



Disruption of male mating strategies in a chemically compromised environment

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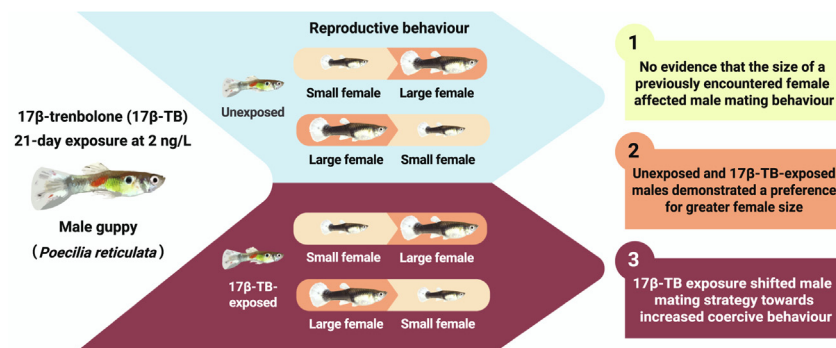
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HIGHLIGHTS

- Contamination of the environment with bioactive pharmaceuticals is a major problem.
- We tested for impacts of the endocrine disruptor 17 β -trenbolone (17 β -TB) on male guppies.
- Exposure altered male mating strategy when females were encountered sequentially.
- Exposed males shifted towards a coercive 'sneaking' reproductive strategy.
- Experience with a previous female was not found to affect male mating behaviour.

GRAPHICAL ABSTRACT



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ABSTRACT

A leading source of endocrine-disrupting chemicals (EDCs) in the environment is run-off of veterinary pharmaceuticals used in agriculture, including hormonal growth promotants (HGP). Despite being banned in various countries, HGP use is still common in beef production around the world. The androgenic steroid 17 β -trenbolone (17 β -TB) is a HGP that commonly enters surface waters via livestock effluent run-off. Here, we used a flow-through system to expose wild-caught adult male guppies (*Poecilia reticulata*) to an environmentally realistic level of 17 β -TB (average measured concentration = 2 ng/L) for 21 days. We then compared the response of exposed and unexposed males to sequentially presented large and small stimulus (unexposed) females. Due to a positive size-fecundity relationship, larger females are generally expected to be preferred by males. While we found no evidence that the size of a previously encountered female affected the amount of courtship or coercive 'sneak' mating behaviour performed by males during the second presentation, males from both exposure treatments conducted more frequent courting events towards larger females during both presentations, suggesting an absolute preference for greater female size. Further, across both presentations, 17 β -TB exposure caused a shift in male mating strategy towards increased coercive sneaking behaviour, although male sequential investment into mating effort was not impacted at the tested dosage. In combination, our findings demonstrate

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that exposure to a field-realistic level of a widespread agricultural pollutant alters male mating strategies in fish, and contribute to a growing understanding of sub-lethal impacts of chemical contaminants on complex behaviours in wildlife.

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1. Introduction

Endocrine-disrupting chemicals (EDCs) are pollutants of particular concern to the scientific community due to their capacity to disturb developmental, physiological and behavioural processes in wildlife at low sub-lethal exposure concentrations (Diamanti-Kandarakis et al., 2009; Godfray et al., 2019). Among the leading transport pathways of EDCs into the environment is run-off of chemicals from agricultural fields into adjacent aquatic ecosystems (Biswas et al., 2017; Kenyon et al., 2019). Moreover, concern is mounting over a group of EDCs known as hormonal growth promoters (HGP), which are highly potent veterinary pharmaceuticals used to promote growth in livestock (Lean et al., 2018). Despite being banned in the European Union (Johnson, 2015), the use of HGPs is common in many of the world's leading beef-producing countries (Hunter, 2010; Kolodziej et al., 2013; Johnson, 2015). For example, in the U.S., the world's leading beef producer, it is estimated that 96% of cattle in concentrated feedlots receive growth-promoting hormones (USDA, 2000). As a result, it is predicted that up to 43,000 kg of growth-promoting hormones per year are released into the environment via feedlot cattle effluent run-off in the U.S. alone (Lange et al., 2002; US EPA, 2002; USDA, 2011; Biswas et al., 2017).

Among the most commonly administered HGPs globally is trenbolone acetate (TBA), a synthetic anabolic steroid with 15–50 times the potency of testosterone (Kolodziej et al., 2013; Lee et al., 2018). After implantation, TBA is hydrolysed to various metabolites, including 17 β -trenbolone (17 β -TB), which is a very high-affinity androgen receptor ligand (Wilson et al., 2002; Kenyon et al., 2019). Given the widespread use of TBA, 17 β -TB has repeatedly been detected in environments impacted by livestock operations (reviewed in Ankley et al., 2018), including in manure (0.5–4.3 μ g/kg; Schiffer et al., 2001), soil (0.5–6.1 μ g/kg; Schiffer et al., 2001; Webster et al., 2012), feedlot run-off and lagoon water (0.0015–270 ng/L; Schiffer et al., 2001; Soto et al., 2004; Durhan et al., 2006; Bartelt-Hunt et al., 2012; Khan and Lee, 2012; Parker et al., 2012; Webster et al., 2012), and dilute river water (0.0013–20 ng/L; Soto et al., 2004; Durhan et al., 2006). This is worrying given that 17 β -TB is temporally persistent (half-life: ~260 days in effluent; Schiffer et al., 2001), can bioconcentrate and bioaccumulate in aquatic species (Ankley et al., 2003; Lagesson et al., 2019), and is known to affect androgen receptor signalling pathways at low environmentally realistic exposure concentrations. A large body of research has demonstrated that 17 β -TB exposure causes changes in endocrine function (e.g. altered sex steroid metabolism, effects on gonadal stage, masculinisation of females), and can produce a variety of adverse apical effects (e.g. skewed sex ratios, impacts on fertility and fecundity) (reviewed in Ankley et al., 2018). Furthermore, over the last five years, it has been revealed that exposure to 17 β -TB can disrupt a range of key behaviours in non-target species, including activity and exploratory behaviour (Bertram et al., 2018a; Lagesson et al., 2019), foraging (Bertram et al., 2018a), shoaling (Bertram et al., 2018a), boldness (Lagesson et al., 2019), risk-taking (Heintz et al., 2015), and mating behaviour (Saaristo et al., 2013; Bertram et al., 2015, 2018b, 2019; Tomkins et al., 2016, 2017; 2018). Such disturbances can drastically alter fitness (reviewed in Saaristo et al., 2018), particularly when

reproductive behaviours are affected (reviewed in Candolin and Wong, 2019).

