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The antidepressant fluoxetine alters mechanisms of pre- and post-copulatory sexual selection in the eastern mosquitofish (*Gambusia holbrooki*)☆

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ABSTRACT

Contamination of aquatic habitats with pharmaceuticals is a major environmental concern. Recent studies have detected pharmaceutical pollutants in a wide array of ecosystems and organisms, with many of these contaminants being highly resistant to biodegradation and capable of eliciting sub-lethal effects in non-target species. One such pollutant is fluoxetine, a widely prescribed antidepressant, which is frequently detected in surface waters globally and can alter physiology and behaviour in aquatic organisms. Despite this, relatively little is known about the potential for fluoxetine to disrupt mechanisms of sexual selection. Here, we investigate the impacts of 30-day exposure to two environmentally realistic levels of fluoxetine (low and high) on mechanisms of pre- and post-copulatory sexual selection in the eastern mosquitofish (*Gambusia holbrooki*). We tested 1) male mating behaviour in the absence or presence of a competitor male, and 2) sperm quality and quantity. We found that high-fluoxetine exposure increased male copulatory behaviour in the absence of a competitor, while no effect was detected under male-male competition. Further, fluoxetine exposure at both concentrations increased total sperm count relative to males from the control group, while no significant change in sperm quality was observed. Lastly, low-fluoxetine males showed a significant reduction in condition index (mass relative to length). Our study is the first to show altered mechanisms of both pre- and post-copulatory sexual selection in an aquatic species resulting from environmentally realistic fluoxetine exposure, highlighting the capacity of pharmaceutical pollution to interfere with sensitive reproductive processes in wildlife.

1. Introduction

Numerous pharmaceutical pollutants are capable of altering ecologically important traits and behaviours in wildlife (Boxall et al., 2012; Arnold et al., 2014; Brodin et al., 2014). Pharmaceutically active compounds enter the environment via multiple pathways, including the excretion of chemicals used for human and veterinary healthcare, discharge from manufacturing and disposal of unused medications (Heberer, 2002). Of the approximately 5000 actively marketed pharmaceutical products, over 600 have now been detected in the environment globally (Küster and Adler, 2014). Worldwide consumption of pharmaceuticals is also increasing due to a growing and ageing human population (Khetan and Collins, 2007; Arnold et al., 2014). Antidepressant pharmaceuticals pose a distinct threat to wildlife as they are specifically designed to induce physiological effects at low concentrations (Khetan and Collins, 2007) and have a particularly strong potential to alter behaviour (Arnold et al., 2014; Brodin et al., 2014). The most frequently

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prescribed class of antidepressants is the selective serotonin reuptake inhibitors (SSRIs) (Fong and Ford, 2014), which act by inhibiting the reuptake of the monoamine neurotransmitter serotonin (5-hydroxytryptamine) by the pre-synaptic nerve cleft, thereby increasing the effect of serotonin on the post-synaptic nerve (Stahl, 1998). Serotonin is a ubiquitous neurotransmitter, present in all phyla possessing nervous systems (Weiger, 1997). As such, SSRIs have the potential to alter a range of ecologically important traits and behaviours in wildlife.

One SSRI of environmental concern is fluoxetine, which is used to treat major depression and other psychiatric disorders in humans, and is among the most commonly prescribed pharmaceuticals (Wong et al., 2005). Present in aquatic environments globally, fluoxetine has been detected in surface waters at concentrations typically ranging from <1 to 100 ng/L (e.g., Kolpin et al., 2002; Fernández et al., 2010; Gardner et al., 2012; Hughes et al., 2013), although levels as high as 596 ng/L have been reported in systems receiving wastewater discharge (Benotti and Brownawell, 2007). In addition, fluoxetine has been found to bioaccumulate in fish tissues, especially in the brain (Brooks et al., 2005; Schultz et al., 2010). Exposure to fluoxetine can influence a range of ecologically important traits, including development (Japanese medaka, *Oryzias latipes*, Foran et al., 2004; western mosquitofish, *Gambusia affinis*, Henry and Black, 2008), reproduction (zebrafish, *Danio rerio*, Lister et al., 2009) and survival (guppy, *Poecilia reticulata*, Pelli and Connaughton, 2015), as well as various morphological and physiological characteristics (e.g., altered growth in *P. reticulata*, Pelli and Connaughton, 2015; impaired cardiovascular and ventilatory response to hypoxia in Gulf toadfish, *Opsanus beta*, Panlilio et al., 2016). Fluoxetine exposure has also been linked with alterations in a variety of behaviours in fish, such as activity (Siamese fighting fish, *Betta splendens*, Kohlert et al., 2012; Arabian killifish, *Aphanius dispar*, Barry, 2013), feeding and foraging (fathead minnow, *Pimephales promelas*, Stanley et al., 2007; *P. promelas*, Weinberger and Klaper, 2014), aggression (*B. splendens,* Lynn et al., 2007; *B. splendens*, Dzieweczynski and Hebert, 2012; *A. dispar,* Barry, 2013), sociability (*A. dispar*, Barry, 2013; *O. latipes*, Ansai et al., 2016) and antipredator behaviour (*P. reticulata*, Pelli and Connaughton, 2015; eastern mosquitofish, *Gambusia holbrooki*, Martin et al., 2017). However, variability in fluoxetine sensitivity reported across studies, model species and biological responses has made ascertaining what fluoxetine concentrations pose a risk to aquatic wildlife challenging (Stewart et al., 2014; Sumpter et al., 2014), highlighting the need for further research investigating the impacts of environmentally realistic concentrations of fluoxetine on ecologically relevant traits. Having received little attention relative to other endpoints, this is especially true for the effects of exposure to fluoxetine on mechanisms of sexual selection.

