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An endocrine-disrupting agricultural contaminant impacts sequential female mate choice in fish☆

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ABSTRACT

The environmental impact of endocrine-disrupting chemicals (EDCs)—compounds that interfere with endocrine system function at minute concentrations—is now well established. In recent years, concern has been mounting over a group of endocrine disruptors known as hormonal growth promotants (HGPs), which are natural and synthetic chemicals used to promote growth in livestock by targeting the endocrine system. One of the most potent compounds to enter the environment as a result of HGP use is 17β-trenbolone, which has repeatedly been detected in aquatic habitats. Although recent research has revealed that 17β-trenbolone can interfere with mechanisms of sexual selection, its potential to impact sequential female mate choice remains unknown, as is true for all EDCs. To address this, we exposed female guppies (*Poecilia reticulata*) to 17β-trenbolone at an environmentally relevant level (average measured concentration: 2 ng/L) for 21 days using a flow-through system. We then compared the response of unexposed and exposed females to sequentially presented stimulus (i.e., unexposed) males that varied in their relative body area of orange pigmentation, as female guppies have a known preference for orange colouration in males. We found that, regardless of male orange pigmentation, both unexposed and exposed females associated with males indiscriminately during their first male encounter. However, during the second male presentation, unexposed females significantly reduced the amount of time they spent associating with low-orange males if they had previously encountered a high-orange male. Conversely, 17β-trenbolone-exposed females associated with males indiscriminately (i.e., regardless of orange colouration) during both their first and second male encounter, and, overall, associated with males significantly less than did unexposed females during both presentations. This is the first study to demonstrate altered sequential female mate choice resulting from exposure to an endocrine disruptor, highlighting the need for a greater understanding of how EDCs may impact complex mechanisms of sexual selection.

1. Introduction

Chemical pollutants are accumulating in environments worldwide at an alarming pace and scale (Kolpin et al., 2002; WHO-UNEP, 2012; Arnold et al., 2014). Of great concern are endocrine-disrupting chemicals (EDCs)—compounds that can alter the endocrine function of organisms at minute concentrations (in the low ng/L range) by interfering with hormonal communication (Kavlock and Ankley, 1996; Lintelmann et al., 2003; Buchanan and Partecke, 2012; Brander, 2013). Endocrine-disrupting chemicals encompass a broad range of both artificial compounds, which include pharmaceuticals, metals, plastics and pesticides (Diamanti-Kandarakis et al., 2009), and natural hormones, such as xenoestrogens (Gore et al., 2015). They can infiltrate ecosystems during their production, use, and/or disposal (WHO-UNEP, 2012), with common sources including wastewater from industry and households, agricultural and suburban run-off, and solid waste (Diamanti-Kandarakis et al., 2009). Once in the environment, many EDCs have a tendency to bioaccumulate (Crews et al., 2007; Walker

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and Gore, 2011), and have therefore continually been detected at elevated concentrations in wildlife tissues, even in the most remote regions on Earth (e.g., polar bears in the Arctic, Letcher et al., 2010; amphipods in the Mariana Trench, Jamieson et al., 2017).

One group of EDCs with the potential to impact wildlife is hormonal growth promotants (HGPs), which are natural and synthetic chemicals used to promote growth in livestock (Hunter, 2010; Sellin Jeffries et al., 2011; Kolodziej et al., 2013; Johnson, 2015). HGPs are used globally, and their use is particularly widespread in several of the world's leading beef-producing nations. For example, in the USA, which is the world's leading beef producer, it is estimated that 20 million cattle (i.e., approximately two thirds of the total livestock in the country) currently receive HGP implants (Johnson, 2015). Although HGPs generally include mixtures of natural and synthetic hormones (Lange et al., 2001; Hunter, 2010), the most commonly administered androgen in HGP implants is trenbolone acetate (Hunter, 2010), which is a highly efficient and potent synthetic steroid (Neumann, 1976). Trenbolone acetate is hydrolysed within implanted cattle to produce the biologically active steroid hormone 17β-trenbolone, which enters the environment via run-off of urine and faeces. Once present in the aquatic environment, 17β-trenbolone has a tendency to accumulate as a result of its long half-life (~260 days measured in animal waste; Schiffer et al., 2001) and has been detected at concentrations ranging from 1 to 20 ng/L in waterways upstream and downstream of cattle farm outflow points (Durhan et al., 2006) to 162 ng/L in tile-drained agroecosystems (Gall et al., 2011).