Considering that sex hormones mediate the expression of a wide range of behaviours under sexual selection (Rubinow and Schmidt, 1996; Cunningham et al., 2012), it is not surprising that exposure to endocrine disruptors such as 17 β -TB can influence these behaviours in wildlife (reviewed in Gore et al., 2018). For example, exposure to EDCs can impact female mating behaviour and investment into reproductive effort in diverse aquatic species, such as palmate newts (*Triturus helveticus*; Secondi et al., 2009), gulf pipefish (*Syngnathus scovelli*; Partridge et al., 2010), sand gobies (*Pomatoschistus minutus*; Saaristo et al., 2009a), mosquitofish (*Gambusia holbrooki*; Saaristo et al., 2013), and guppies (*Poecilia reticulata*; Tomkins et al., 2016, 2018). However, in many species, males can also allocate their reproductive effort strategically if females differ in reproductive quality, if chances of encountering mates are high and/or if investment in reproductive behaviour comes at the cost of future mating opportunities (Wong et al., 2004). For example, males of some species suffer costs through energetically expensive courtship displays (e.g. Hoefler, 2008) and sexual ornamentation (e.g. Fitzpatrick et al., 1995), and indiscriminate expenditure of ejaculate (e.g. Friesen et al., 2015). For such species, costs associated with male reproduction make strategic allocation of mating effort an essential part of maximising reproductive fitness (reviewed in Edward and Chapman, 2011). Interestingly, despite established effects of EDCs on female reproductive behaviour and strategic investment into mating effort (see above), comparatively little is known about potential impacts of exposure on these traits in males (but see Saaristo et al., 2009b; Bertram et al., 2015, 2018b). Further, of the handful of studies that have been conducted, the majority have focused on scenarios where males can make direct comparisons between reproductive options simultaneously. This is true even though, in nature, males of many species often encounter females sequentially (Real, 1990). This is an important distinction, as the type of mating encounter can have major implications for mating strategy (e.g. Head et al., 2015). Despite this, the potential of 17 β -TB—and EDCs more generally—to impact male mating behaviour and investment into mating effort when females are encountered sequentially remains unknown.

The guppy, a small viviparous freshwater fish species (Houde, 1997), represents an excellent model for investigating potential impacts of 17 β -TB on male reproductive behaviour and mating effort when females are encountered sequentially. This is, in part, because a wealth of knowledge exists on the mating system of guppies (e.g. Bischoff et al., 1985; Kodric-Brown, 1985), including in terms of disturbance by chemical pollution (e.g. Bertram et al., 2015, 2018b; Fursdon et al., 2019). Male guppies engage in elaborate sigmoid courtship displays to gain solicited copulations from females or, alternatively, perform coercive 'sneak' mating attempts, involving a male coercively approaching a female from behind and thrusting his modified anal fin (i.e. gonopodium) towards the female's genital pore (Houde, 1997). Although the guppy mating system is primarily driven by female choice, males can also be choosy, and have been shown to prefer larger females (Houde, 1997; Dosen and Montgomerie, 2004; Bertram et al., 2018b), which are generally more fecund (Herdman et al., 2004).

Therefore, guppies can be used to further our understanding male strategic allocation of mating effort, in addition to examining effects of endocrine disruptors (if any) on this process.

Accordingly, the aims of this experiment were threefold. Firstly, we tested whether male guppies demonstrate strategic investment in mating effort when females are encountered sequentially. Second, we investigated whether 17 β -TB at a field-detected level (average measured concentration: 2 ng/L) can disrupt patterns (if any) of male reproductive behaviour and strategic mating effort. Third, we examined whether exposure to 17 β -TB impacted a suite of male morphological characteristics, including weight, standard length, and condition index (weight relative to length).

2. Methods

2.1. Fish collection and housing

Guppies were sourced from a wild population at Alligator Creek, Queensland, Australia (19°26'17" S, 146°57'01" E), which is a pristine, rainforest-fed site situated within Bowling Green Bay National Park. Water samples taken from this site over consecutive years (2015–2018) have revealed no contamination from 17 β -TB (Envirolab Services, unpublished data; see water testing details below). Fish were transported in aerated tanks to Monash University, where they were acclimated to laboratory conditions for three months in mixed-sex glass aquaria (24 °C, 12:12 h light:dark regime). Fish were fed *ad libitum* once daily (Otohime Hirame larval diet; 580–910 μ m). Only sexually mature fish were used in experiments, as determined by the development of breeding colouration and a well-defined gonopodium in males, and a gravid spot and fan-like anal fin in females (Houde, 1997).

2.2. Exposure and water testing

We used a flow-through system to expose 120 male fish to 17 β -TB for 21 days. This system design and exposure period are consistent with previous studies investigating impacts of 17 β -TB on poeciliid behaviour (Saaristo et al., 2013; Bertram et al., 2015, 2018a, 2018b, 2019; Tomkins et al., 2016, 2017, 2018). Males were randomly distributed between six 54 L glass aquaria (length \times width \times height: 60 \times 30 \times 30 cm, water depth: 25 cm; three control tanks and three 17 β -TB-exposed tanks; 20 fish per tank). The flow-through system was fed with aged carbon-filtered tap water, with the exposed tanks then being dosed with 17 β -TB at a nominal concentration of 8 ng/L (see below for details). This exposure level is consistent with 17 β -TB concentrations detected in river water impacted by livestock effluent discharge (Durhan et al., 2006). Over the duration of the exposure period, aquaria within the flow-through system were housed under the same light, temperature, and feeding conditions as described above. Flow-rates were maintained at \sim 1.67 L/h using flow meters (BES, MPB Series 1200), ensuring that water in each tank was fully cycled once per day. Each aquarium contained 2 cm of gravel substrate, an airstone, and a glass heater (Aqua One, 55 W).

To create a stock solution, 17 β -TB (17 β -hydroxyestra-4,9,11-trien-3-one; CAS: 10161–33-8; Novachem, Germany) was firstly dissolved in ethanol (HPLC grade, \geq 99.99%) at 400 mg/L. This stock solution was then diluted to 400 μ g/L using deionised water, and again within the flow-through system to achieve the desired nominal exposure level of 8 ng/L (average measured concentration = 1.89 ng/L, SD = 0.69, n = 9). Each week, 100 mL water samples were drawn from exposed and control tanks and analysed by the commercial environmental testing company Envirolab Services, using gas chromatography–tandem mass spectrometry (7000C Triple Quadrupole GC–MS/MS, Agilent Technologies,

Delaware, USA). For a detailed description of this analysis, see Tomkins et al. (2018). No 17 β -TB was detected in the control tanks throughout the exposure period (i.e. < 1 ng/L, n = 9).

