The endocrine disruptor 17β-trenbolone alters the relationship between pre- and post-copulatory sexual traits in male mosquitofish (*Gambusia holbrooki*)

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HIGHLIGHTS

- An endocrine-disrupting chemical affects mechanisms of sexual selection.
- An anabolic steroid disrupts relationships between pre- and post-copulatory traits.
- Sperm motility was increased in fish exposed to an anabolic steroid.
- Reproductive behaviour was affected by pollutant exposure.

GRAPHICAL ABSTRACT
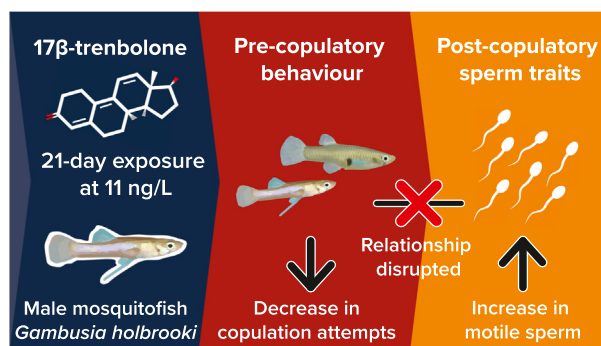
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ABSTRACT

It is now well-established that reproduction in wildlife can be disrupted by anthropogenic environmental changes, such as chemical pollution. However, very little is known about how these pollutants might affect the interplay between pre- and post-copulatory mechanisms of sexual selection. Here, we investigated the impacts of 21-day exposure of male eastern mosquitofish (*Gambusia holbrooki*) to a field-realistic level (average measured concentration: 11 ng/L) of the endocrine-disrupting chemical 17β-trenbolone (17β-TB) on pre- and post-copulatory reproductive traits. We examined male reproductive behaviour by testing the time spent near a female behind a partition, as well as the number of copulation attempts made, and the time spent chasing a female in a free-swimming context. Sperm traits were also assayed for all males. We found that exposure of male fish to 17β-TB altered the relationship between key pre- and post-copulatory reproductive traits. Furthermore, 17β-TB-exposed males had, on average, a higher percentage of motile sperm, and performed fewer copulation attempts than unexposed males. However, there was no overall effect of 17β-TB exposure on either the time males spent associating with or chasing females. Taken together, our findings demonstrate the potential for chemical pollutants to affect both pre- and post-copulatory sexual traits, and the interplay between these mechanisms of sexual selection in contaminated wildlife.

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1. Introduction

In many species, reproductive outcomes are largely determined by competition among males for access to reproductive opportunities with females (Darwin, 1871). This competition can act as a powerful selective force shaping the evolution of male traits, including behaviours that maximise mating and fertilisation success (Andersson, 1994). Males, for instance, may be selected for pre-copulatory behaviours that enhance detection of potential mates, or engage in elaborate courtship displays to entice choosy females (Wong and Candolin, 2005). Males can also adopt behavioural strategies that bypass female mate choice entirely through, for example, coercive matings (Taru et al., 2002).

When females are capable of mating with multiple males (Jennions and Petrie, 2000), sexual selection can also act on male reproductive traits post-copulation (Birkhead and Pizzari, 2002). For instance, in situations where the sperm of several males compete to fertilise a female's eggs, this can give rise to sperm competition (Parker, 1970). Here, both the amount and quality of sperm produced can strongly influence male fertilisation success (Parker, 1970; Thornhill, 1983). Indeed, evidence suggests that males are capable of strategically allocating more sperm in the presence of sperm competition (Birkhead and Pizzari, 2002). Sperm competition can also favour, for example, the production of more viable sperm (Birkhead and Pizzari, 2002), and faster-swimming sperm (Malo et al., 2006).

It is important to note that a male's overall fitness is dependent on the competitiveness of both his pre- and post-copulatory sexual traits, with an investment trade-off often seen between these two mechanisms of sexual selection (Andersson and Simmons, 2006; Lüpold et al., 2014). Therefore, due to potential allocation trade-offs, males that are more successful in pre-copulatory sexual selection may incur higher costs of sperm production, thus limiting sperm quality and number and, in doing so, reducing competitive fertilisation success (Andersson and Simmons, 2006; Evans, 2010). In this regard, even though it is now well-established that reproductive performance can be disrupted by anthropogenic changes to the environment (Aulsebrook et al., 2020; Candolin and Wong, 2012; Candolin and Wong, 2019), we still know very little about how such changes might affect the interplay between pre- and post-copulatory mechanisms of sexual selection, despite its role in mediating reproductive outcomes.