A growing number of studies have demonstrated that exposure to 17β-trenbolone can have alarming impacts on wildlife, particularly in aquatic environments. Exposure has been linked with severe morphological and physiological abnormalities in fish, including abnormal gonadal development (Örn et al., 2006), reduced reproductive output (Ankley et al., 2003), irreversible masculinisation (Baumann et al., 2014), and even complete and functional sex reversal (Larsen and Baatrup, 2010; Morthorst et al., 2010). We now know that 17β-trenbolone can also induce behavioural abnormalities, with recent research revealing that environmentally realistic exposure concentrations can affect risk-taking behaviour in guppies (*Poecilia reticulata*; Heintz et al., 2015), as well as reproductive behaviour and sexual selection processes in both guppies (Bertram et al., 2015; Tomkins et al., 2016, 2017) and eastern mosquitofish (*Gambusia holbrooki*; Saaristo et al., 2013). However, the potential impacts of 17β-trenbolone on more complex mechanisms of sexual selection remain poorly understood, as is also true for EDCs generally.

Sexual selection, by directly influencing mating outcomes, has important consequences for reproductive success, population dynamics and broader evolutionary processes (Candolin and Wong, 2012). Because sex hormones regulate the expression of a range of behaviours under sexual selection (Beyer et al., 1976; Munakata and Kobayashi, 2010), exposure to endocrine disruptors is likely to influence sexual selection processes. Indeed, recent research has revealed that, in simultaneous mate choice experiments (i.e., when females are presented with two or more males at the same time), exposure to environmentally relevant concentrations of endocrine-disrupting chemicals can impair female mate choice in sand gobies (*Pomatoschistus minutus*; Saaristo et al., 2009) and guppies (Tomkins et al., 2016). However, in nature, opportunities for females to make direct comparisons between suitors are often limited (Jennions and Petrie, 1997). In many species, it is more common for females to encounter mates sequentially (Bradbury and Andersson, 1987), making investigating the effects of EDCs on sequential female mate choice more ecologically relevant.

Guppies are a small, freshwater fish that occur in contaminated environments around the world (e.g., López-Rojas and Bonilla-Rivero, 2000; Widianarko et al., 2000). They are an ideal species for investigating the impacts of endocrine disruptors on the mechanisms of sexual selection as their mating system is driven primarily by female choice. Males compete for the attention of females, achieving copulations via two contrasting mating strategies. Briefly, males either mate consensually with females following successful courtship displays (termed 'sigmoid displays'), or gain copulations by sneaking up behind females and attempting to mate with them coercively (termed 'sneak' attempts) (Houde, 1997). Previous research investigating female mate choice in guppies has demonstrated that females show a strong preference for males with relatively large areas of orange pigmentation on their bodies (e.g., Houde, 1987; Kodric-Brown, 1989; Long and Houde, 1989; Endler, 1995; Grether, 2000; Kodric-Brown and Nicoletto, 2001). Orange colouration is an honest indicator of male quality in guppies, correlating positively with swimming performance (Nicoletto, 1993), foraging ability (Endler, 1980; Karino and Shinjo, 2007; Karino et al., 2007), sperm quality (Locatello et al., 2006; Pitcher et al., 2007) and sperm load size (Pitcher and Evans, 2001; Pitcher et al., 2007), as well as parasite resistance (Houde and Torio, 1992). However, these studies have relied almost exclusively on experimental set-ups in which females are able to make direct comparisons between males. This is true, despite the fact that, in the wild, female guppies will often have to make reproductive decisions based on sequential encounters with potential suitors (Houde, 1997; Pitcher et al., 2003). Guppies, therefore, provide an excellent opportunity to further our understanding of the impacts of EDCs on sexual selection by investigating the hitherto unknown impact of EDCs on sequential female mate choice.