Sexual selection can occur both before (i.e., pre-copulatory) and after (i.e., post-copulatory) mating (Andersson and Simmons, 2006), with both of these processes being vulnerable to disruption by pharmaceutical pollution. Studies using pharmacological dosages have demonstrated that treatment with fluoxetine can induce male sexual dysfunction in humans (Gregorian et al., 2002; Serretti and Chiesa, 2009) and rodents (Taylor et al., 1996; Matuszcyk et al., 1998). However, findings from the handful of studies that have examined the impacts of environmentally realistic concentrations of fluoxetine on reproductive behaviour have been mixed. Specifically, while some studies have reported an increase in certain reproductive behaviours following fluoxetine exposure (Weinberger and Klaper, 2014), others have reported a decrease (Forsatkar et al., 2014), or no significant effect (Schultz et al., 2011; Dzieweczynski and Hebert, 2012). Further, the effects of fluoxetine on male mating behaviour under male-male competition, where males compete for the opportunity to reproduce, has received very little attention, despite being a central component of pre-copulatory sexual selection (Andersson, 1994). Clearly, the potential impacts of fluoxetine on mating and reproductive behaviours in wildlife require further investigation.

In species where females mate multiply (polyandry), an important component of post-copulatory sexual selection is sperm competition, where the sperm of multiple males compete to fertilise available ova (Andersson and Simmons, 2006). In polyandrous species, a key predictor of each male's fertilisation success is his proportional contribution to the sperm pool (Parker, 1998), with elevated sperm production allowing males to copulate more often and allocate more sperm to each ejaculate (Parker, 1982). Sperm quality traits such as viability and speed can also influence fertilisation success under sperm competition (Snook, 2005). Because treatment with SSRIs, including fluoxetine, can reduce fertility in human males (reviewed in Brezina et al., 2012; Nørr et al., 2016), considerable attention has been paid to the impacts of fluoxetine at pharmacological levels on fertility in rodent models (e.g., Bataineh and Daradka, 2007; Alzahrani, 2012; Monteiro Filho et al., 2014). In addition, research in aquatic species has reported reproductive dysfunction in species as diverse as male goldfish (*Carassius auratus*, Mennigen et al., 2010) and zebra mussels (*Dreissena polymorpha*, Fong, 1998). Despite this, the potential effects of exposure to environmentally realistic levels of fluoxetine on both sperm quality and quantity remain to be investigated in *any* aquatic vertebrate.

The eastern mosquitofish is a small, internally fertilising poeciliid fish with a widespread geographic distribution (Pyke, 2005, 2008) that is attracting increased interest as a model for investigating the impacts of chemical pollutants (e.g., Saaristo et al., 2013, 2014; Magellan et al., 2014; Martin et al., 2017; Melvin et al., 2017). Mosquitofish have a coercive mating system, where males copulate with females by 'sneaking' from behind and thrusting the tip of their gonopodium—a modified anal fin used for internal fertilisation—into the female's genital pore (Bisazza et al., 2001). No courtship occurs and, although females may exert some control over the outcome of unsolicited mating attempts by spending more time associating with preferred males, male sexual coercion and male-male competition are the primary modes of pre-copulatory sexual selection in this species (Bisazza et al., 2001). Wild mosquitofish females are typically inseminated by multiple males (Zane et al., 1999) and are capable of storing sperm for several months (Evans et al., 2003), putting the sperm of multiple males in direct competition. Further, in this species, approximately ninety-percent of all broods are sired by multiple males, making sperm competition a major source of post-copulatory sexual selection (Zane et al., 1999). These attributes make mosquitofish an excellent system for investigating the effects of pollutants on sexually selected traits and behaviours.

Here, we investigated the effects of 30-day exposure to two environmentally realistic levels of fluoxetine—nominal low and high concentrations of 40 and 400 ng/L, respectively—on mechanisms of pre- and post-copulatory sexual selection in mosquitofish. Utilising two separate flow-through exposures, we experimentally investigated the impact of fluoxetine on 1) male mating behaviour in the absence or presence of a competitor, and 2) total sperm count and sperm quality. In addition, all fluoxetine-exposed and control (i.e., unexposed) males were tested for differences in their morphological characteristics.

2. Materials and methods

2.1. Animal collection and housing

Mosquitofish were wild-caught from the Science Centre Lake at Monash University (37° 54′ 28″ S, 145° 08′ 16″ E), Victoria, Australia.

Analysis of water samples from the site of fish collection indicated no contamination with fluoxetine (Envirolab Services, unpublished data; see below for details of water testing). Sexually mature fish were acclimated to laboratory conditions in single-sex aquaria for 1 month prior to experimentation (12:12 h light:dark cycle; 24–26 °C; 128 L; 80 × 45 × 45 cm). Fish were fed *ad libitum* once daily with commercial fish food (Otohime Hirame larval diet; 580–910 μm).