One anthropogenic change that is threatening wildlife and the ecosystems they inhabit is the accumulation of chemical pollutants in the environment (Schwarzenbach et al., 2006). While studies of chemical pollution have conventionally focused on acutely toxic contaminants such as heavy metals and pesticides (Travis and Hester, 1991), a rapidly growing body of research is drawing attention to the impacts of emerging contaminants, such as pharmaceuticals. These pollutants have garnered attention as they have the potential to exert sub-lethal effects on development, physiology, and behaviour (Aulsebrook et al., 2020; Brodin et al., 2014; Saaristo et al., 2018). A chemical pollutant class of particular concern is endocrine-disrupting chemicals (EDCs), due to their extreme potency at low exposure levels (Gore et al., 2015). These compounds have the capacity to disrupt the natural hormonal functioning and gene–environment interactions of exposed wildlife at extremely low concentrations (i.e. pg/L or ng/L; Colborn et al., 1993; Gore et al., 2015), via physiological, cellular, molecular, and epigenetic changes (Gore et al., 2015). Although conventional studies exploring the effects of chemical pollutants on wildlife have largely focused on lethality, development, and reproduction (Melvin and Wilson, 2013), pollutants such as EDCs can also significantly alter ecologically important behaviours (Arambula and Patisaul, 2018). This is concerning given that behavioural disruptions can adversely affect individual fitness and survival (Sih et al., 2004), and may also have ecological and evolutionary consequences (Wong and Candolin, 2015). As such, there has been increasing interest in behavioural ecotoxicology as a tool for assessing the adverse impacts of chemical pollutants on wildlife (Melvin and Wilson, 2013; Saaristo et al., 2018).

One EDC of particular concern is 17β-trenbolone (17β-TB), a synthetic androgen with a potency 15 to 50 times greater than testosterone (Neumann, 1976). 17β-TB is the most bioactive metabolite of trenbolone acetate, an anabolic steroid that is commonly administered to livestock as a hormonal growth promotant in beef-producing countries, such as the United States and Australia (Johnson and Hanrahan, 2010; Stephany, 2010). Due to the widespread use of trenbolone acetate, 17β-TB has repeatedly been detected in the environment, especially in aquatic habitats impacted by effluent run-off from agricultural activity (reviewed in Ankley et al., 2018). Environmental concentrations of 17β-TB typically range from 0.0015 to 270 ng/L in feedlot run-off and lagoon water (Bartelt-Hunt et al., 2012; Challis et al., 2021; Durhan et al., 2006; Khan and Lee, 2012; Parker et al., 2012; Schiffer et al., 2001; Soto et al., 2004; Webster et al., 2012), and 0.0013 to 20 ng/L in lake and river water (Durhan et al., 2006; Soto et al., 2004; Zhou et al., 2016). Upon exposure, 17β-TB has been shown to rapidly bioaccumulate in fish (Ankley et al., 2003; Lagesson et al., 2019; Schultz et al., 2013). Notable effects of 17β-TB exposure on animals observed in past studies include alteration of sex ratios towards a greater proportion of males, increases in growth, impacts on fertility and fecundity, and changes in gonadal histopathology (reviewed in Ankley et al., 2018). Moreover, recent studies have demonstrated that 17β-TB can also disrupt key behaviours such as activity and exploration (Bertram et al., 2018c; Lagesson et al., 2019), foraging (Bertram et al., 2018c), sociality (Bertram et al., 2018c), boldness (Heintz et al., 2015; Lagesson et al., 2019), risk-taking (Heintz et al., 2015; Lagesson et al., 2019), and sexual behaviours (Bertram et al., 2019; Bertram et al., 2015; Bertram et al., 2018b; Bertram et al., 2020; Saaristo et al., 2013; Tomkins et al., 2016; Tomkins et al., 2018; Tomkins et al., 2017). Because 17β-TB is expected to induce effects primarily by activating the androgen receptor (Ankley et al., 2018), it is likely that reproductive traits are one of the most sensitive biological endpoints affected by exposure. Furthermore, as 17β-TB has been shown to affect both behaviour and physiology, there is a potential for both pre- and post-copulatory sexual mechanisms to be affected by exposure to 17β-TB.

Worryingly, there is mounting evidence that EDCs, including 17β-TB, have the potential to disrupt pre-copulatory reproductive behaviours across a wide range of species, including impairment of male courtship, and changes in male and female mating behaviour (reviewed in Aulsebrook et al., 2020; Gore et al., 2018). Similarly, studies have shown that EDCs can affect post-copulatory traits, from reduction in sperm quality in brown trout (*Salmo trutta* f. *fario*; Lahnsteiner et al., 2005) to alterations in a multitude of functionally essential sperm parameters in mammals (reviewed in Tavares et al., 2016). Interestingly, histopathological studies have found that exposure to 17β-TB increased testes size and spermatozoa quantity in male zebrafish (*Danio rerio*; Baumann et al., 2014; Örn et al., 2006), and resulted in a greater proportion of spermatozoa in exposed male medaka (*Oryzias latipes*) compared to controls (Park et al., 2009). Although the balance of this relationship between investment in pre- and post-copulatory sexual traits is important for overall reproductive function and the fitness of males under male-male competition (Lüpold et al., 2014), it is rare to find studies that have used a multi-pronged approach to investigate the effects of EDC exposure on both pre- and post-copulatory sexual traits contemporaneously. As such, the potential effects of 17β-TB on both male pre-copulatory behaviour and post-copulatory sperm performance warrant further investigation.

Accordingly, we tested the hypothesis that exposure to 17β-TB exerts effects on both pre-copulatory reproductive behaviours and post-copulatory sperm traits in wild-caught male eastern mosquitofish (*Gambusia holbrooki*). On the basis of previously reported effects of 17β-TB exposure on fish (Ankley et al., 2018), we hypothesised that 17β-TB would alter pre-copulatory behaviour in male eastern mosquitofish and increase sperm function and quality, leading to disruptions in the relationship between pre- and post-copulatory reproductive traits.