Here, we test the hypothesis that short-term exposure to an environmentally realistic concentration of 17β-trenbolone will impact sequential female mate choice in guppies. Given that 17β-trenbolone has been shown to affect reproductive behaviour in guppies and other Poeciliids, we expected exposure to also disrupt female mate choice processes when males are encountered sequentially, which is often the more environmentally realistic scenario.

2. Methods

2.1. Fish collection and housing

Guppies were collected from Alligator Creek in Queensland, Australia (19° 26′ 17" S, 146° 57′ 01" E), where a wild population has established itself as a result of deliberate and/or accidental introductions from the pet trade. The sampling site is located inside the Bowling Green Bay National Park, and is thus thought to be a pristine location. Indeed, we have taken water samples from this site over consecutive years and found no presence of 17β-trenbolone (ALS Group, unpublished data). Fish were actively collected using dip nets and brought back to Monash University in aerated containers, where they were acclimated to laboratory conditions (25–27 °C, 12:12 h light:dark regime) in sex-specific tanks for three months prior to exposure to ensure sexual receptivity during behavioural trials. Fish were fed *ad libitum* once daily with a commercial fish pellet (Otohime Hirame larval diet, 580–910 μm).

2.2. Chemical exposure and water testing

Female guppies were exposed to 17β-trenbolone for 21 days via a flow-through system adapted from previous studies (Saaristo et al., 2013; Bertram et al., 2015; Tomkins et al., 2016, 2017). The system was comprised of six 54 L aquaria, consisting of three unexposed tanks and three 17β-trenbolone-exposed tanks. A total of 120 females were randomly distributed between these six tanks

(i.e., 20 fish per tank). Fish in the exposed aquaria received an average measured concentration of 2 ng/L of 17β-trenbolone (see below for details), which is consistent with concentrations detected in freshwater systems affected by agricultural activity (Durhan et al., 2006), while the unexposed tanks received fresh water only. Throughout the flow-through exposure, all fish were maintained under the same housing conditions as those described above.

The stock solution was created by dissolving 17β-trenbolone (17β-hydroxyestra-4,9,11-trien-3-one, CAS: 10161-33-8; Novachem, Germany) in ethanol (HPLC grade, ≥99.99%) to create a stock standard of 400 mg/L. This stock solution was diluted to 400 μg/L using deionised water, before being further diluted in the flow-through system to achieve a 17β-trenbolone exposure concentration of 2 ng/L (average measured concentration = 1.67 ng/L, SD = 0.56, $n = 9$). Water samples (200 mL) were collected from each of the 17β-trenbolone-exposed and unexposed tanks weekly and analysed using gas chromatography–tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware, USA). Analysis was conducted by Envirolab Services (MPL Laboratories, Perth; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025). No contamination with 17β-trenbolone was detected in the unexposed tanks throughout the exposure period (limit of quantification: 1 ng/L, $n = 9$). For a detailed description of the collection and analysis of water samples, see 'Supplementary Methods' in Supplementary material.