2.3. Behavioural trials

To investigate potential impacts of 17 β -TB exposure on strategic investment of mating effort and reproductive behaviour in male guppies, we carried out behavioural trials in two stages. In the first, a single male and a single stimulus (unexposed) female were placed in a 21 L tank (30 \times 30 \times 24 cm; water depth: 20 cm) in separate 500 mL containers. After a 5 min acclimation period, the fish were released and allowed to interact freely for 15 min. The stimulus female was then removed and replaced with a second (unexposed) stimulus female. After another 5 min acclimation period in a 500 mL container, the second female was released and allowed to interact freely for 15 min with the focal male. Stimulus females used in both stages of each trial were drawn randomly from one of eight 21 L holding tanks (30 \times 30 \times 24 cm; water depth: 20 cm), having been housed for 24 h under the same temperature, light, and feeding conditions as the experimental males. Females were unexposed to ensure 17 β -TB-induced changes in male behaviour (if any) were not confounded by effects of exposure on the stimulus fish (*sensu* Tomkins et al., 2017, 2018; Bertram et al., 2018b, 2018c, 2019). Males and females were allowed to interact freely during behavioural trials (as opposed to being separated by a divider) to ensure males could conduct their full suite of reproductive behaviours (i.e. courtship and sneaks) towards the stimulus females.

Due to a known male preference for larger (and, hence, generally more fecund) females (Dosen and Montgomerie, 2004), focal males were presented sequentially with 'small' and/or 'large' females. The total length of small females ranged from 16.59 to 22.95 mm (mean \pm SD = 19.52 \pm 1.86 mm, Table S1), while the total length of large females ranged from 24.13 to 29.94 mm (mean \pm SD = 27.13 \pm 1.77 mm, Table S1). Females were presented to males in four different combinations (first female/second female): small/small, large/large, small/large, and large/small. These treatments allowed us to disentangle whether males were showing an absolute preference for large females, or if their responsiveness to sequentially presented females varied depending on previous female experience (i.e. a male's experience with a previously encountered female; Pitcher et al., 2003; Wong et al., 2004; Wong and Svensson, 2009). These four treatments were repeated for both exposed males (small/small: n = 15; large/large: n = 15; small/large: n = 15; and large/small: n = 15) and unexposed males (small/small: n = 15; large/large: n = 15; small/large: n = 15; and large/small: n = 15). All male and female fish were tested once only.

Behavioural trials were video-recorded (Canon PowerShot S120) and the subsequent videos analysed using JWatcher V1.0 (Blumstein and Daniel, 2007). As a measure of male preference, we quantified the total number of courtship bouts and sneak mating attempts performed towards each female. Previous research on reproductive behaviour in guppies has shown that, when males and females are allowed to interact freely, both courtship and sneak mating behaviour are accurate indicators of male mating intent (Herdman et al., 2004). Courtship bouts describe a male orienting his body towards a female and performing sigmoid displays, while sneak attempts involve a male surreptitiously approaching a non-receptive female from behind and attempting to mate coercively (as described in Houde, 1997). Experimenters were blind to treatment while conducting behavioural trials and throughout video analysis.

2.4. Morphological analysis

Immediately after each behavioural trial, all fish were euthanised with anaesthetic clove oil (40 mg/L) before being weighed (± 0.0001 g) and measured for total length (± 0.01 mm). We then calculated condition index (i.e. a fish's relative mass) as the residuals of a least-squares regression line of each male's total length (mm) against its weight (g) (i.e. $\text{weight} = -0.0067 + 0.0038 \times \text{total length}$).

2.5. Statistical analysis

Data were analysed in R version 3.3.2 (R Development Core Team, 2016), with statistical significance being assigned at $\alpha = 0.05$. Because assuming homogeneity of slopes can lead to misleading results (Engqvist, 2005), we adopted a conservative approach and investigated interaction terms where they were significant at $\alpha = 0.1$. Residuals were checked for normality (Shapiro-Wilk test) and homogeneity of variance (Fligner-Killeen test), where appropriate.

We first examined potential effects of exposure treatment (i.e. unexposed or 17 β -TB exposed) and female body length (i.e. small or large) on the frequency of male courtship events and sneak mating attempts during the first female presentation. For courtship events, we used a generalised linear model (GLM) with a Gaussian error distribution. For sneak mating attempts, we used a negative binomial distribution. All models included exposure treatment, female size and their interaction term as fixed effects.

Next, we determined if males adjusted their reproductive behaviour in the second female presentation based on the size of the female they encountered in the first presentation, and whether this response was influenced by 17 β -TB exposure. To achieve this, we ran separate GLM models for number of courtship events and sneak mating attempts, with Gaussian and negative binomial error distributions, respectively. In these models, exposure treatment, female size in the first presentation (i.e. previous female experience), female size in the second presentation (i.e. present female experience), and their interaction terms, were treated as fixed effects. *F* tests were used to calculate the *p*-values of fixed effects and the interaction terms (Bolker et al., 2009).

Mann-Whitney *U* tests were used to evaluate whether exposure to 17 β -TB altered male weight, total length or condition index.

3. Results

3.1. Courtship displays during the first female presentation

In analysing the first female presentation, we found no interaction between exposure treatment and female size on the frequency of courtship events ($F_{1,116} = 0.35$, $p = 0.555$). A significant effect of female size was detected on male courtship, with males from both exposure treatments conducting more frequent courtship bouts towards large females than small females ($t = 3.72$, $p < 0.001$; Fig. 1a). However, no significant effect of exposure treatment was seen on male courtship behaviour ($t = 0.30$, $p = 0.764$; Fig. 1b).