2. Materials and methods

2.1. Study species

The eastern mosquitofish is a small freshwater fish native to south-eastern North America, with an extensive global distribution as a result of human-mediated introductions (Pyke, 2008). Due to its widespread distribution and occurrence in habitats disturbed by human activity, the species has received growing interest as a model for investigating the effects of chemical pollutants on wildlife (e.g., Bertram et al., 2019; Bertram et al., 2018c; Lee et al., 2018; Martin et al., 2019b; Martin et al., 2020; Martin et al., 2019c). In this study, we focus on the behavioural and reproductive traits of male eastern mosquitofish, with female eastern mosquitofish acting only as stimulus fish. This is due to the species' mating system, which is dominated by male sexual coercion of females (Pilastro et al., 1997). Specifically, male eastern mosquitofish do not engage in courtship displays but instead chase and harass females incessantly for mating opportunities (at up to one mating attempt per minute; Wilson, 2005). As the species is internally fertilised, the male achieves copulations by approaching the female from behind and then darting, at speed, to insert his gonopodium—a modified anal fin that serves as an intromittent organ—into the female's genital pore (Bisazza et al., 2001).

2.2. Animal collection and housing

Wild sexually mature male and female eastern mosquitofish used in this study were collected from the Science Centre Lake (37°54′28″ S, 145°08′16″ E), Monash University, Victoria, Australia. Water sampling at this site over consecutive years, including at the time of fish collection, has indicated no contamination with 17β-TB (Envirolab Services, unpublished data). Following collection, fish were transported back to Monash University and acclimated to laboratory conditions for 1 month prior to exposure and experimentation in mixed-sex glass holding tanks (81 L; 60 × 45 × 30 cm; 24–26 °C; 12:12 h light:dark regime; 50:50 sex ratio), with 30% water changes performed for all housing tanks once per week. During the housing, exposure, and experimentation periods, fish were fed *ad libitum* once daily with commercial pellets (Otohime Hirame larval diet 580–910 μm).

2.3. Exposure regime

Male eastern mosquitofish were exposed for 21 days to 17β-trenbolone (nominal concentration: 25 ng/L) via a flow-through system, following Saaristo et al. (2013) and Bertram et al. (2015). In total, 162 male fish were randomly assigned to one of four flow-through 17β-TB exposure aquaria (54 L; 60 × 30 × 30 cm) or one of four identical unexposed (i.e. control) aquaria (54 L; 60 × 30 × 30 cm) containing only freshwater, with approximately equal densities per aquaria. The exposure regime lasted for 21 days because past research has shown that 21-day exposure to 17β-TB at environmentally relevant levels was sufficient to elicit behavioural responses in fish (Bertram et al., 2018b; Bertram et al., 2020; Tomkins et al., 2018), including eastern mosquitofish (Bertram et al., 2018c; Saaristo et al., 2013). Each aquarium contained an air stone for aeration, a large rock for refuge, an aquarium heater, and a 2 cm-deep layer of gravel as substrate.

Throughout the exposure period, each replicate flow-through aquaria was measured once per day for temperature (unexposed tanks: 24.02 ± 0.23 °C, mean $\pm$ SD, $n = 84$; exposed tanks: 24.03 ± 0.24 °C, mean $\pm$ SD, $n = 84$), and flow-through rates (BES, MPB Series 1200; unexposed tanks: 18.53 ± 0.33 mL/min, mean $\pm$ SD, $n = 84$; exposed tanks: 18.54 ± 0.38 mL/min, mean $\pm$ SD, $n = 84$). Mean temperature and flow-through rates were consistent within and between exposed and unexposed treatment tank groups (temperature: two-way ANOVA, $F = 1.14$, $df = 7$, $p = 0.343$; flow-through rates: two-way ANOVA, $F = 0.21$, $df = 7$, $p = 0.983$).

2.4. Exposure monitoring and GC–MS/MS analysis

The 17β-TB exposure concentration (measured concentration: 10.93 ± 10.24 ng/L, mean $\pm$ SD, $n = 16$) was achieved using methods described in Bertram et al. (2018c). This involved first dissolving 17β-TB (17β-hydroxyestra-4,9,11-trien-3-one; CAS: 10161–33–8; Novachem, Germany) in ethanol to produce a stock solution (400 mg/L). This solution was diluted with deionised water (4 μg/L), and then diluted into the flow-through system which was fed with aged carbon-filtered tap water to achieve the final average exposure concentration of 11 ng/L.

Concentrations of 17β-TB were measured weekly in exposed tanks, as well as unexposed tanks to ensure the absence of contamination. Briefly, this involved collecting 200 mL water samples in amber glass bottles from each tank and storing samples at 4 °C until analysis. Water samples were tested using gas chromatography-tandem mass spectrometry (7000C Triple Quadrupole GC–MS/MS, Agilent Technologies, Delaware, USA) by Envirolab Services (MPL Laboratories, Perth; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025). The limit of quantification was 1 ng/L and no contamination of unexposed tanks was detected throughout the exposure period ($n = 16$). For a detailed description of the GC–MS/MS protocol, see Tomkins et al. (2018).