2.3. Behavioural trials

To investigate the impact of exposure to 17β-trenbolone on sequential female mate choice in guppies, a 27 L trial tank (30 × 30 × 30 cm) was separated into two compartments using a transparent perforated divider to allow full visual and chemical communication. A single experimental (i.e., unexposed or 17β-trenbolone-exposed) female was placed into one compartment (20 × 30 × 30 cm) in a 500 mL holding container and a single stimulus (i.e., unexposed) male placed into the other compartment (10 × 30 × 30 cm) in an identical holding container. Stimulus males were not subjected to the flow-through exposure, instead being drawn randomly from one of eight 27 L same-sex holding tanks (30 × 30 × 30 cm), having been housed under the same temperature, light and feeding conditions as females from the flow-through exposure. Stimulus males were unexposed to ensure 17β-trenbolone-induced changes in male behaviour did not influence the behaviour of females (Saaristo et al., 2013). After a 5 min acclimation period, both fish were released from their holding containers into their respective compartments and allowed to interact for 15 min through the divider. The first stimulus male was then removed and replaced with a second stimulus male, which was again subject to a 5 min acclimation period in a holding container before being released and allowed to interact with the female through the divider for a further 15 min.

Our experimental design required two categories of stimulus males, those with a high percentage body area of orange pigmentation (i.e., 'high-orange' males) and those with a low percentage body area of orange pigmentation (i.e., 'low-orange' males). This is because a strong female preference for males with relatively large areas of orange pigmentation on their bodies has been documented in many guppy populations (e.g., Kodric-Brown, 1985; Houde, 1987; Long and Houde, 1989; Endler, 1995; Kodric-Brown and Nicoletto, 2001), including in guppies from the Alligator Creek population used in our study (e.g., Gamble et al., 2003; Bertram et al., 2015). Male percentage body area of orange pigmentation was judged visually at the beginning of the exposure period and males were separated accordingly. Immediately following behavioural trials, males were photographed and the subsequent images used to quantify their percentage body area of orange pigmentation using digital colouration analysis (see 'Morphological analysis' below). Low-orange males possessed a percentage body area of orange pigmentation ranging from 3.16 to 8.22% (mean = 5.21%, SD = 1.32%), while high-orange males ranged from 12.05 to 19.66% (mean = 15.31%, SD = 1.75%) (Table S1). These values are comparable to those reported in previous research investigating sequential female mate choice in guppies by Pitcher et al. (2003), both in terms of the mean percentage body area of orange pigmentation in each stimulus male group, as well as the degree of separation between the group means. Further, Karino and Shinjo (2004) demonstrated that female guppies show a preference for males bearing as little as 2.0% more orange colouration than relatively dull males, indicating that the minimum difference of 3.83% orange pigmentation in our study between low- and high-orange groups is a sufficient gap for females to exercise choice.

Stimulus males were presented to females in four combinations (first male/second male): low-orange/low-orange, high-orange/high-orange, low-orange/high-orange, and high-orange/low-orange. These treatments allowed us to disentangle whether females were simply showing an absolute preference for males with increased orange pigmentation, or if their responsiveness to sequentially presented males varied depending on previous male experience. These four presentation combinations were repeated for both unexposed females (low-orange/low-orange: $n = 16$; high-orange/high-orange: $n = 16$; low-orange/high-orange: $n = 15$; high-orange/low-orange: $n = 16$) and exposed females (low-orange/low-orange: $n = 16$; high-orange/high-orange: $n = 16$; low-orange/high-orange: $n = 15$; high-orange/low-orange: $n = 15$). All male and female fish were tested once only. Female preference for both the first and second male was determined by quantifying the amount of time spent within a 5 cm 'preference zone' abutting the male compartment. Association time is commonly used as a measure of female mating preference in guppies (e.g., Kodric-Brown, 1985, 1989; Karino and Shinjo, 2004; Pilastro et al., 2004; Tomkins et al., 2016) and has been shown to be an accurate indicator of female mate choice in Poeciliid fish (Walling et al., 2010). Female behaviour was quantified using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007).

2.4. Morphological analysis

Immediately following behavioural trials, all fish were weighed (±0.0001 g) and measured for total length (±0.01 mm). Stimulus males were also photographed immediately after behavioural trials on their right side in a standardised fashion (Nikon D90, shutter speed = 1/250, Nikon AF Micro-Nikkor 60 mm, f/2.8D) and the resultant images analysed using Photoshop (CS6 version 13.0 Extended) to determine the percentage of each male's body area containing orange pigmentation. See Bertram et al. (2015) for details.