3.2. Sneak mating attempts during the first female presentation

Similar to courtship displays, no significant interaction was observed between exposure treatment and female size on the frequency of male sneak mating attempts ($F_{1,116} = 0.04$, $p = 0.843$). Further, there was no significant effect of female size on the number of sneak mating attempts performed by males ($z = 1.48$, $p = 0.139$; Fig. 1c). We did detect an effect of exposure treatment, however, with males exposed to 17 β -TB conducting more frequent

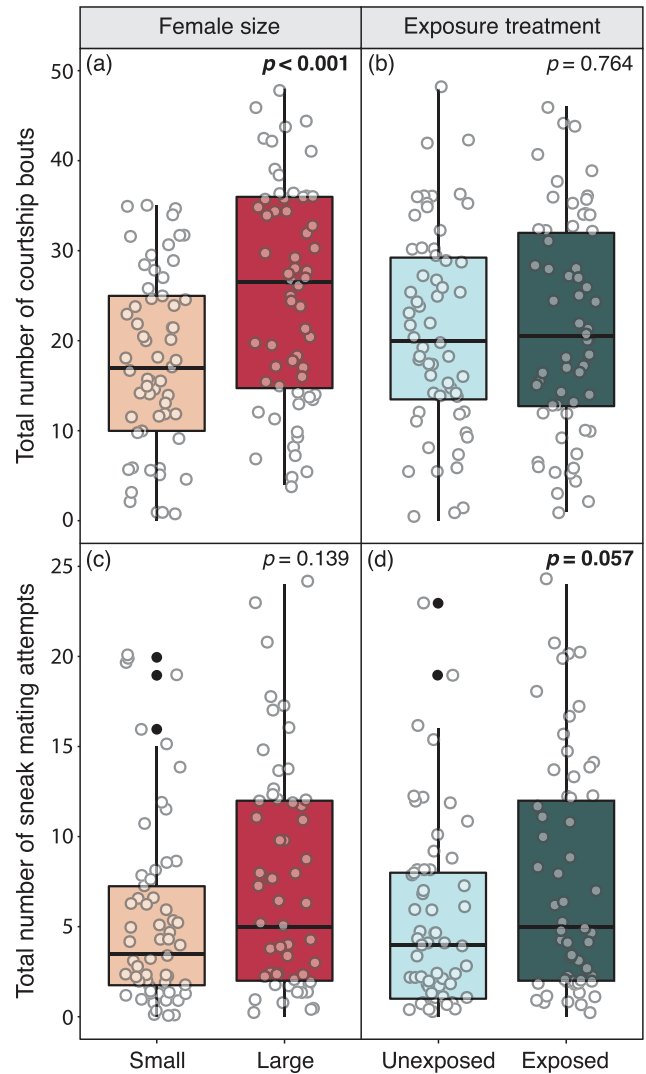


Fig. 1. Frequency of male reproductive behaviours performed during the first female presentation, including the number of courtship bouts conducted based on (a) female size class, and (b) exposure treatment, as well as the number of sneak mating attempts carried out based on (c) female size class, and (d) exposure treatment. Box plots show twenty-fifth, fiftieth (median) and seventy-fifth percentiles with horizontal lines. Outliers are represented by filled circles. Stimulus female size class: small $n = 60$, large $n = 60$. Male 17 β -TB exposure treatment: unexposed $n = 60$, exposed $n = 60$.

sneak mating attempts than unexposed males, although this difference was marginally non-significant ($z = 1.91$, $p = 0.057$; Fig. 1d).

3.3. Courtship displays during the second female presentation and across presentations

We found no evidence that a male's previous experience with a female (i.e. the size of the female encountered in the first presentation) influenced the frequency of courtship behaviour performed during the second female presentation ($F = 0.54$, $p = 0.465$; Table S2). However, consistent with the first presentation, there was a significant effect of female size on the courtship behaviour of males, with both exposed and unexposed males conducting more frequent courtship bouts towards large females compared to small females ($t = 3.71$, $p < 0.001$; Fig. 2a). There was, however, no significant effect of exposure treatment on the frequency of male courtship behavior ($t = 0.08$, $p = 0.935$; Fig. 2b).

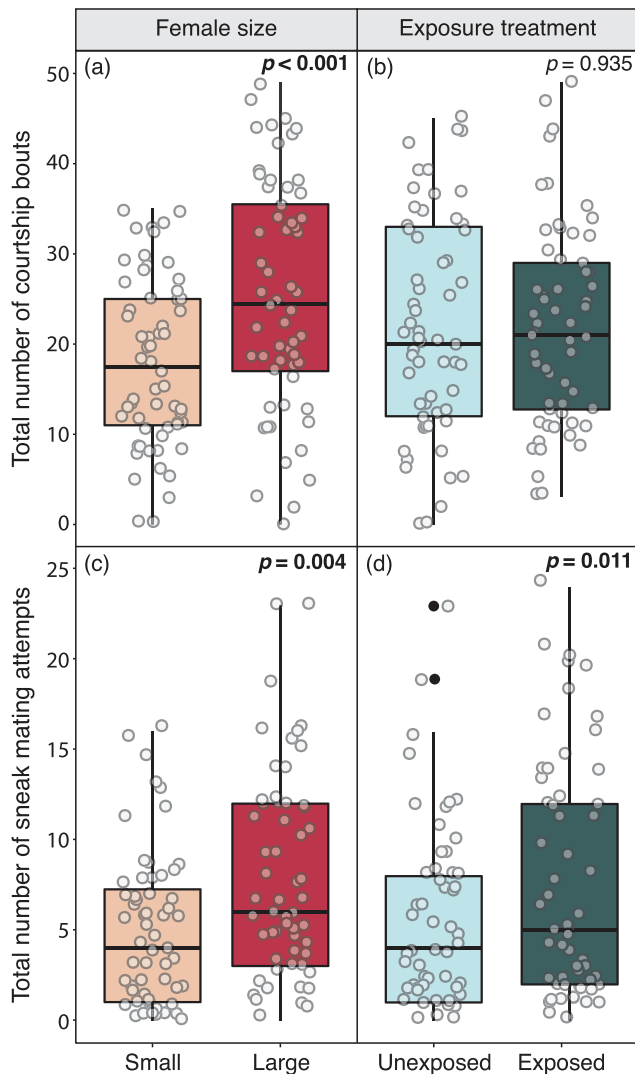


Fig. 2. Frequency of male reproductive behaviours conducted during the second female presentation, including the number of courtship bouts carried out based on (a) female size class, and (b) exposure treatment, and the number of sneak mating attempts performed based on (c) female size class, and (d) exposure treatment. Box plots show twenty-fifth, fiftieth (median) and seventy-fifth percentiles with horizontal lines. Outliers are represented by filled circles. Stimulus female size class: small $n = 60$, large $n = 60$. Male 17 β -TB exposure treatment: unexposed $n = 60$, exposed $n = 60$.

3.4. Sneak mating attempts during the second female presentation and across presentations

We detected no evidence that the size of the female encountered in the first presentation influenced the frequency of sneak mating attempts conducted by males during the second female presentation ($F < 0.01$, $p = 0.964$; Table S2). We did, however, observe a significant effect of both female size and exposure treatment on the frequency of male sneak mating attempts, with males directing more attempts towards larger females ($z = 2.86$, $p = 0.004$; Fig. 2c), and exposed males performed more frequent sneak mating attempts than unexposed males ($z = 2.55$, $p = 0.011$; Fig. 2d).