2.2. Flow-through chemical exposures

Male mosquitofish were exposed to fluoxetine using two separate flow-through systems that were identical in design, comprising fish to be tested for 1) reproductive behaviour and 2) sperm traits. Separate exposures were conducted to ensure that males tested for sperm traits would not have the opportunity to expend ejaculate in free-swimming behavioural trials. In either system, males were randomly allocated to one of three exposure treatments: freshwater control, low fluoxetine or high fluoxetine (see below). Fish were subjected to a 30-day exposure period. This length of exposure was chosen because clinical trials in humans suggest that fluoxetine does not exhibit its full therapeutic (anxiolytic-like) effects for 2–4 weeks after the initiation of treatment (e.g., Gardier et al., 1996; Matuszcyk et al., 1998), fluoxetine exposure periods ranging from 28 to 35 days are sufficient to induce behavioural changes in a variety of fish species (e.g., Pelli and Connaughton, 2015; Martin et al., 2017; McCallum et al., 2017; Saaristo et al., 2017), and 30 days is the duration of one spermatogenic cycle in mosquitofish (Koya and Iwase, 2004). Further, mosquitofish are non-migratory and individuals generally have a relatively small home range (several meters, Noggle et al., 2004; Pyke, 2005), meaning that populations living in contaminated systems are likely to be exposed for prolonged periods. Each exposure involved three identical flow-through systems, one per treatment, which followed the design of previous experiments (Bertram et al., 2015; Martin et al., 2017; Saaristo et al., 2017; Tomkins et al., 2017, 2018), with some modifications. For each treatment, a mixing tank (182 L; 90 × 45 × 45 cm) fed into four exposure tanks (54 L; 60 × 30 × 30 cm), each of which housed 35 males. Exposure aquaria were equipped with 2 cm of natural gravel substrate, a large stone for refuge, an airstone, and an aquarium heater. Exposure tanks were kept on a 12:12 h light:dark cycle and were monitored daily for temperature (first exposure: mean = 25.19 °C, SD = 0.66 °C, $n = 360$; second exposure: mean = 25.21 °C, SD = 0.57 °C, $n = 360$) and flow-through rates (24 h cycling, ~1.67 L/h per tank).

To achieve the nominal low- and high-fluoxetine treatment concentrations—40 and 400 ng/L, respectively—used in each flow-through system, stock solutions were prepared as follows. Every third day, 1 mL of fluoxetine hydrochloride (CAS: 56296-78-7; Sigma-Aldrich, St Louis, MO) dissolved in methanol (HPLC grade, ≥99.9%) (low: 0.1 mg/mL, high: 1 mg/mL) was evaporated to dryness under a gentle nitrogen stream, before being diluted with Milli-Q water to form a 1 L solution. Every 24 h, a 180 mL aliquot of this solution was further diluted to produce a 3 L stock solution for each exposure level. Fluoxetine concentrations in each exposure tank were measured weekly, as well as being randomly sampled in half of the control (i.e., unexposed) aquaria, to ensure the absence of contamination. Analysis was performed by Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025), using gas chromatography–tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware, USA), following methods adapted from Papoutsis et al. (2012). For additional detail on the collection and analysis of water samples, see electronic supplementary material, 'Supplementary methods'.

2.3. Male reproductive behaviour

Males from the first flow-through exposure were used to test the impacts of fluoxetine on mechanisms of pre-copulatory sexual selection in two separate behavioural experiments. Two days prior to behavioural trials, males in all exposure tanks had a small portion of either the top or bottom of their caudal fin clipped for identification during competitive mating trials—a common method of fish identification (Ricker, 1949). Both non-competitive and competitive mating trials involved males being drawn at random from exposure tanks and allocated to one of 16 observation tanks (54 L; 60 × 30 × 30 cm) filled to a depth of 20 cm with aged water. Unexposed stimulus females were used in both behavioural experiments to avoid any potential influence of female fluoxetine exposure on male behaviour and were drawn randomly from four holding tanks containing fresh water only (54 L; 60 × 30 × 30 cm). Males and stimulus females were tested in one trial only and were not retested across behavioural experiments to control for any potential order effects.

In the first behavioural experiment, the effects of fluoxetine on male reproductive behaviour were tested in a non-competitive setting. This involved quantifying the behaviour of a single control ($n = 33$), low-fluoxetine ($n = 39$) or high-fluoxetine ($n = 37$) male when paired with an unexposed stimulus female. Free-swimming behavioural trials were preceded by a 5 min acclimation period, after which both fish were simultaneously released from their holding containers and allowed to freely interact for 15 min. Behaviours quantified included the number of male copulation attempts performed, involving a male approaching a female from behind and attempting to insert his gonopodium into her gonoduct (Bisazza et al., 2001), as well as the duration of time spent by the male actively following the female (within 5 cm).

In the second behavioural experiment, the impact of fluoxetine on male mating performance was tested under male-male competition. This involved quantifying the sum of the combined reproductive behaviours of two rival males when allowed to freely interact with, and compete over, a single unexposed stimulus female. Each trial was comprised of males both from either the control ($n = 37$), low-fluoxetine ($n = 43$) or high-fluoxetine ($n = 35$) treatments. Males from the same treatment were paired because wild males are likely to experience similar levels of environmental contamination. For each trial, males were drawn from separate exposure tanks within the same treatment to ensure that competing males had no recent experience with one another (i.e., no interaction for ≥31 days prior to behavioural trials). Again, after a 5 min acclimation period, the fish were released into the trial tank and allowed to freely interact for 15 min. The sum of the number of copulation events directed by both males towards the female was quantified, as well as the cumulative amount of time spent by both males following the female.

Subsequent to all trials, males were euthanised with an overdose of anaesthetic clove oil (40 mg/L) and were subject to morphological analysis (see below). Behavioural trials were video-recorded and quantified using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007). Quantification of video recordings was performed blind to treatment, with competitive mating trials being scored twice (once per male).

2.4. Sperm traits

Males from the second flow-through exposure were used to investigate the effects of fluoxetine exposure on mechanisms of post-copulatory sexual selection. Here, experimental (control, low-fluoxetine or high-fluoxetine) males were tested for total sperm count and sperm quality traits, with males tested for total sperm

count being separate from those analysed for sperm quality.