2.5. Statistical analysis

Data were analysed in R version 3.3.2 (R Core Development Team, 2016). Tests of normality (Shapiro-Wilk test; Royston, 1995) and homogeneity of variance (Fligner-Killeen test; Conover et al., 1981) were performed, where appropriate. Association time was square-root transformed prior to analysis to normalise residual errors. Statistical significance was assigned at $\alpha = 0.05$.

Firstly, we examined whether female association time differed due to treatment (i.e., unexposed versus 17β-trenbolone-exposed) and/or male percentage body area of orange pigmentation during the first presentation using a generalised linear model (GLM).

Treatment, male percentage body area of orange pigmentation and the interaction term were treated as fixed effects. Secondly, a linear mixed-effects model (*lme* function, *nlme* package; Pinheiro et al., 2017) with a Gaussian error distribution was used to determine whether females altered their response to males based on previous male experience. Treatment, male percentage body area of orange pigmentation, presentation order and the interaction terms were entered as fixed effects, with male ID entered as a random effect. Likelihood ratio tests (G^2) were then used to calculate the *p*-values of interaction terms (Bolker et al., 2009). Lastly, another GLM was used to test whether female association time differed due to treatment and/or male percentage body area of orange pigmentation during the second male presentation. In this instance, treatment, presentation order and the interaction term were entered as fixed effects. Presentation order was entered as a fixed effect to account for previous male experience. Mann-Whitney *U* tests were used to evaluate whether exposure to 17β-trenbolone altered female weight or total length, and independent samples *t*-tests were used to compare the orange pigmentation of males.

3. Results

3.1. Female association time during first male presentation

We found no interaction between treatment and male orange pigmentation on female association time ($F_{3,116} = 0.20$, $p = 0.660$). Regardless of their own exposure status, we found no difference in the total time that females spent associating with low- and high-orange males ($F_{1,117} = 2.85$, $p = 0.094$). However, in general, unexposed females spent more time associating with males than exposed females, irrespective of male orange pigmentation ($F_{1,117} = 45.17$, $p < 0.001$; Fig. 1).

3.2. Sequential female choice

We found a significant three-way interaction between treatment, male orange pigmentation and presentation order ($G^2 = 9.94$, $p = 0.019$). To account for this complex interaction, we analysed each treatment group separately.

For unexposed females, we found an interaction between male orange pigmentation and presentation order on female association time ($G^2 = 31.39$, $p < 0.001$). Specifically, unexposed females that were initially offered a high-orange male reduced their association time when subsequently presented with a low-orange male ($t_{28} = 3.49$, $p < 0.001$; Fig. 2A). However, in all other presentation combinations, there were no significant differences in the total time that unexposed females spent associating with the first and second male (low/low: $t_{14} = 1.73$, $p = 0.10$; high/high: $t_{14} = 1.42$, $p = 0.178$; low/high: $t_{14} = -1.99$, $p = 0.066$).

Contrasting with unexposed females, for exposed females we found no interaction between male percentage body area of orange pigmentation and presentation order on female association time ($G^2 = 3.81$, $p = 0.283$; Fig. 2B).

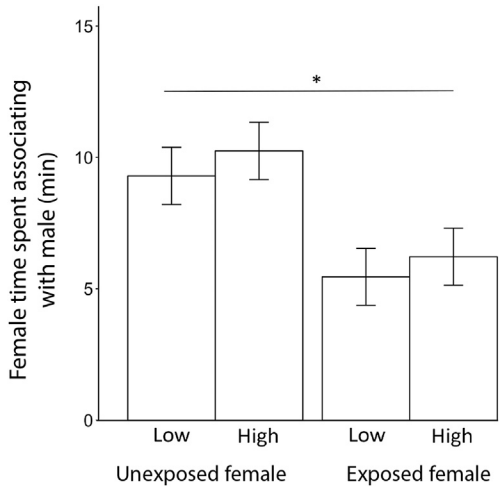

Fig. 1. Mean (±SE) time spent by unexposed and 17β-trenbolone-exposed females associating with low- and high-orange males during the first male presentation. The asterisk indicates a significant difference between groups ($p < 0.05$) obtained from ANOVA.