2.5. Behavioural trials

To test for impacts of 17β-trenbolone on the full array of male eastern mosquitofish reproductive behaviours (i.e., association, chasing, mating attempts), two separate experiments were conducted (see below). For both experiments, male eastern mosquitofish were selected randomly from unexposed or 17β-TB-exposed tanks, and allocated to a trial tank (54 L; 60 × 30 × 30 cm; water depth: 20 cm) containing fresh water (i.e., water free from 17β-TB; *sensu* Bertram et al., 2019; Bertram et al., 2020; Tomkins et al., 2016; Tomkins et al., 2017). Each individual male was only subjected to one of the two experiments. Stimulus female conspecifics (not exposed to 17β-TB) were randomly drawn from one of two holding tanks (54 L; 60 × 30 × 30 cm). Stimulus females were left unexposed to avoid any potential effects of stimulus fish exposure on focal (male) fish behaviour (*sensu* Bertram et al., 2018a; Bertram et al., 2019; Martin et al., 2019a). Males and females were acclimated for 5 min in opaque containers within the trial tank before being released and video-recorded for 15 min. To avoid chemical cross-contamination between trials, observation tanks were drained and re-filled with clean water from eight reservoir tanks (180 L; 90 × 45 × 45 cm) upon completion of each trial. The temperature of the trial tank was recorded for each trial to ensure that temperature was kept consistent between trials and housing.

The first experiment involved examining the tendency for an exposed ($n = 28$) or unexposed ($n = 36$) male eastern mosquitofish to associate with a stimulus (unexposed) conspecific female, which was performed in one of ten identical trial tanks (Fig. 1a). Each trial tank was divided transversely into two compartments, a larger central compartment (49.5 L; 55 × 30 × 30 cm) where the male fish was placed, and a smaller compartment (4.5 L; 5 × 30 × 30 cm) where the stimulus female fish was placed. The dividing partition was transparent with small perforations to allow for visual and chemical communication, whilst preventing physical interaction between the male and female. Upon commencement of the trial, the focal male and stimulus female were released from their acclimation containers into the centre of their respective tank compartments, and the focal male was allowed to freely explore the tank for 15 min. Using external markings on the trial tank, a 5 cm zone in the larger male compartment directly adjacent to the smaller female compartment was demarcated, and the total time spent by the focal male inside this zone was used to quantify the amount of time the focal male spent associating with the female (Fig. 1a). This method for quantifying reproductive association behaviour is a commonly used measure of mating intent in poeciliids (e.g., Bierbach et al., 2011), including mosquitofish (Pyke, 2005).
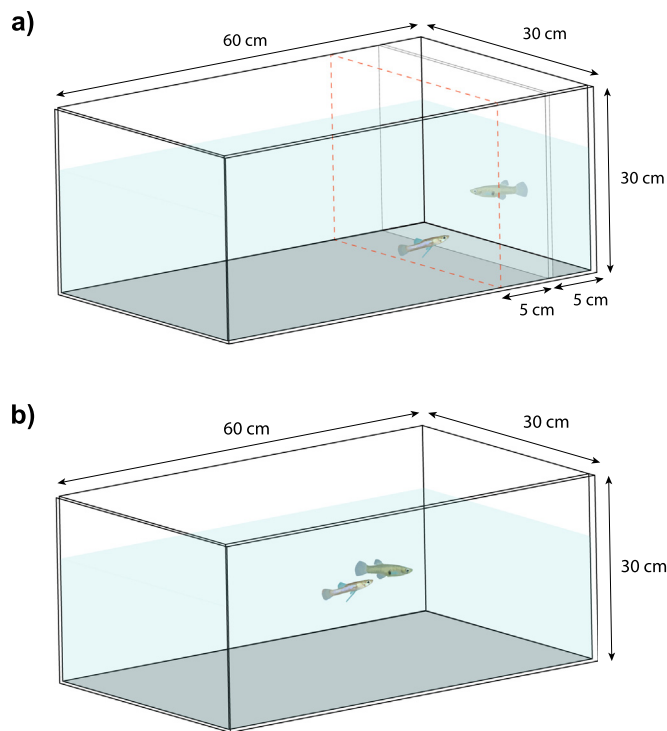
Fig. 1. (a) Association assay, in which males were assessed for the total time spent associating with a stimulus female, quantified as time spent within a 5 cm association zone (red dotted line) directly adjacent to the stimulus female compartment. (b) Mating effort assay, in which males were allowed to freely interact with a stimulus female and were assessed for the number of copulation attempts made and the total time spent chasing the female. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The second experiment involved examining the mating effort of an exposed ($n = 28$) or unexposed ($n = 41$) male eastern mosquitofish that was allowed to freely interact with a stimulus (unexposed) female. Upon commencement of the trial, the focal male and stimulus female fish were released from their respective acclimation containers into the centre of a trial tank (Fig. 1b) and allowed to interact freely for 15 min. The behaviours of the focal male quantified during this experiment include the number of sneak copulation attempts performed and the total time spent chasing the stimulus female (within 5 cm). Sneak copulation attempts were identified by observing the male approaching the female from behind and attempting to insert his gonopodium into her gonoduct (Bisazza et al., 2001).

Reproductive behaviours video-recorded in both experiments were quantified blind to treatment post-experiment using JWatcher V1.0 (Blumstein and Daniel, 2007).