Total sperm count was estimated in control ($n = 32$), low-fluoxetine ($n = 29$) and high-fluoxetine ($n = 29$) males following Evans et al. (2003), with some modifications. Briefly, after being euthanised as described above, males were dabbed dry and placed on a glass Petri dish under a dissection microscope (Leica MZ9.5), before being covered with 2 mL of activation solution (150 mM KCl with 2 mg/mL bovine serum albumin). To release spermatozeugmata (sperm bundles), the gonopodium was swung forward three times before gentle pressure was applied to the abdomen, slightly anterior to the base of the gonopodium. After repeating this action to ensure the release of the entire ejaculate, the evacuated spermatozeugmata were immediately recovered using a micropipette and made up to a volume of 1 mL with activation solution. This solution was then gently resuspended 100 times with a pipette to aid in breaking up spermatozeugmata. The sperm were then killed with 20 μL of 35% formalin and stained with 10 μL of trypan blue. Using Milli-Q water, a 3.5-fold dilution was produced to create an appropriate cell concentration for counting. This solution was vortexed to produce a homogeneous suspension, with a 10 μL aliquot being loaded into each well of an improved Neubauer haemocytometer (Blaubrand, Germany). Sperm in ten 200 × 200 μm squares, five per haemocytometer chamber, were counted under × 40 magnification (Olympus B×60). Total sperm count was estimated by multiplying the mean of these ten counts by the sample dilution factor and the initial sample volume. Sperm counts were performed blind of treatment, as is also true for the following assays.

Sperm quality was measured using computer-assisted sperm analysis (CASA) software (v. 14, CEROS, Hamilton-Thorne Biosciences, Beverly, MA) in control ($n = 51$), low-fluoxetine ($n = 50$) and high-fluoxetine ($n = 53$) males. Briefly, this involved euthanised males being covered in 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), in which sperm remain quiescent. Sperm were then extracted (as above) and a 5 μL aliquot of sperm in extender medium collected. The sperm were activated with 20 μL of activation solution and gently resuspended 100 times using pipette action to break up the spermatozeugmata. A 3 μL drop of this solution was placed into the well of a 12-well multitest slide (MP Biomedicals, Irvine, CA) and a coverslip gently placed on top. To avoid sperm sticking, all slides and coverslips were dipped in 1% polyvinyl alcohol (Sigma-Aldrich) solution for 3 min and air-dried prior to use (Wilson-Leedy and Ingermann, 2007), as well as being warmed to 25 °C (LEC Warm Stage). A minimum of 1000 sperm were tracked per male (mean = 1116.54, SE = 6.09, $n = 154$) using a video camera (XC-ST50, Sony, Tokyo, Japan) coupled to a negative phase-contrast microscope (Olympus CX41) with a 10 × objective. Measurements of sperm function included: average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), path linearity (LIN, %) and motility (%) (see electronic supplementary material, Table S1 for detailed descriptions).

A second sub-sample of ejaculate was collected from males analysed using CASA, which was simultaneously tested for the proportion of live sperm (control: $n = 51$, low-fluoxetine: $n = 50$, high-fluoxetine: $n = 53$), following Evans (2009), with some modifications. A live/dead Sperm Viability Kit (L-7011; Molecular Probes Inc., OR, USA) was used, which firstly involved a 10 μL sample of ejaculate in extender solution being collected and gently resuspended 30 times with pipette action to break up the spermatozeugmata. Sperm were stained with 10 μL of a 1:50 dilution of membrane-permeant nucleic acid stain (1 mM SYBR 14), which stains live sperm green under fluorescent light. The sample was then vortexed and incubated at 25 °C in the dark for 10 min, before being counter-stained with 2 μL of 2.4 mM propidium iodide, which stains dead sperm red, and incubated for a further 10 min. After again being vortexed, 4 μL of the solution was placed onto a slide and viewed under a fluorescence microscope (Leica DFC425C). A minimum of 200 cells were counted per male (mean = 256.01, SE = 4.80, $n = 154$).

2.5. Morphological analysis

Males were measured subsequent to both reproductive behaviour and sperm analysis trials. Euthanised males were dabbed dry and measured for standard length (snout to caudal peduncle) (±0.01 mm), weight (±0.0001 g) and gonopodium length (±0.01 mm). An index of male body condition was calculated by plotting mass (g) against standard length (mm) to produce a least-squares regression line (i.e., weight = −0.440 + 0.029 × length). Condition index was calculated as the residuals of this regression line. All relevant morphological measures were also recorded for stimulus females.

2.6. Statistical analysis

Data were analysed in R version 3.2.3 (R Development Core Team, 2015). Where appropriate, data were checked for normality (visual inspection of standard diagnostic plots) and homogeneity of variance (Fligner-Killeen test). Vuong tests (*vuong* function, *pscl* package; Jackman, 2012) indicated zero-inflation of the number of copulation attempts performed by males towards females, both in the absence and presence of a competitor, which was accounted for by fitting zero-inflated Poisson (ZIP) generalised linear models (GLMs) (*zeroinfl* function, *pscl* package; Zeileis et al., 2008). To test for overdispersion in the number of copulation attempts performed, zero-inflated negative binomial (ZINB) GLMs (*zeroinfl* function) were then also fitted and compared with their respective ZIP GLM alternatives using likelihood-ratio tests (*lrtest* function, *lmtest* package; Zeileis and Hothorn, 2002). In both competitive and non-competitive trials, this procedure indicated overdispersion and, thus, ZINB GLMs were favoured (Zuur et al., 2009). For all models, predictors were selected based on their biological relevance (see electronic supplementary material, Table S2 for a summary of model parameters). General linear hypothesis tests (GLHTs; *glht* function, *multcomp* package; Hothorn et al., 2008) were used for post-hoc comparison of mean responses across treatment levels. Partial Wald tests were used to assess whether coefficients, or pairwise differences between treatment levels, were significantly different from zero (at $\alpha = 0.05$). The impact of fluoxetine on the amount of time males spent following females, both in the absence and presence of a competitor, was tested using analysis of covariance (ANCOVA). To approximate normality, following time in non-competitive trials was cube root transformed, while following time in the competitive trials underwent a rank normal transformation.