3.3. Female association time during second male presentation

Overall, unexposed females spent more time associating with males than did exposed females during the second male presentation ($F_{1,112} = 33.47$, $p < 0.001$). However, when unexposed females were presented with a low-orange male after having first observed a high-orange male, their association time reduced to a level that was comparable to that of exposed females in all presentation combinations ($F_{1,28} = 0.14$, $p = 0.712$; Fig. 3).

3.4. Morphology

There was no significant difference in the weight ($U = 870$, $p = 0.605$) or total length ($U = 587$, $p = 0.592$) of unexposed and exposed females.

4. Discussion

This is the first study to demonstrate that exposure to an endocrine-disrupting chemical (EDC) at an environmentally relevant concentration can influence female mate choice when males are encountered sequentially. We found that, during their first male encounter, both unexposed and 17β-trenbolone-exposed females associated with males indiscriminately, although exposed females spent significantly less time associating with males overall than did unexposed females. During their second male encounter, unexposed females that were presented with a low-orange male significantly reduced their association time if they had previously encountered a high-orange male. Conversely, exposed females associated indiscriminately with males during their second male encounter, and again associated with males significantly less overall than did unexposed females. These findings demonstrate the profound influence that a widespread androgenic EDC can have on sexual selection processes at environmentally realistic exposure concentrations.

Both unexposed and exposed female guppies showed no preference for greater orange colouration during their first male encounter. It is well established that female guppies prefer males with increased orange pigmentation (Endler, 1980; Houde, 1997), including in the population used in this research (Gamble et al., 2003; Bertram et al., 2015). However, the vast majority of studies that have investigated female mate choice in guppies have done so using simultaneous choice experiments, where the female is able to make direct comparisons between males. To our knowledge, only one study has investigated female mate choice in guppies when males are encountered sequentially, which, in accordance with our results, found that virginal female guppies showed no preference for greater orange colouration during their first male encounter (Pitcher et al., 2003). While virgin females were not used in this study, females were sexually isolated for three months prior to exposure, as well as throughout the 21-day exposure period, which likely explains why they associated with males indiscriminately

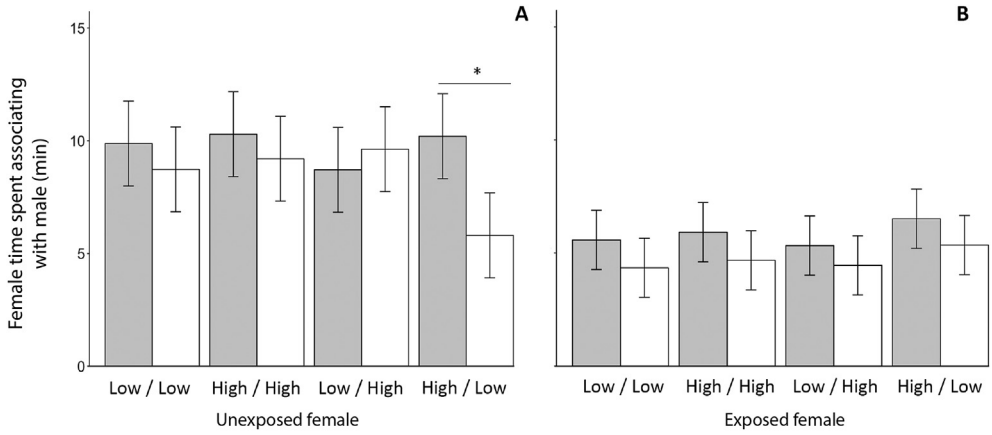

Fig. 2. Mean (±SE) time that (A) unexposed and (B) 17β-trenbolone-exposed females spent associating with males in each trial combination. Grey bars represent the first male presentation and white bars represent the second male presentation. The asterisk indicates a significant difference between groups ($p < 0.05$) obtained from Tukey's tests of simplified linear mixed-effects models.

