic EDCs may result in the 'hyper-masculinisation' of
288 these traits in males. This phenomenon has been documented previously. For
289 example, exposure of African clawed frogs (*Xenopus laevis*) to androgenic endocrine
290 disruptors intensified androgen-dependent male mate calling (Hoffmann and Kloas,
291 2012), and increased the intensity of male sexual behaviours in various cyprinid fish
292 species (Belanger et al., 2010). The present results demonstrate that, in guppies, the
293 relative use of alternate reproductive strategies by males can be altered by exposure to
294 17β-trenbolone. Specifically, despite 17β-trenbolone having no significant effect on
295 mate solicitation by males (i.e., courtship), the increased number of sneaking attempts
296 performed by exposed males toward unexposed females (relative to the control group)
297 suggests that exposed males may favour this coercive reproductive strategy.
298 Interestingly, this finding was reversed given female exposure, with unexposed males
299 sneaking upon exposed females more than exposed males, possibly indicating a

300 greater capacity of unexposed males to take advantage of female exposure (although
301 the mechanisms underlying this possible phenomenon are not presently considered).
302 Disruption of the relative usage of alternative male reproductive strategies has
303 implications for male reproductive success, as sneaking behaviour is associated with
304 reduced insemination efficiency relative to copulations preceded by courtship
305 (Pilastro and Bisazza, 1999). Although sneaking behaviour is a viable sperm transfer
306 method, sperm transfer rates are approximately three times higher when delivered
307 after courtship (Pilastro and Bisazza, 1999). Further, postcopulatory female choice
308 may hamper the average reproductive success of males engaging in sneaking
309 behaviour. Such directional postcopulatory sexual selection has been documented in
310 female guppies, which have been shown to bias fertilisation in favour of more
311 colourful males (Pilastro et al., 2004).
312 Female exposure to 17β-trenbolone led to a decrease in the total duration and
313 frequency of male courtship behaviour, and an increase in male sneaking behaviour
314 (relative to the control group). Male guppies typically have very high levels of sexual
315 activity, meaning that females receive continual mating attempts that are mostly
316 unwanted (Houde, 1997). Typically, females actively avoid these incessant mating
317 attempts by swimming away from pursuing males (Houde, 1997). The present
318 findings suggest that the impact of female exposure to 17β-trenbolone on male
319 reproductive behaviour was male-driven, as female association time (indicative of
320 receptivity) was not influenced by exposure. This implies that exposure to 17β-
321 trenbolone inhibited the ability of females to actively avoid males. Regarding
322 courtship-initiated mating, females are able to exercise mating preferences — via both
323 precopulatory and postcopulatory mechanisms — by choosing which males to mate
324 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 17β-trenbolone-exposure may directly interfere with sexual selection in guppies.

No significant effect of 17β-trenbolone exposure on female reproductive behaviour was detected. 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 production of male reproductive behaviour (Howell et al., 1980). Further, exposure of fathead minnow (*Pimephales promelas*) to 17β-trenbolone has been shown to severely alter female reproductive biology and supress 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), regardless of exposure to 17β-trenbolone.

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 exposing juvenile guppies to trenbolone acetate for 60 days. In that study, however, fish were exposed dietarily to 300 mg/kg of trenbolone acetate (Zamora et al., 2008), which is neither an environmentally relevant mode, nor concentration, of exposure. 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). Thus, 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), meaning that interactions between exposed and unexposed individuals are 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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Appendix A. Supplementary materials

Supplementary data to this article can be found online at

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Figure legends

Fig. 1. Mean (± SE) of the (A) duration (seconds) of male courting behaviour (orienting and sigmoid behaviours), (B) number of male courting events (orienting and sigmoid behaviours) and (C) 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.

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). Treatments indicate unexposed (U) and exposed (E) fish, with male treatment followed by female treatment.

Fig. 3. Female association time (seconds) given male orange pigmentation (% of body area) across treatments (n = 75). Solid black line indicates least squares regression line and shaded region indicates 95% confidence interval.

Fig. 4. Boxplots of male Condition Index for unexposed males (n = 37) and those exposed to 17β-trenbolone (n = 38).