2.6. Sperm analysis

Following behavioural trials, the post-copulatory reproductive performance of males from both experiments was examined using computer-assisted sperm analysis (CASA). This involved first euthanising male fish with an overdose (40 mg/L) of clove oil. Euthanised males were placed with their right lateral side facing upwards on a petri dish under a dissection microscope, and were covered with 500 µL of extender solution (207 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 10 mM Tris, pH 7.5; Locatello et al., 2006) to render sperm inactive. To release the ejaculate of the fish, a metal probe was used to swing the gonopodium forward three times, and gentle pressure was applied anterior to the base of the gonopodium, on the abdomen. A 5 µL aliquot of ejaculate in extender solution was collected and added to 20 µL of activation solution (150 mM KCl and 2 mg/mL bovine serum albumin) and gently resuspended 100 times to separate spermatozeugmata

(sperm bundles). A 4 µL aliquot of this solution was then loaded onto a 12-cell multitest slide (MP Biomedicals, Aurora, OH, USA) that had first been coated with 1% polyvinyl alcohol to prevent sperm from sticking (Wilson-Leedy and Ingermann, 2007). The multitest slide was covered with a coated coverslip and placed under a negative phase-contrast microscope coupled with a CASA system (v. 14, CEROS, Hamilton-Thorne Biosciences, Beverly, MA) for assessment of sperm traits. A minimum of 1000 sperm were tracked per male (mean ± SE: 1127.40 ± 8.85, $n = 133$ fish). Protocols for sperm collection and analysis followed Bertram et al. (2018a). Sperm function traits assessed included average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), path linearity (LIN, %), and proportion of motile sperm (MOT, %). Full descriptions of the sperm traits examined are provided in Table S1.

2.7. Morphological analysis

Following sperm collection, males were measured for standard length (±0.01 mm) and weight (±0.0001 g). A condition index for male fish was calculated by first plotting mass (g) against standard length (mm) to produce a least squares regression line (i.e., line of best fit) and calculating the residuals of this regression line (i.e., the difference between the actual mass at a given standard length and the mass estimated from the regression line). All relevant morphological measures were recorded for stimulus females following behavioural trials.

2.8. Statistical analysis

Statistical analyses were conducted using R version 4.0.2 (R Core Team, 2020), with statistical significance assigned at $\alpha = 0.05$. Data were tested for normality (Shapiro-Wilk test; Royston, 1995) and homogeneity of variance (Fligner-Killeen test; Conover et al., 1981), and were transformed to approximate a Gaussian distribution (detailed in Supplementary Material, Section 1.2 Statistical analysis), where appropriate.

To test for effects of 17β-TB on the relationships between pre-copulatory behaviours and post-copulatory sperm traits, and the effects of 17β-TB on individual pre-copulatory behaviours, separate models were run for each behavioural trait (i.e. association time, copulation attempts, chasing time). These models included focal male weight (g), stimulus female weight (g), sperm average path velocity (VAP), proportion of motile sperm (MOT), and exposure treatment as predictor variables. More specifically, we were interested in possible two-way interactions between sperm traits (VAP and MOT) and exposure treatment, with a significant interaction term indicating that the relationship between pre- and post-copulatory traits had been altered by 17β-TB exposure. The sperm traits VAP and MOT were chosen to be included in analyses based on results from a principal component analysis (PCA) assessing the inter-correlation between the five sperm traits (VAP, VSL, VCL, LIN, MOT) that were quantified with CASA (see Supplementary Material Section, 1.2 Statistical analysis; Fig. S1). For association behaviour quantified in the first experiment, an analysis of covariance (ANCOVA) model was used to test the total time the focal male spent associating with the stimulus female. For mating effort behaviours quantified in the second experiment, a zero-inflated negative binomial generalised linear model (zinbGLM) was fitted to analyse the number of copulation attempts performed by the focal male towards the stimulus female, and an ANCOVA model was used to analyse the total time the focal male spent chasing the stimulus female.

Furthermore, to test for the mean effect of 17β-TB exposure on sperm traits (i.e. VAP, VSL, VCL, LIN, MOT), each individual sperm trait was analysed separately using ANCOVA. All models included male condition index, exposure treatment, and their interaction term, as well as the behavioural experiment type (association or mating effort) as predictor variables. Where a significant interaction between exposure treatment and male condition index was not detected, the interaction term was dropped from the model.

Male standard length, weight, and condition index of fish from each experiment were compared between exposure treatment groups using ANCOVA. These models included exposure treatment and behavioural experiment type (association or mating effort) as predictor variables.

An extended description of the statistical methods used, including variable transformations and determining models of best fit, can be found in the Supplementary Material, Section 1.2 Statistical analysis.

3. Results

3.1. Effects of 17β-trenbolone on relationships between pre-copulatory behaviours and post-copulatory sperm traits

A significant interaction between proportion of motile sperm (MOT) and exposure treatment was observed when analysing the total time spent by the focal male associating with the female ($F_{1,57} = 5.21$, $p = 0.026$). Specifically, a positive relationship between proportion of motile sperm and association time with the female was seen in unexposed male fish ($b = 21.5$ s/% motile sperm; Fig. 2) but this relationship was negative in male fish exposed to 17β-TB ($b = -19.7$ s/% motile sperm; Fig. 2). Additionally, a significant positive relationship was observed between sperm average path velocity (VAP) and the total time the focal male spent associating with the female, regardless of exposure treatment ($F_{1,57} = 7.88$, $p = 0.007$; Fig. 2). However, there was no general effect of 17β-TB trenbolone exposure on the total time spent by the focal male associating with the female ($F_{1,57} = 1.78$, $p = 0.188$; Fig. S2).