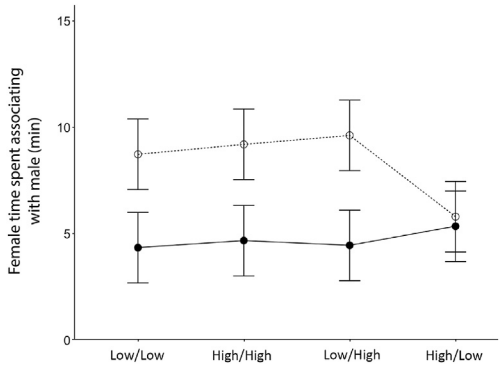

Fig. 3. Interaction plot showing the mean (±SE) time that unexposed females (open circles) and 17β-trenbolone exposed females (closed circles) from each presentation combination spent associating with males during the second male encounter. Plot displays the interaction between treatment and presentation order.

during their first male encounter. Further, although female guppies are able to store sperm for several months (Houde, 1997; Gasparini et al., 2012; López-Sepulcre et al., 2013), it is possible that the sperm storages of females used in this experiment were diminished during the extended isolation period preceding and during our exposure, which may have contributed to the lack of choosiness observed in females during their first male encounter.

Our results demonstrate that female preference can be influenced by previous male experience. Time spent by unexposed females associating with males during the first and second presentation did not differ in the low-orange/low-orange, low-orange/high-orange and high-orange/high-orange trial combinations. However, when unexposed females were presented with a low-orange male after having first observed a high-orange male, the amount of time they spent associating with the second male reduced significantly. This suggests that females were not simply showing an overall preference for increased orange colouration, but were adjusting their mate choice decisions based on previous experience with potential suitors (i.e., 'previous male effect' *sensu* Bakker and Milinski, 1991). Considering that females increase their reproductive success by maximising the quality of their mating partners (Bateman, 1948), this strategy reduces the likelihood of females mating with low-quality males in a population containing males that differ in quality (Bakker and Milinski, 1991; Milinski, 2001), and has previously been demonstrated in zebra finches (*Taeniopygia guttata*; Collins, 1995), smooth newts (*Lissotriton vulgaris*; Gabor and Halliday, 1997), crickets (*Gryllus bimaculatus*; Bateman et al., 2001) and guppies (Pitcher et al., 2003).

In contrast to unexposed females, we found no evidence of a previous male effect in females exposed to 17β-trenbolone. Moreover, exposed females showed no preference for increased orange colouration in either their first or second male presentation, indicating a breakdown of sexual selection processes. Further, exposed females spent significantly less time associating with males overall than did unexposed females during both their first and second male encounter, indicating that not only were exposed females less choosy, they were also generally less interested in mating. This finding is in agreement with research by Saaristo et al. (2013), where female mosquitofish exposed to 17β-trenbolone at 6 ng/L for 21 days approached males less, and spent more time swimming away from males, than did unexposed females. This result is also consistent with work by Tomkins et al. (2016), where 21-day exposure at 4 ng/L resulted in guppy females being less choosy and performing less association behaviour when presented with two males simultaneously. Interestingly, when unexposed females in the present study exhibited reduced interested in a male (i.e., during the second male presentation in the high-orange/low-orange combination), their association time reduced to a level that was comparable to—i.e., not significantly different from—the time spent by exposed females associating with males in all presentation combinations. This is important as it demonstrates that, regardless of male quality, females exposed to 17β-trenbolone behave similarly to unexposed females that are relatively disinterested in mating. To understand why 17β-trenbolone impacts choosiness in females, its mode of action must be considered.