Total sperm count was compared between treatments using a GLM with a quasipoisson distribution to accommodate overdispersion, after which post-hoc comparisons were made using partial Wald tests through a GLHT, with *p*-values adjusted based on the joint normal distribution of the linear function. The effect of fluoxetine on sperm quality was assessed using ANCOVAs. To approximate normality, a rank normal transformation was applied to sperm path linearity (LIN) and the proportion of live sperm, while a folded root transformation was applied to the proportion of motile sperm. Male condition index and standard length were included as predictors in all models analysing sperm quality and quantity, as male body size is known to affect sperm traits in mosquitofish (O'Dea et al., 2014; see electronic supplementary material, Table S2 for further details).

The impact of fluoxetine on male morphology was assessed using ANCOVA, with post-hoc GLHT evaluation across fluoxetine treatments where appropriate, and with p-values adjusted as above. Standard length, weight and condition index were rank normal transformed, while gonopodium length was cube root transformed, in order to approximate normality. For all models, preliminary ANCOVAs revealed no significant interaction between fluoxetine treatment and chemical exposure system (i.e., males tested for either reproductive behaviour or sperm analysis) (ANCOVA: standard length: $F_{2,577} = 0.52$, $p = 0.594$; weight: $F_{2,577} = 0.79$, $p = 0.457$; condition index: $F_{2,577} = 0.37$, $p = 0.693$; gonopodium length: $F_{2,577} = 0.25$, $p = 0.776$). Morphological measurements from males across reproductive behaviour and sperm analysis experiments were therefore pooled within treatment levels ($n = 583$).

3. Results

3.1. Chemical analyses

During the first flow-through exposure—comprising males to be tested for reproductive behaviour—mean measured exposure concentrations in the low- and high-fluoxetine treatments were 41.68 ng/L (SD = 25.87, $n = 20$) and 478.50 ng/L (SD = 121.71, $n = 20$), respectively. Mean exposure concentrations within the second flow-through system—comprising males to be examined for sperm traits—were 29.51 ng/L (SD = 6.22, $n = 20$) and 379.50 ng/L (SD = 69.01, $n = 20$) in the low- and high-fluoxetine treatments, respectively. Fluoxetine concentrations measured within both flow-through exposures are environmentally realistic, with each of the low concentrations falling within the range of levels detected in surface waters (e.g., Fernández et al., 2010; Gardner et al., 2012; Hughes et al., 2013), while each of the high concentrations are within the range of levels measured in receiving waters (Benotti and Brownawell, 2007; Lara-Martín et al., 2015). The observed variation in measured exposure concentrations from the nominal levels for each of the low- and high-fluoxetine treatments—40 and 400 ng/L, respectively—is likely explained by the scale and ecological realism of the flow-through systems used, with numerous adult fish being exposed simultaneously in large aquaria containing a gravel substrate and stones for refuge. While these factors likely contributed somewhat to the observed variability in exposure concentrations, they were utilised to more closely reflect environmental conditions.

3.2. Male reproductive behaviour

Fluoxetine impacted the number of copulation attempts performed by male mosquitofish. In the absence of a competitor, high-fluoxetine males attempted to mate with females more often than did control (i.e., unexposed) males ($z = 2.02$, $p = 0.043$; Fig. 1). Specifically, high-fluoxetine males performed an average of 3.22 [1.81, 5.75] (where values in brackets indicate one standard error below, and one standard error above the mean, respectively) times the number of copulation attempts performed by control males. No significant differences were detected in the number of copulation events performed by control and low-fluoxetine males ($z = 1.07$, $p = 0.284$), nor by low- and high-fluoxetine males ($z = 1.14$, $p = 0.255$). More generally, male condition index was positively associated with the number of copulation attempts performed ($z = 2.22$, $p = 0.026$), with a one standard deviation (i.e., 0.012) increase in condition index resulting in, on average, 1.80 [1.38, 2.35] times as many copulations. A non-significant positive trend was also detected between the number of copulation attempts performed by males and female standard length ($z = 1.70$, $p = 0.089$).

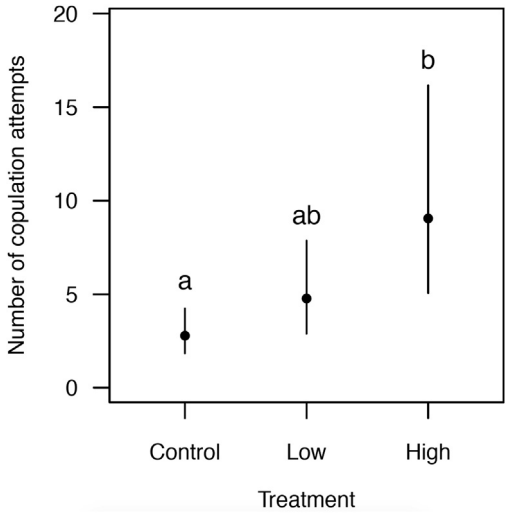

Fig. 1. Mean (±SE) number of copulation attempts performed by control (0 ng/L; $n = 33$), low-fluoxetine (42 ng/L, $n = 39$) and high-fluoxetine (479 ng/L, $n = 37$) males towards a stimulus (i.e., unexposed) female in the absence of a competitor male.

The amount of time males spent following females was not affected by treatment ($F_{2,103} = 0.94$, $p = 0.395$).