3.5. Morphology

We found no significant effect of 17 β -TB exposure on male weight (Mann-Whitney $U = 1486.5$, $p = 0.100$) or total length

(Mann-Whitney $U = 1823.5$, $p = 0.9039$). However, exposure to 17 β -TB was associated with a non-significant marginal increase in male condition index (Mann-Whitney $U = 1433$, $p = 0.054$).

4. Discussion

Here, we investigated whether short-term (21-day) exposure to a low environmentally realistic level (average measured concentration = 2 ng/L) of the veterinary steroid and widespread pharmaceutical pollutant 17 β -trenbolone (17 β -TB) affected male sequential mating effort in fish. We did not find evidence that previous female experience (i.e. the size the female encountered in the first presentation) impacted the amount of courting or sneaking behaviour performed by males during the second female presentation. However, regardless of exposure treatment, males carried out more frequent courting behaviour towards large females during both presentations, suggesting an absolute preference for greater female size. In addition, across both presentations, exposure to 17 β -TB resulted in a shift in male mating strategy towards increased sneaking behaviour. This is the lowest 17 β -TB concentration (i.e. 2 ng/L) shown to elicit this effect to date, although no effect of exposure was observed on male sequential investment into mating effort at this dosage. Lastly, exposure to 17 β -TB was associated with a non-significant marginal increase in male condition index (i.e. relative mass).

Both exposed and unexposed males showed a preference for greater female size during the first and second female presentation by courting larger females more frequently. Male preference for larger females has previously been demonstrated in guppies using simultaneous choice experiments (i.e. when males are able to make direct comparisons between potential suitors; [Dosen and Montgomerie, 2004](#); [Herdman et al., 2004](#)). Our study, however, demonstrates that male guppies can strategically allocate reproductive effort when females are encountered sequentially. In addition, we found no effect of previous female experience on the behaviour of exposed and unexposed males, which suggests that males were strategically investing their effort based on an absolute preference for larger females, as opposed to altering their behaviour based on previous female experience ([Wong et al., 2004](#)).

Exposure to 17 β -TB did not significantly affect the courtship behaviour of males in either the first or second female presentation. Although previous research has shown that exposure to androgenic EDCs can amplify the expression of male sexual behaviours (e.g. [Belanger et al., 2010](#); [Hoffmann and Kloas, 2012](#); [Marteinson et al., 2015](#)), this result is consistent with the majority of previous studies investigating impacts of 17 β -TB on courtship in male guppies. Specifically, [Bertram et al. \(2015\)](#) and [Bertram et al. \(2018b\)](#) both found no effect of 17 β -TB on male courting behaviour in a one-on-one scenario (i.e. a single male paired with a single female), and [Tomkins et al. \(2016\)](#) observed no difference in the courtship behaviour of exposed and unexposed males in a dichotomous choice experiment. However, recent research has revealed that 17 β -TB can influence the courtship behaviour of male guppies in a competitive setting, with [Tomkins et al. \(2017\)](#) reporting that exposed males courted females less than unexposed males when in the presence of a rival male. Interestingly, this decrease in courtship behaviour was also associated with an increase in aggression in exposed males, which may have limited the amount of time available for these males to court ([Tomkins et al., 2017](#)). This suggests that 17 β -TB-induced changes in male courtship behaviour may be dependent on social context, manifesting only under male-male competition. However, this does not appear to be the case for sneak mating behaviour.

Males exposed to 17 β -TB conducted more frequent coercive copulatory behaviour (i.e. sneak mating attempts) than unexposed

males during both the first and second female presentations—although this effect was marginally non-significant in the first presentation. This is consistent with the findings of Bertram et al. (2015, 2018b), who observed an increase in male sneak mating behaviour amongst 17 β -TB-exposed guppy males in a one-on-one scenario, and Tomkins et al. (2017), who found that exposed males performed more frequent sneak mating attempts towards females when in the presence of a rival male. However, the exposure concentration of 2 ng/L employed in the present study is the lowest dosage of 17 β -TB shown to induce this behavioural response to date (4 ng/L, Bertram et al., 2018b; 8 ng/L, Tomkins et al., 2017; 22 ng/L, Bertram et al., 2015). This increase in unsolicited male mating behaviour is not surprising given that 17 β -TB is a high-affinity ligand for the vertebrate androgen receptor (Wilson et al., 2002), of which two isoforms, AR α and AR β , have been characterised in teleost fish (Harbott et al., 2007). By binding to ARs, 17 β -TB mimics the effects of endogenous androgens such as testosterone and 11-ketotestosterone (Wilson et al., 2002; Larsen and Baatrup, 2010; Munakata and Kobayashi, 2010), inducing behavioural changes via the transcriptional modulation of downstream genes and related neural pathways (Hau, 2007). Indeed, endogenous androgens are involved in molecular processes mediating the development and manifestation of various behaviours in teleost fishes, including sexual behaviours (Hau, 2007). For example, a recent study involving genetic editing of the androgen receptor (AR) gene in zebrafish (*Danio rerio*) revealed that males lacking this gene performed significantly less mating behaviour towards females (Yong et al., 2017). Because such behaviours are modulated by endogenous androgens, they are potentially vulnerable to disruption by exposure to exogenous androgens (reviewed in Gore et al., 2018). How, then, might this behavioural change impact reproductive fitness in exposed populations?

An increase in coercive male mating behaviour is likely to influence both male and female fitness. Previous research has shown that successful sneaks transfer approximately one third as many sperm into the female's gonoduct compared to consensual matings (Pilastro and Bisazza, 1999), meaning that sneak copulations have a significantly lower probability of successful insemination compared to copulations preceded by courtship. Further, sexual selection processes that occur either during or after copulation (i.e. cryptic female choice) may also disadvantage sneaking males. For example, Pilastro et al. (2004) found that female guppies preferentially transfer sperm from males they perceive as more desirable (i.e. more colourful males). Although female cryptic preference for courting over sneaking males has not been specifically tested in guppies, cryptic preference for courting males has been documented in various other species (e.g. Edvardsson and Arnqvist, 2000; Pizzari and Birkhead, 2000), which, combined with evidence of cryptic female choice in guppies, suggests that female guppies may also preferentially transfer sperm from courting over sneaking males. An increase in sneaking behaviour is also likely to impact female fitness, as previous research has shown that coercive mating behaviour in guppies can physically damage the female genital pore (Constantz, 1989), increase the risk of disease transmission (Bisazza et al., 2001), and reduce female foraging efficiency (Pilastro et al., 2003).