For the number of copulation attempts made by the focal male, there was a significant interaction between VAP and exposure treatment ($z = -2.19$, $p = 0.028$), with a positive relationship between VAP and number of copulation attempts seen in unexposed males ($b = 0.196$ copulation attempts/μm/s average path velocity), which was not observed in exposed males. There was also an overall effect of exposure treatment on copulation attempts ($z = 2.64$, $p = 0.008$), with unexposed fish performing more copulation attempts than exposed fish (Fig. S3).

Exposure to 17β-TB did not significantly affect the relationships between sperm traits and the time the focal male spent chasing the stimulus female (VAP: $F_{1,61} < 0.01$, $p = 0.964$; MOT: $F_{1,61} = 1.19$, $p = 0.279$), nor was there a general effect of 17β-TB exposure on time spent chasing the stimulus female ($F_{1,61} = 2.52$, $p = 0.118$; Fig. S4). However, overall, there was a marginally non-significant positive relationship between VAP and time spent chasing the stimulus female ($F_{1,61} = 3.34$, $p = 0.073$; Fig. S5).

3.2. Sperm analysis

The proportion of motile sperm (MOT) was found to be significantly higher in males exposed to 17β-TB compared to unexposed males in fish from both behavioural experiments (Fig. 3; Table S2). However, exposure to 17β-TB did not affect other sperm traits, including VAP, VSL, VCL, and LIN (Table S2). Male condition index did not have a significant effect on any of the measured sperm traits (Table S3). Males subjected to the mating effort behavioural trial had higher average values of VAP, VSL, and VCL than males subjected to the association behaviour trial (Table S4).

3.3. Morphology

Male standard length, weight, and condition index did not differ significantly between 17β-TB-exposed and unexposed fish across both experiments (Table S5).
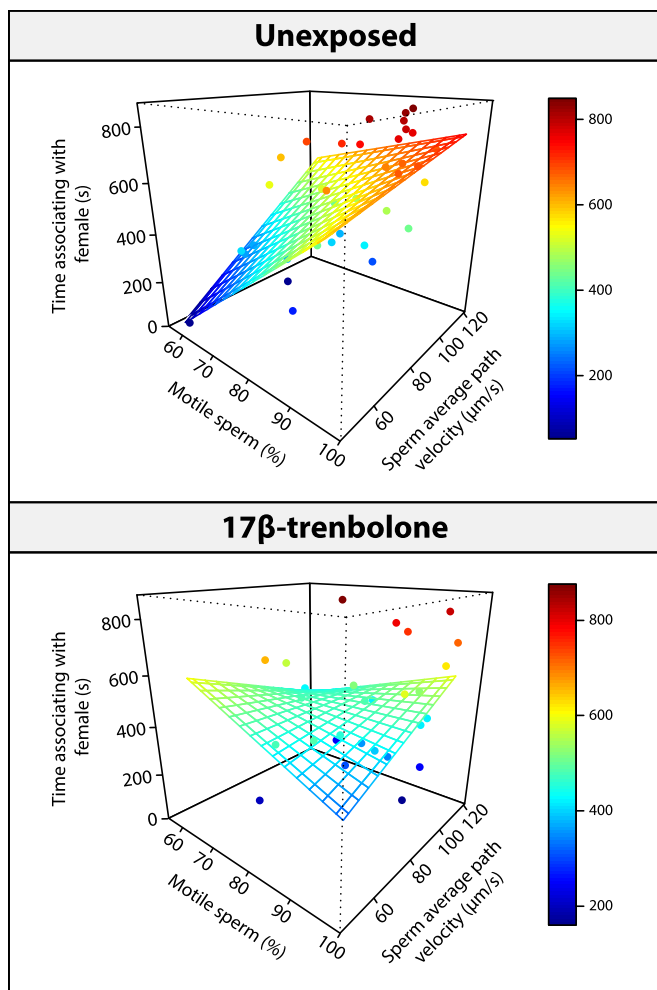

Fig. 2. The three-way association between sperm average path velocity (VAP, μm/s), proportion of motile sperm (MOT, %), and total time the focal male associating with the stimulus female (s), split by 17β-trenbolone exposure treatment (unexposed or exposed). Colours on each plot correspond to values along the y-axes (s) as indicated by the colour bar.
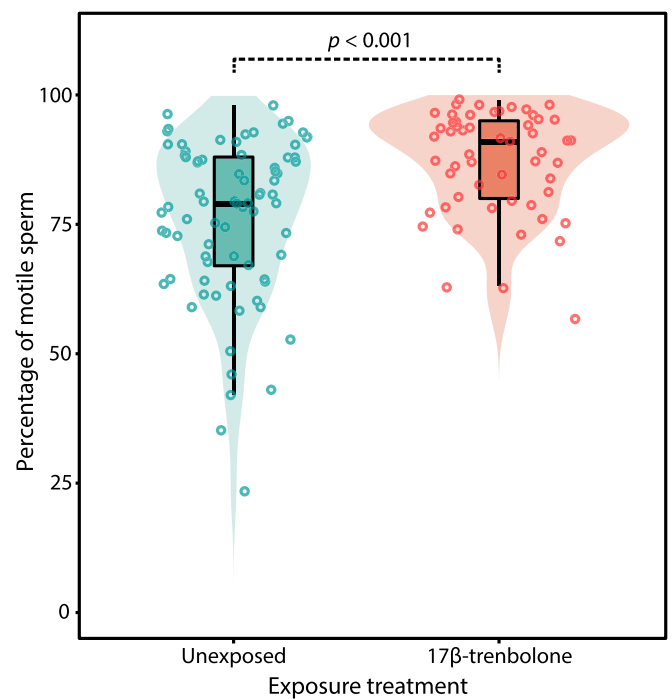