Under male-male competition, no significant effect of fluoxetine was detected on the total number of copulation attempts performed by rival males (all $p > 0.05$; Fig. 2). The combined number of copulation attempts performed by competing males was not significantly affected by the absolute difference in their condition index, nor standard length ($z = -0.10$, $p = 0.920$ and $z = 0.24$, $p = 0.813$, respectively). Longer females attracted more copulation attempts ($z = 2.49$, $p = 0.013$), with males together performing an average of 1.36 [1.20, 1.54] times as many attempts per standard deviation (i.e., 1.91 mm) increase in female length. Similar to the results of the non-competitive mating trials, no impact of treatment was detected on the combined amount of time males spent following females ($F_{2,108} = 1.22$, $p = 0.299$). Further, following time was not significantly affected by the absolute difference in condition index, nor standard length, between rival males ($F_{1,108} = 1.23$,

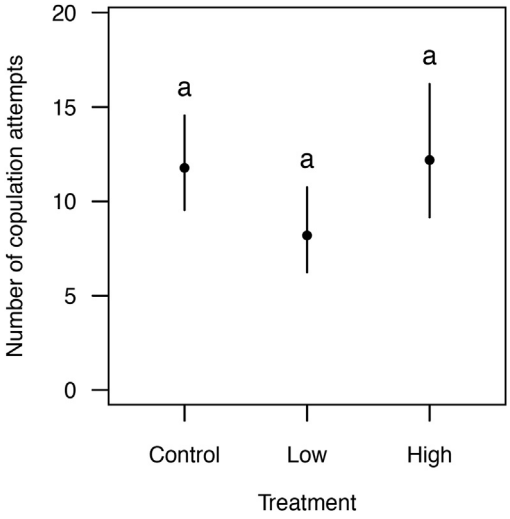

Fig. 2. Mean (±SE) of the combined number of copulation attempts performed by two competing males in the control (0 ng/L; $n = 37$), low-fluoxetine (42 ng/L, $n = 43$) and high-fluoxetine (479 ng/L, $n = 35$) treatments, towards a stimulus (i.e., unexposed) female.

$p = 0.270$ and $F_{1,108} = 2.34$, $p = 0.129$, respectively). Following time was, however, affected by female standard length, with males spending more time following longer females ($F_{1,108} = 13.80$, $p < 0.001$).

3.3. Sperm traits

Fluoxetine significantly affected sperm count ($F_{2,85} = 8.61$, $p < 0.001$), with males from both the low- and high-fluoxetine treatments having higher sperm counts than control fish (Fig. 3). For a given condition and standard length, low-fluoxetine males were predicted to have 1.45 [1.32, 1.59] times as many sperm as control males ($z = 3.98$, $p < 0.001$). High-fluoxetine males were predicted to have 1.31 [1.19, 1.44] times as many sperm as control males with similar condition and length ($z = 2.77$, $p = 0.016$). Standard length was positively associated with sperm count ($t = 3.74$, $p < 0.001$; Fig. S1), with a one standard deviation (i.e., 1.42 mm) increase in standard length corresponding to 1.14 [1.10, 1.18] times as many sperm.

Fluoxetine did not significantly impact any CASA parameters or sperm viability (all $p > 0.05$; electronic supplementary material, Table S3). Further, no measures of sperm quality were influenced by male standard length, condition index or weight (all $p > 0.05$).

3.4. Morphological analysis

Male standard length, weight and gonopodium length were not significantly affected by fluoxetine ($F_{2,579} = 1.04$, $p = 0.355$, $F_{2,579} = 2.93$, $p = 0.054$ and $F_{2,579} = 0.81$, $p = 0.447$, respectively). However, fluoxetine did impact condition index ($F_{2,579} = 5.16$, $p = 0.006$; Fig. 4). Specifically, low-fluoxetine males showed a significant reduction in condition index compared to control males ($t = -3.18$, $p = 0.004$). There was, however, no difference in condition index between males in low-fluoxetine and high-fluoxetine treatments ($t = -1.92$, $p = 0.135$), or between males in high-fluoxetine and control treatments ($t = -1.25$, $p = 0.423$, respectively).

4. Discussion

We found that exposure to environmentally realistic levels of fluoxetine can alter sexually selected traits and behaviours in male mosquitofish. Fluoxetine influenced mechanisms of pre-copulatory sexual selection through changed male mating behaviour, although these effects were dependent on both exposure concentration and the absence or presence of a competitor. Further, fluoxetine influenced mechanisms of post-copulatory sexual selection through increased sperm counts, while sperm quality was unaffected. Finally, exposure to fluoxetine resulted in reduced body condition in males from the low treatment.

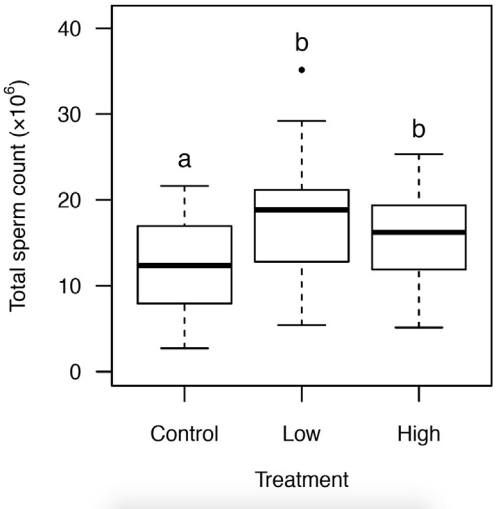

Fig. 3. Total sperm count ($\times 10^6$) of control (0 ng/L; $n = 32$), low-fluoxetine (30 ng/L, $n = 29$) and high-fluoxetine (380 ng/L, $n = 29$) males.