Exposure to 17 β -TB was associated with a non-significant marginal increase in male condition index (i.e. relative mass), although no such change was observed in terms of either weight or length alone. Hence, this increase in body condition was the result of these males having, on average, slightly greater masses, as well as slightly smaller lengths. This effect is most likely driven by 17 β -TB-induced weight gain as skeletal morphogenesis is complete in adults (Pandey, 1969; Baatrup and Junge, 2001). This relative weight gain was anticipated given 17 β -TB's potent growth-promoting activity and is consistent with previous studies linking

21-day 17 β -TB exposures at a range of environmentally realistic concentrations with increased relative mass in male poeciliids (i.e. 2.6 and 3.3 ng/L, Lagesson et al., 2019; 4 ng/L, Bertram et al., 2018b; 16 ng/L, Bertram et al., 2019). Moreover, 21-day exposure of guppies to a higher concentration of 17 β -TB (i.e. 22 ng/L) resulted in increases in both male condition index and weight (Bertram et al., 2015), suggesting a stronger effect at higher dosage levels.

In conclusion, our findings demonstrate that exposure to an environmentally realistic level of a potent and widespread agricultural contaminant alters male mating strategy in a fish when females are encountered sequentially. The increased sneaking behaviour observed in 17 β -TB-exposed males is likely to impact both male and female fitness, which, combined with circumvention of female mate choice resulting from this coercive mating strategy, may have consequences at the population level. More broadly, our findings emphasise the potential for sub-lethal concentrations of pervasive chemical pollutants to alter complex behavioural processes and mechanisms of sexual selection in wildlife.

5. Ethics

This study was approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2013/09) and is compliant with all relevant State and Federal laws of Australia.

6. Authors' contributions

M.G.B., P.T., M.S. and B.B.M.W. conceived and designed the study. M.G.B., P.T. and R.B.T. collected the data, which was analysed by M.G.B., M.M. and J.M.M. The manuscript was written by M.G.B. and P.T. All authors contributed to revising the manuscript and gave their final approval for publication.

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Declaration of Competing Interest

The authors declare that we have no competing interests.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2019.134991>.

References

- Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, M.W., Gray, L.E., 2003. Effects of the androgenic growth promoter 17 β -trenbolone on fecundity and reproductive endocrinology of the fathead minnow. *Environ. Toxicol. Chem.* 22, 1350–1360.
- Ankley, G.T., Coady, K.K., Gross, M., Holbech, H., Levine, S.L., Maack, G., Williams, M., 2018. A critical review of the environmental occurrence and potential effects in aquatic vertebrates of the potent androgen receptor agonist 17 β -trenbolone. *Environ. Toxicol. Chem.* 37, 2064–2078.