Fig. 3. Percentage of motile sperm plotted by exposure treatment (unexposed or 17β-trenbolone exposed) in male eastern mosquitofish. Box plots show 25th, 50th (median), and 75th percentiles.

4. Discussion

Here, we investigated whether 21-day exposure to an environmentally realistic level (average measured concentration = 10.93 ± 10.24 ng/L, mean ± SD) of the synthetic veterinary steroid and environmental pollutant 17β-trenbolone (17β-TB) affected pre-copulatory reproductive behaviours and post-copulatory sperm traits in male fish. We found evidence for disruption of the relationship between pre- and post-copulatory reproductive traits in male fish due to 17β-TB exposure. We also found that 17β-TB-exposed fish had, on average, a higher percentage of motile sperm than unexposed fish. However, there was no overall effect of 17β-TB exposure on either the total time males spent associating with or chasing females. Lastly, we found that males exposed to 17β-TB performed significantly fewer copulation attempts than unexposed males.

Exposure to 17β-TB disrupted the relationship between pre- and post-copulatory reproductive traits in male eastern mosquitofish. In unexposed males, we found a positive correlation between the total time spent associating with a female and the percentage of motile sperm (MOT) belonging to those males. Similarly, there was a positive correlation in unexposed males between the number of copulation attempts made and sperm velocity (VAP). However, these correlations were both negative in fish exposed to 17β-TB, indicating that contamination disrupted the relationships between these key pre- and post-copulatory traits. The positive correlation between number of copulation attempts and sperm velocity seen in unexposed males is an adaptation to maximise fertilisation success, given that in species such as eastern mosquitofish, males perform sneak copulations and routinely face intense post-copulatory sperm competition (Evans, 2010; Lüpold et al., 2014). Similarly, males with a relatively higher proportion of motile sperm may be bolder and associate with females more often when under intense sperm competition in order to maximise fertilisation success (Birkhead and Pizzari, 2002; Gasparini et al., 2019). Therefore, the alteration of these adaptive relationships by exposure to 17β-TB is expected to reduce the reproductive success of males under male-male competition. The disruption of relationships between pre- and post-copulatory reproductive traits caused by exposure to 17β-TB suggests that investments in pre-copulatory traits over post-copulatory traits (or *vice versa*) becomes more important when attempting to maximise fertilisation success under such conditions (Lüpold et al., 2014).

Exposure to 17β-TB was also associated with a significant increase in sperm motility in male eastern mosquitofish, indicating an increase in sperm quality (Rurangwa et al., 2004). Due to the mode of action of 17β-TB (a potent androgen receptor agonist), gonads are expected to be a primary organ affected by exposure (Ankley et al., 2018). Indeed, previous studies have shown that exposure to 17β-TB can lead to increases in spermatozoa numbers in male zebrafish (Baumann et al., 2014; Örn et al., 2006) and medaka (Park et al., 2009)—a general increase in sperm-related traits that is concordant with our findings. However, contrary to our study, Bertram et al. (2019) found that exposure to 17β-TB did not affect sperm traits in male eastern mosquitofish. Sperm motility is a crucial factor in determining fertilisation success (Rurangwa et al., 2004), and general sperm quality—of which sperm motility is a commonly used indicator—is crucial in predicting fertilisation success under sperm competition (Boschetto et al., 2011; García-González and Simmons, 2005). Indeed, sperm motility and fertilisation capacity are correlated in various fish species such as rainbow trout (*Oncorhyncus mykiss*; Lahnsteiner et al., 1996), carp (*Cyprinus carpio*; Magyary et al., 1996), African catfish (*Clarias gariepinus*; Rurangwa et al., 2001), and Atlantic halibut (*Hippoglossus hippoglossus*; Tvedt et al., 2001). Therefore, alterations in the relationship between pre- and post-copulatory reproductive traits seen in 17β-TB-exposed fish may be driven by the observed increase in sperm motility caused by 17β-TB exposure. Furthermore, there were no differences in sperm functional traits (VAP, VSL, and VCL) between 17β-TB-exposed and unexposed fish, indicating that there was no trade-off between sperm quality and sperm function

in exposed fish. Taken together, a disruption in the relationship between pre- and post-copulatory reproductive traits does not necessarily indicate an overall reduction in competitive reproductive success in 17β-TB-exposed individuals. It appears that due to an overall increase in sperm motility in 17β-TB-exposed fish, there may be less importance in investing in pre-copulatory mating behaviours in order to maximise fertilisation success (Lüpold et al., 2014). As reproductive behaviours such as associating and copulating with females may increase an individual's risk of predation (Magnhagen, 1991), investing in pre-copulatory reproductive behaviours may become less important once post-copulatory sexual traits are optimised.