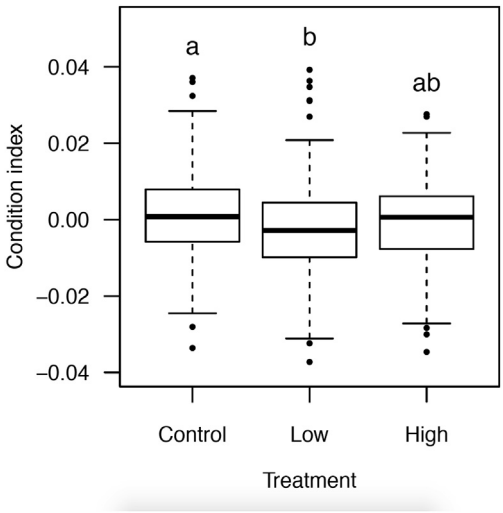

Fig. 4. Condition index of control ($n = 184$), low-fluoxetine ($n = 209$) and high-fluoxetine ($n = 190$) males. Males within each treatment were pooled across chemical exposures.

4.1. Pre-copulatory sexual selection: male reproductive behaviour

Fluoxetine affected the number of copulation attempts performed by males in the absence of a competitor. Specifically, while low-fluoxetine exposure (42 ng/L) did not significantly alter the number of mating attempts performed, high-fluoxetine (479 ng/L) males performed a greater number of attempts than males in the control treatment. To date, this is the lowest measured concentration of waterborne fluoxetine shown to alter reproductive behaviour in fish. This increase in copulatory behaviour in high-fluoxetine males can be expected to result in enhanced reproductive fitness as higher mating effort (i.e., number of mating attempts made) by eastern mosquitofish males has been shown to increase the likelihood of successful insemination (Evans et al., 2003). Further, mating effort is likely to be a strong predictor of actual reproductive success, with Deaton (2008) reporting that the number of mating attempts performed by male western mosquitofish explained approximately 67% of the variation in the proportion of offspring sired. However, the increased frequency of male sexual acts seen in high-fluoxetine males is expected to increase sexual conflict, as male sexual harassment is known to interfere with female foraging efficiency in eastern mosquitofish (Pilastro et al., 2003). Therefore, high-fluoxetine males may also experience reduced reproductive success if females employ strategies to minimise the costs of male sexual harassment, including, for example, by preferentially associating with males performing less frequent copulatory attempts (Pilastro et al., 2003).

Through what pathways may fluoxetine be altering male mating behaviour in fish? Although the effects of SSRIs—including fluoxetine—on reproductive behaviour in fish are not yet wholly understood (reviewed in Fent et al., 2006; Prasad et al., 2015), it is established that serotonin has an important role in modulating

various reproductive functions in fish and does so via multiple pathways, at both central (i.e., preoptic-hypothalamic area and pituitary) and peripheral (i.e., gonads) levels (Prasad et al., 2015; Dorelle et al., 2017). One key pathway by which SSRIs can alter reproductive behaviour in fish is by influencing the hypothalamus-pituitary-gonadal (HPG) and/or hypothalamus-pituitary-interrenal (HPI) axes (reviewed in Kreke and Dietrich, 2008), including by affecting the secretion of gonadotropin-releasing hormone (GnRH) and luteinising hormone (LH) from the hypothalamus and pituitary, respectively (Yaron and Sivan, 2006; Kreke and Dietrich, 2008). Further, SSRIs may disrupt reproductive function in fish by influencing the production of androgens, which are known to regulate sexual behaviours in fish (Borg, 1994; Munakata and Kobayashi, 2010) and are influenced by fluoxetine treatment (Mennigen et al., 2010, 2011; Fernandes et al., 2011). To date, few studies have tested the effects of environmentally realistic concentrations of fluoxetine on reproductive behaviour. However, consistent with the present findings, research on fathead minnows has reported increased reproductive behaviour (i.e., nest maintenance) in fluoxetine-exposed fish (at 1 μg/L and 100 μg/L; Weinberger and Klaper, 2014), with no effect being observed at lower levels (2.5 ng/L and 28 ng/L, Schultz et al., 2011; 100 ng/L, Weinberger and Klaper, 2014). Further, as was also seen in the present study, fluoxetine exposure (0.1 μg/L and 1 μg/L) did not affect the amount of following behaviour performed by male fathead minnows towards females (Weinberger and Klaper, 2014). In contrast, Siamese fighting fish exposed to fluoxetine were found to decrease male territorial defence during parental care (540 ng/L; Forsatkar et al., 2014). Overall, the differential sensitivities of reproductive behaviours between these studies may be due to differences in the reproductive behaviours tested (e.g., territorial defence during parental care and mating behaviours), dissimilar methods of reproduction (e.g., internal versus external fertilisation), incompatible exposure dosages and durations, or, perhaps, interspecific differences in fluoxetine sensitivity (e.g., differences in fluoxetine metabolism or bioavailability) (Gust et al., 2009).

No effect of fluoxetine was detected on the total number of copulation attempts performed by males under male-male competition, at either dosage (42 ng/L or 479 ng/L). Copulating multiple times with individual females is a means by which males can adjust their sperm allocation in a competitive mating situation (Parker, 1998). Indeed, male mosquitofish are known to adjust their mating effort based on perceived sperm competition risk and should generally perform higher levels of mating activity with increasing competition (Evans et al., 2003). This result, therefore, indicates that males exposed to fluoxetine were able to appropriately adjust their reproductive behaviour in the presence of a rival. Considering the seemingly limited scope of male eastern mosquitofish to adjust the size of individual ejaculates between copulations (Evans et al., 2003), fluoxetine exposure at the dosages tested is also not expected to alter the amount of sperm transferred to the female by competing males. To date, the effects of environmentally realistic concentrations of fluoxetine on male mating behaviour under male-male competition have been examined in only one other study. Concordant with our findings, Dzieweczynski and Hebert (2012) reported that 3-day exposure of male Siamese fighting fish to fluoxetine (540 ng/L) did not affect the amount of time spent by males performing female-directed courtship behaviour when encountering models of a male and female conspecific simultaneously. However, fluoxetine exposure also decreased male-directed aggressive behaviours in Siamese fighting fish, which was not the case in mosquitofish, with very few overtly aggressive interactions (e.g., fin nips) observed in our study, regardless of treatment.