- Baatrup, E., Junge, M., 2001. Antiandrogenic pesticides disrupt sexual characteristics in the adult male guppy (*Poecilia reticulata*). *Environ. Health Perspect.* 109, 1063–1070.
- Bartelt-Hunt, S.L., Snow, D.D., Kranz, W.L., Mader, T.L., Shapiro, C.A., Donk, S.J., et al., 2012. Effect of growth promotants on the occurrence of endogenous and synthetic steroid hormones on feedlot soils and in runoff from beef cattle feeding operations. *Environ. Sci. Technol.* 46, 1352–1360.
- Belanger, R.M., Pachkowski, M.D., Stacey, N.E., 2010. Methyltestosterone-induced changes in electro-olfactogram responses and courtship behaviors of cyprinids. *Chem. Senses.* 35, 65–74.
- Bertram, M.G., Saaristo, M., Baumgartner, J.B., Johnstone, C.P., Allinson, M., Allinson, G., Wong, B.B.M., 2015. Sex in troubled waters: widespread agricultural contaminant disrupts reproductive behaviour in fish. *Horm. Behav.* 70, 85–91.
- Bertram, M.G., Saaristo, M., Martin, J.M., Ecker, T.E., Michelangeli, M., Johnstone, C.P., Wong, B.B.M., 2018a. Field-realistic exposure to the androgenic endocrine disruptor 17 β -trenbolone alters ecologically important behaviours in female fish across multiple contexts. *Environ. Pollut.* 243, 900–911.
- Bertram, M.G., Saaristo, M., Ecker, T.E., Baumgartner, J.B., Wong, B.B.M., 2018b. An androgenic endocrine disruptor alters male mating behavior in the guppy (*Poecilia reticulata*). *Behav. Ecol.* 29, 1255–1263.
- Bertram, M.G., Ecker, T.E., Wong, B.B.M., O'Bryan, M.K., Baumgartner, J.B., Martin, J.M., Saaristo, M., 2018c. The antidepressant fluoxetine alters mechanisms of pre- and post-copulatory sexual selection in the eastern mosquitofish (*Gambusia holbrooki*). *Environ. Pollut.* 238, 238–247.
- Bertram, M.G., Martin, J.M., Saaristo, M., Ecker, T., Michelangeli, M., Deal, N.D.S., Wong, B.B.M., 2019. Context-specific behavioural changes induced by exposure to an androgenic endocrine disruptor. *Sci. Total Environ.* 664, 177–187.
- Bisazza, A., Vaccari, G., Pilastro, A., 2001. Female mate choice in a mating system dominated by male sexual coercion. *Behav. Ecol.* 12, 59–64.
- Bischoff, R.J., Gould, J.L., Rubenstein, D.L., 1985. Tail size and female choice in the guppy (*Poecilia reticulata*). *Behav. Ecol. Sociobiol.* 17, 253–255.
- Biswas, S., Kranz, W.L., Shapiro, C.A., Snow, D.D., Bartelt-Hunt, S.L., Mamo, M., et al., 2017. Effect of rainfall timing and tillage on the transport of steroid hormones in runoff from manure amended row crop fields. *J. Hazard Mater.* 324, 436–447.
- Blumstein, D.T., Daniel, J.C., 2007. Quantifying Behaviour the JWatcher Way. Sinauer Associates, USA.
- Bolker, B.M., Brooks, M.E., Clark, C.J., Geange, S.W., Poulsen, J.R., Stevens, M.H.H., White, J.S.S., 2009. Generalized linear mixed models: a practical guide for ecology and evolution. *Trends Ecol. Evol.* 24, 127–135.
- Candolin, U., Wong, B.B.M., 2019. Mate choice in a polluted world: consequences for individuals, populations and communities. *Phil. Trans. R. Soc. B* 374, 20180055.
- Conzant, G.D., 1989. Reproductive biology of poeciliid fishes. In: Meffe, G.K., Snelson, F.F. (Eds.), *Ecology and Evolution of Livebearing Fishes (Poeciliidae)*. Prentice Hall, Englewood Cliffs, NJ, pp. 33–50.
- Cunningham, R.L., Lumia, A.R., McGinnis, M.Y., 2012. Androgen receptors, sex behavior, and aggression. *Neuroendocrinology* 96, 131–140.
- Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Gore, A.C., 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr. Rev.* 30, 293–342.
- Dosen, L.D., Montgomerie, R., 2004. Female size influences mate preferences of male guppies. *Ethology* 110, 245–255.
- Durhan, E.J., Lambright, C.S., Makynen, E.A., Lazorchak, J., Hartig, P.C., Wilson, V.S., Ankley, G.T., 2006. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. *Environ. Health Perspect.* 114, 65–68.
- Edward, D.A., Chapman, T., 2011. The evolution and significance of male mate choice. *Trends Ecol. Evol.* 26, 647–665.
- Engqvist, L., 2005. The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Anim. Behav.* 70, 967–971.
- Edvardsson, M., Arnqvist, G., 2000. Copulatory courtship and cryptic female choice in red flour beetles *Tribolium castaneum*. *Proc. R. Soc. B* 267, 559–563.
- Fitzpatrick, S., Berglund, A., Rosenqvist, G., 1995. Ornaments or offspring: costs to reproductive success restrict sexual selection processes. *Biol. J. Linn. Soc.* 55, 251–1226.
- Friesen, C.R., Powers, D.R., Copenhaver-Perry, E., Mason, R.T., 2015. Size dependence in non-sperm ejaculate production is reflected in daily energy expenditure and resting metabolic rate. *J. Exp. Biol.* 218, 1410–1418.
- Fursdon, J.B., Martin, J.M., Bertram, M.G., Lehtonen, T.K., Wong, B.B.M., 2019. The pharmaceutical pollutant fluoxetine alters reproductive behaviour in a fish independent of predation risk. *Sci. Total Environ.* 650, 642–652.
- Godfray, H.C.J., Stephens, A.E.A., Jepson, P.D., Jobling, S., Johnson, A.C., Matthiessen, P., McLean, A.R., 2019. A restatement of the natural science evidence base on the effects of endocrine disrupting chemicals on wildlife. *Proc. R. Soc. B* 286, 20182416.
- Gore, A.C., Holley, A.M., Crews, D., 2018. Mate choice, sexual selection, and endocrine-disrupting chemicals. *Horm. Behav.* 101, 3–12.
- Harbott, L.K., Burmeister, S.S., White, R.B., Vagell, M., Fernald, R.D., 2007. Androgen receptors in a cichlid fish, *Astatotilapia burtoni*: structure, localization, and expression levels. *J. Comp. Neurol.* 504, 57–73.
- Hau, M., 2007. Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. *BioEssays* 29, 133–144.
- Head, M.L., Jacomb, F., Vega-Trejo, R., Jennions, M.D., 2015. Male mate choice and insemination success under simultaneous versus sequential choice conditions. *Anim. Behav.* 103, 99–105.
- Heintz, M.M., Brander, S.M., White, J.W., 2015. Endocrine disrupting compounds alter risk-taking behavior in guppies (*Poecilia reticulata*). *Ethology* 121, 480–491.
- Herdman, E.J.E., Kelly, C.D., Godin, J.G.J., 2004. Male mate choice in the guppy (*Poecilia reticulata*): Do males prefer larger females as mates? *Ethology* 110, 97–111.
- Hoefler, C.D., 2008. The costs of male courtship and potential benefits of male choice for large mates in *Phidippus clarus* (Araneae, Salticidae). *J. Arachnol.* 36, 210–212.
- Hoffmann, F., Kloas, W., 2012. Effects of environmentally relevant concentrations of the xeno-androgen, methyl-dihydrotestosterone, on male and female mating behavior in *Xenopus laevis*. *Chemosphere* 87, 1246–1253.
- Houde, A.E., 1997. Sex, Color, and Mate Choice in guppies. Princeton University Press, Princeton, NJ.
- Hunter, R.A., 2010. Hormonal growth promotant use in the Australian beef industry. *Anim. Prod. Sci.* 50, 637–659.
- Johnson, R., 2015. The U.S.-EU beef hormone dispute. Congressional Research Service.
- Kenyon, P.T., Zhao, H., Yang, X., Wu, C., Cwiertny, D.M., Kolodziej, E.P., 2019. Detection and quantification of metastable photoproducts of trenbolone and altrenogest using liquid chromatography–tandem mass spectrometry. *J. Chromatogr. A* 1603, 150–159.
- Khan, B., Lee, L.S., 2012. Estrogens and synthetic androgens in manure slurry from trenbolone acetate/estradiol implanted cattle and in wastereceiving lagoons used for irrigation. *Chemosphere* 89, 1443–1449.
- Kodric-Brown, A., 1985. Female preference and sexual selection for male coloration in the guppy (*Poecilia reticulata*). *Behav. Ecol. Sociobiol.* 17, 199–205.
- Kolodziej, E.P., Qu, S., Forsgren, K.L., Long, S.A., Gloer, J.B., Jones, G.D., Cwiertny, D.M., 2013. Identification and environmental implications of photo-transformation products of trenbolone acetate metabolites. *Environ. Sci. Technol.* 47, 5031–5041.
- Lagesson, A., Saaristo, M., Brodin, T., Fick, J., Klaminder, J., Martin, J.M., Wong, B.B.M., 2019. Fish on steroids: temperature-dependent effects of 17 β -trenbolone on predator escape, boldness, and exploratory behaviors. *Env. Poll.* 245, 243–252.
- Lange, I.G., Daxenberger, A., Schiffer, B., Witters, H., Ibarreta, D., Meyer, H.H.D., 2002. Sex hormones originating from different livestock production systems: fate and potential disrupting activity in the environment. *Anal. Chim. Acta* 473, 27–37.
- Larsen, M.G., Baatrup, E., 2010. Functional behavior and reproduction in androgenic sex reversed zebrafish (*Danio rerio*). *Environ. Toxicol. Chem.* 29, 1828–1833.
- Lean, I.J., Golder, H.M., Lees, N.M., McGilchrist, P., Santos, J.E.P., 2018. Effects of hormonal growth promotants on beef quality: a meta-analysis. *J. Animal Sci.* 96, 2675–2697.
- Lee, H.S., Jung, D.W., Han, S., Kang, H.S., Suh, J.H., Oh, H.S., Koo, Y.E., 2018. Veterinary drug, 17 β -trenbolone promotes the proliferation of human prostate cancer cell line through the Akt/AR signaling pathway. *Chemosphere* 198, 364–369.
- Marteinson, S.C., Letcher, R.J., Fernie, K.J., 2015. Exposure to the androgenic brominated flame retardant 1,2-dibromo-4-(1,2-dibromoethyl)-cyclohexane alters reproductive and aggressive behaviors in birds. *Environ. Toxicol. Chem.* 34, 2395–2402.
- Munakata, A., Kobayashi, M., 2010. Endocrine control of sexual behavior in teleost fish. *Gen. Comp. Endocrinol.* 165, 456–468.
- Pandey, S., 1969. Effects of methyl testosterone on testis and secondary sex characters of hypophysectomized adult guppy *Poecilia reticulata* Peters. *Can. J. Zool.* 47, 783–786.
- Parker, J.A., Webster, J.P., Kover, S.C., Kolodziej, E.P., 2012. Analysis of trenbolone acetate metabolites and melengestrol in environmental matrices using gas chromatography–tandem mass spectrometry. *Talanta* 99, 238–246.
- Partridge, C., Boettcher, A., Jones, A.G., 2010. Short-term exposure to a synthetic estrogen disrupts mating dynamics in a pipefish. *Horm. Behav.* 58, 800–807.
- Pilastro, A., Bisazza, A., 1999. Insemination efficiency of two alternative male mating tactics in the guppy *Poecilia reticulata*. *Proc. R. Soc. B* 266, 1887–1891.
- Pilastro, A., Benetton, S., Bisazza, A., 2003. Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. *Anim. Behav.* 65, 1161–1167.
- Pilastro, A., Simonato, M., Bisazza, A., Evans, J.P., 2004. Cryptic female preference for colourful males in guppies. *Evolution* 58, 665–669.
- Pitcher, T.E., Neff, B.D., Rodd, F.H., Rowe, L., 2003. Multiple mating and sequential mate choice in guppies: females trade up. *Proc. R. Soc. B* 270, 1623–1629.
- Pizzari, T., Birkhead, T.R., 2000. Female feral fowl eject sperm of subordinate males. *Nature* 405, 787–789.
- R Development Core Team, 2016. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
- Real, L., 1990. Search theory and mate choice. I. Models of single-sex discrimination. *Am. Nat.* 136, 376–404.
- Rubinow, D.R., Schmidt, P.J., 1996. Androgens, brain, and behavior. *Am. J. Psych.* 153, 974–984.
- Saaristo, M., Craft, J.A., Lehtonen, K.K., Björk, H., Lindström, K., 2009a. Disruption of sexual selection in sand gobies (*Pomatoschistus minutus*) by 17-ethinyl estradiol, an endocrine disruptor. *Horm. Behav.* 55, 530–537.
- Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K., 2009b. Sand goby (*Pomatoschistus minutus*) males exposed to an endocrine disrupting chemical fail in nest and mate competition. *Horm. Behav.* 56, 315–321.
- Saaristo, M., Tomkins, P., Allinson, M., Allinson, G., Wong, B.B.M., 2013. An androgenic agricultural contaminant impairs female reproductive behaviour in a freshwater fish. *PLoS One* 8, e62782.
- Saaristo, M., Brodin, T., Balshine, S., Bertram, M.G., Brooks, B.W., Ehlman, S.M., et al., 2018. Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife. *Proc. R. Soc. B* 285, 20181297.