The agricultural contaminant 17β-trenbolone is a potent, non-aromatisable androgen receptor agonist (Rogozkin, 1991; Hotchkiss et al., 2008). It binds with high affinity to available androgen receptors, mimicking the effects of androgens such as testosterone and 11-ketotestosterone (Wilson et al., 2002). It is also hypothesised that 17β-trenbolone indirectly inhibits the production of 17β-estradiol by limiting the production of testosterone and, thus, restricting the aromatisation of testosterone to 17β-estradiol (Zhang et al., 2008). As a result, 17β-trenbolone can suppress estrogenic activity in female fish. Ankley et al. (2003) observed reduced plasma concentrations of vitellogenin and 17β-estradiol in 17β-trenbolone-exposed female fathead minnows (*Pimephales promelas*), which was linked with the development of male morphological characteristics. Exposure to 17β-trenbolone has also

been found to cause varying levels of masculinisation in female mosquitofish (Sone et al., 2005; Brockmeier et al., 2012), zebrafish (*Danio rerio*; Morthorst et al., 2010; Baumann et al., 2014) and Japanese medaka (*Oryzias latipes*, Seki et al., 2006). It is likely that, despite our low exposure concentration and relatively short exposure period, 17β-trenbolone-exposed females in our experiment experienced some degree of masculinisation, which may have reduced their desire to mate and, in turn, made them less choosy. Further research in this area is needed to gain a better understanding of the underlying mode of action of 17β-trenbolone.

We found no effect of 17β-trenbolone exposure on female weight or length, despite the anabolic potency of 17β-trenbolone (Neumann, 1976). This result is consistent with previous research examining the morphological impacts of 17β-trenbolone-exposure at environmentally realistic concentrations. Specifically, 17β-trenbolone had no impact on the weight or length of female guppies at 4 ng/L (Tomkins et al., 2017), 8 ng/L (Tomkins et al., 2016) or 22 ng/L (Bertram et al., 2015), and had no influence on the morphology of female fathead minnows at 5 ng/L or 50 ng/L (Ankley et al., 2003). However, at 22 ng/L, 17β-trenbolone resulted in an increase in the weight and condition index of male guppies (Bertram et al., 2015), while at 4 ng/L, exposure resulted in an increase in male condition index, but not weight (M.G. Bertram et al., unpublished data). This suggests that male morphology is more sensitive to 17β-trenbolone-exposure than female morphology. However, more research is required to disentangle these dose-dependent and sex-specific effects.

In conclusion, this is the first study to show altered sequential female mate choice resulting from exposure to an endocrine disruptor. We found that, during a second male encounter, unexposed females altered the amount of time they spent associating with males depending on the orange colouration of a previously encountered male. Exposed females, on the other hand, associated with males indiscriminately during both the first and second male presentations. Further, exposed females spent less time associating with males overall than did unexposed females, indicating a decrease in mating interest. Considering that orange colouration is an honest indicator of male quality in guppies (Endler, 1980; Houde and Torio, 1992; Nicoletto, 1993; Pitcher and Evans, 2001; Locatello et al., 2006; Karino and Shinjo, 2007; Karino et al., 2007; Pitcher et al., 2007), the 17β-trenbolone-induced behavioural shifts observed in this study are expected to result in exposed females mating with inferior suitors. In nature, it is often more common for female guppies to encounter males sequentially, meaning the indirect costs associated with this breakdown in sexual selection processes could have population-level impacts by influencing the quality and quantity of offspring produced (reviewed in Candolin and Heuschele, 2008; Candolin and Wong, 2012; Wong and Candolin, 2015). Thus, this study highlights the need for a greater understanding of the potential impacts of EDCs on complex sexual selection processes, and how these changes may, in turn, influence population dynamics, ecosystem function, and broader evolutionary processes.

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.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.02.046.

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