4.2. Post-copulatory sexual selection: sperm traits

Exposed males from both low- and high-fluoxetine treatments (30 ng/L and 380 ng/L, respectively) had higher average sperm counts than control males. Sperm number is the strongest predictor of the outcome of sperm competition in poeciliids (Boschetto et al., 2011) and, as aforementioned, male mosquitofish likely have minimal scope for adjusting the size of ejaculates between copulations (Evans et al., 2003). However, males with larger sperm reserves may be able to increase their number of sperm allocations (over multiple copulations) and, thereby, fertilise a greater number of females (O'Dea et al., 2014). While our study is the first to test the effects of environmentally realistic levels of fluoxetine on total sperm count in an aquatic organism, previous studies have, for example, demonstrated that exposure to fluoxetine can reduce basal milt volume (54 μg/L) and decrease pheromone-stimulated milt volume in goldfish (540 ng/L and 54 μg/L; Mennigen et al., 2010), as well as decrease spermatozoan density in zebra mussels (20 ng/L and 200 ng/L; Lazzara et al., 2012). However, exposure to lower levels of fluoxetine did not significantly impact spermatogenesis in fathead minnows (2.5 ng/L and 28 ng/L; Schultz et al., 2011). Nevertheless, in fiddler crabs (*Uca pugilator*), administration of fluoxetine at pharmacological levels can stimulate testicular development (Sarojini et al., 1993).

The divergence in observed impacts of fluoxetine on sperm production and performance may be due to contrasting serotonergic regulation of reproductive processes in different species. For example, in female fish, administration of serotonin prevents steroid-induced maturation of oocytes in mummichog (*Fundulus heteroclitus*, Cerdà et al., 1998) but induces oocyte maturation in Japanese medaka (Iwamatsu et al., 1993). These alterations are predicted to reduce fecundity, and increase reproductive output, respectively. In the present study, increased sperm production resulting from fluoxetine exposure is likely driven by changes to androgen signalling within the HPG axis, as androgens—namely 11-ketotestosterone—are responsible for the induction of spermatogenesis in mosquitofish (Edwards et al., 2013). However, the specific mechanisms by which fluoxetine increases sperm counts require further investigation. More generally, across all treatments, standard length was positively associated with sperm count, as is an established relationship in mosquitofish (O'Dea et al., 2014).

This is the first study to have tested the effects of fluoxetine at environmentally relevant levels on sperm quality in an aquatic organism, and found no significant effect of exposure. This result contrasts with studies on rodents, where fluoxetine administration has been associated with impaired sperm motility (Batatineh and Daradka, 2007; Alzahrani, 2012). Further, although it has been suggested that fluoxetine exhibits spermicidal properties (Kumar et al., 2006; Alzahrani, 2012), these toxic effects may only be seen in response to exposure at higher dosages and were, therefore, not presently observed.

4.3. Morphology

Low-fluoxetine males showed a reduction in condition index, while the body condition of high-fluoxetine males did not differ significantly from the control. This represents a non-monotonic dose-response relationship, a phenomenon commonly reported in fluoxetine exposures (e.g., Guler and Ford, 2010; Bossus et al., 2014; Martin et al., 2017), as well as in pharmaceutical exposures more generally (reviewed in Vandenberg et al., 2012; Fong and Ford, 2014; Wilkinson et al., 2016). Reduced body condition in response to fluoxetine exposure has also been reported in various fish species, including convict cichlids (*Amatitlania nigrofasciata*, Latifi et al., 2015), goldfish (Mennigen et al., 2009) and hybrid

striped bass (*Morone saxatilis* × *M. chrysops*, Gaworecki and Klaine, 2008), albeit at higher concentrations than those used in the present study. Reduced body condition may be explained by a reduction in food intake, an effect previously observed in fluoxetine-exposed fish (Gaworecki and Klaine, 2008; Mennigen et al., 2009; Weinberger and Klaper, 2014). This suppression of appetite may be driven by fluoxetine-induced neuroendocrine disruption of the hypothalamic-pituitary-adrenal axis or through direct action on liver metabolism (Mennigen et al., 2009).

5. Conclusion

Here, we report that 30-day exposure to environmentally realistic concentrations of the pervasive pharmaceutical contaminant fluoxetine altered mechanisms of both pre- and post-copulatory sexual selection in mosquitofish. Further research is needed to better understand the governing mechanisms underpinning these effects as well as the potential for fluoxetine at environmentally realistic exposure concentrations to influence pre- and post-copulatory reproductive processes in other species. Taken together, the present findings highlight the complex and ecologically important effects of psychotherapeutic drugs on aquatic organisms and emphasise the need for continued investigation into their potential sub-lethal impacts.

Ethics

This research was approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2015/02) and complied with all relevant State and Federal laws of Australia.

Authors' contributions

M.G.B., T.E.E., B.B.M.W. and M.S. conceived and designed the experiments, which M.G.B., T.E.E. and J.M.M. conducted. M.G.B., T.E.E. and J.B.B. carried out statistical analysis. M.K.O.B. coordinated all sperm analysis, which M.G.B., T.E.E. and J.M.M. performed. M.G.B. and T.E.E. drafted the manuscript. All authors contributed to manuscript preparation and gave final approval for publication.

Conflicts of interest

The authors declare that we 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.2018.03.006.

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