- Schiffer, B., Daxenberger, A., Meyer, K., Meyer, H.H., 2001. The fate of trenbolone acetate and melengestrol acetate after application as growth promoters in cattle: environmental studies. *Environ. Health Perspect.* 109, 1145–1151.
- Secondi, J., Hinot, E., Djalout, Z., Sourice, S., Jadas-Hécart, A., 2009. Realistic nitrate concentration alters the expression of sexual traits and olfactory male attractiveness in newts. *Funct. Ecol.* 23, 800–808.
- Soto, A.M., Calabro, J.M., Precht, N.V., Yau, A.Y., Orlando, E.F., Daxenberger, A., Sonnenschein, C., 2004. Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in eastern Nebraska, USA. *Environ. Health Perspect.* 112, 346–352.
- Tomkins, P., Saaristo, M., Allinson, M., Wong, B.B.M., 2016. Exposure to an agricultural contaminant, 17 β -trenbolone, impacts female mate choice in a freshwater fish. *Aquat. Toxicol.* 170, 365–370.
- Tomkins, P., Saaristo, M., Bertram, M.G., Tomkins, R.B., Allinson, M., Wong, B.B.M., 2017. The agricultural contaminant 17 β -trenbolone disrupts male-male competition in the guppy (*Poecilia reticulata*). *Chemosphere* 187, 286–293.
- Tomkins, P., Saaristo, M., Bertram, M.G., Michelangeli, M., Tomkins, R.B., Wong, B.B.M., 2018. An endocrine-disrupting agricultural contaminant impacts sequential female mate choice in fish. *Environ. Pollut.* 237, 103–110.
- United States Department of Agriculture, 2000. A.a.P.H.I.S, Implant usage by U.S. feedlot, in: Info Sheet, Veterinary Services.
- United States Department of Agriculture, 2011. Cattle, N.A.S.S.
- United States Environmental Protection Agency, 2002. Economic Analysis of the Final Revisions to the National Pollutant Discharge Elimination System Regulation and the Effluent Guidelines for Concentrated Animal Feeding Operations. Washington, DC, Office of Water.
- Webster, J.P., Kover, S.C., Bryson, R.J., Harter, T., Mansell, D.S., Sedlak, D.L., Kolodziej, E.P., 2012. Occurrence of trenbolone acetate metabolites in simulated confined animal feeding operation (CAFO) runoff. *Environ. Sci. Technol.* 46, 3803–3810.
- Wilson, V.S., Lambright, C., Ostby, J., Gray Jr, L.E., 2002. *In vitro* and *in vivo* effects of 17 β -trenbolone: a feedlot effluent contaminant. *Toxicol. Sci.* 70, 202–211.
- Wong, B.B.M., Jennions, M.D., Keogh, J.S., 2004. Sequential male mate choice in a fish, the Pacific blue-eye *Pseudomugil signifier*. *Behav. Ecol. Sociobiol.* 56, 253–256.
- Wong, B.B.M., Svensson, P.A., 2009. Strategic male signaling effort in a desert-dwelling fish. *Behav. Ecol. Sociobiol.* 63, 543–549.
- Yong, L., Thet, Z., Zhu, Y., 2017. Genetic editing of the androgen receptor contributes to impaired male courtship behavior in zebrafish. *J. Exp. Biol.* 220, 3017–3021.