Exposure to 17β-TB did not affect the time spent by focal males associating with or chasing females. In agreement with this finding, studies on guppies (*Poecilia reticulata*) have reported that 17β-TB exposure did not affect the time males spent associating with (Tomkins et al., 2017) or following females (Bertram et al., 2018b), and had no effect on male courtship behaviour (Bertram et al., 2015; Bertram et al., 2018b; Bertram et al., 2020; Tomkins et al., 2016), which is a key indicator of male reproductive interest in guppies (Houde, 1997). However, contrary to our study, Bertram et al. (2019) found that male eastern mosquitofish exposed to 17β-TB spent less time associating with females. Taken together, there does not seem to be a pronounced effect of 17β-TB exposure at low field-realistic concentrations on association and following reproductive behaviours of male fish, with the exception of Bertram et al. (2019) showing a reduction in association behaviour.

In contrast to our finding that 17β-TB exposure did not affect association and chasing behaviours exhibited by male fish, we did find a general effect of 17β-TB exposure on sneak copulation attempts, with exposed males performing less copulation attempts than unexposed males. Here, the exposure status of females could play an important role in determining male copulatory behaviour under contaminant exposure. In our study, stimulus females were not exposed to 17β-TB to avoid any potential effects of stimulus fish exposure on focal (male) fish behaviour. This was because Bertram et al. (2015) found that different combinations of male and female exposure resulted in different outcomes in terms of male mating behaviour. In contrast to our study, Saaristo et al. (2013) paired male and female eastern mosquitofish from the same exposure treatment groups, and found that there were no differences between the number of copulation attempts performed by unexposed or exposed males. Therefore, it is possible that differences in results between this study and those found in Saaristo et al. (2013) were due to differences in the exposure status of the females. Interestingly, Saaristo et al. (2013) found that female eastern mosquitofish exposed to 17β-TB spent less time associating with males. Further studies looking at the sex-specific effects of 17β-TB on females may, therefore, provide greater insight into the overall effects of 17β-TB on wildlife reproductive behaviour. Additionally, the study species' mating system may be important in determining how contaminant exposure affects male mating behaviour. This is because, in a different poeciliid species, the guppy (*Poecilia reticulata*), fish exposed to 17β-TB have repeatedly been shown to perform more sneak copulation attempts than unexposed fish (Bertram et al., 2015; Bertram et al., 2018b; Bertram et al., 2020; Tomkins et al., 2017). It is important to note that in some poeciliid species, such as guppies, males employ two different tactics to gain copulations, either by courting females or through forced copulation (Bisazza, 1993; Bisazza et al., 2001). However, in approximately half of the poeciliid species, including in eastern mosquitofish, males never court and coercive mating attempts are the only way to achieve fertilisation (Bisazza, 1993; Farr, 1989). This fundamental difference in mating systems between guppies and mosquitofish may, in part, explain why there are differences seen in the effects of 17β-TB on copulatory behaviour between these poeciliid species.

Alterations in the mating behaviour of male fish due to 17β-TB exposure is unsurprising as the endocrine system plays an important role in regulating behavioural processes (Norris and Carr, 2014). 17β-trenbolone is a high-affinity ligand for the vertebrate androgen receptor

and it is the activation of these receptors that generate its anabolic properties, including influencing reproductive behaviour and sperm output (reviewed in Ankley et al., 2018). Importantly, two androgen receptor isoforms (ARα and ARβ) have been characterised in teleost fish (Harbott et al., 2007), and male sexual behaviours have been shown to be mediated by testicular androgens that bind to these receptors (Borg, 1994; Munakata and Kobayashi, 2010). As such, exogenous androgens such as 17β-TB can mimic the effect of these endogenous androgens by binding to the same receptors (Wilson et al., 2004; Wilson et al., 2002), altering the sexual behaviour of fish exposed to such compounds in the environment (Ankley et al., 2018; Larsen and Baatrup, 2010). Therefore, the effects of 17β-TB, and endocrine-disrupting chemicals in general, on reproductive behaviours in wildlife warrant further investigation given that there are potential mechanistic pathways that facilitate these effects, and because behavioural alterations may vary across species.

5. Conclusion

In conclusion, we report that exposure to an environmentally realistic level of the potent agricultural pollutant 17β-trenbolone disrupts the adaptive relationship between pre- and post-copulatory reproductive traits in male eastern mosquitofish. This may be driven, in part, by the general increase in sperm motility seen in males exposed to 17β-TB in this study. Alterations in copulatory behaviour were also observed in 17β-TB-exposed male fish, which was unsurprising as 17β-TB affects androgen receptors that mediate male sexual behaviour in fish. More broadly, our findings demonstrate the potential for chemical pollutants to affect both pre- and post-copulatory sexual traits, and the interplay between these mechanisms of sexual selection in wildlife.

Ethical statement

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

Data accessibility

All data and R code associated with this article can be accessed at https://doi.org/10.26180/14633436.

CRediT authorship contribution statement

Hung Tan: Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Michael G. Bertram:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Jake M. Martin:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Tiarne E. Ecker:** Investigation, Writing – review & editing. **Stephanie L. Hannington:** Investigation, Writing – review & editing. **Minna Saaristo:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Moira K. O'Bryan:** Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Bob B.M. Wong:** Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.148028.

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