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The pharmaceutical pollutant fluoxetine alters reproductive behaviour in a fish independent of predation risk

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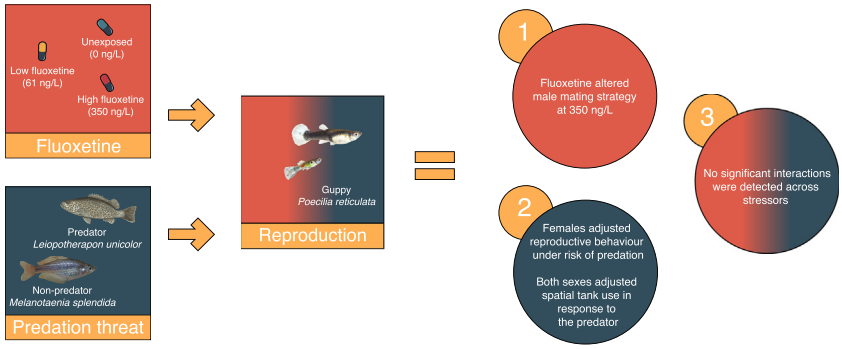
HIGHLIGHTS

- Pharmaceutical pollution represents a major global threat to wildlife and ecosystems.
- Guppies (*P. reticulata*) were exposed to fluoxetine at two field-realistic levels.
- Male and female guppy reproductive behaviour was assessed under predation risk.
- High fluoxetine (350 ng/L) increased male coercive mating behaviour, independent of a predatory threat.
- Highlights importance of considering interactions between natural stressors and pharmaceutical pollutants.

GRAPHICAL ABSTRACT



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ABSTRACT

Pharmaceutical pollutants constitute a major threat to wildlife because of their capacity to induce biological effects at low doses. One such pollutant is the antidepressant fluoxetine, which has been detected in surface waters globally at levels that recent studies suggest can alter physiology and behaviour in aquatic organisms. However, wildlife exposed to pharmaceutical contaminants are typically confronted with multiple stressors simultaneously, including predation risk, which is a particularly important natural stressor that can have direct (e.g. mortality) and indirect (e.g. changed prey behaviour) fitness effects. Accordingly, we investigated potential impacts of environmentally realistic fluoxetine exposure on reproductive behaviour in the guppy (*Poecilia reticulata*) under predation risk. Specifically, we tested whether fluoxetine exposure altered mating behaviour in male and female guppies in the presence of either a predatory spangled perch (*Leiopotherapon unicolor*) or a non-predatory rainbowfish (*Melanotaenia splendida*) control. We found that fluoxetine and the presence of a predatory spangled perch did not interact to affect reproductive behaviour. We also found that, independent of a predatory threat, fluoxetine exposure altered male mating strategy, with males in the high treatment conducting significantly more coercive 'sneak' copulations, whereas the number of courtship displays performed was not significantly affected. Moreover, while fluoxetine exposure did not significantly affect the amount of time that males and females spent following one another, we found that females, but not males, followed a potential partner less when in the presence of the predatory fish. Finally, both sexes reacted to the risk of predation by spending less time in close proximity to a predator than a non-predator. In combination, our findings highlight the capacity of fluoxetine to influence processes of sexual selection at field-realistic concentrations and emphasise the importance of considering multiple stressors when assessing impacts of pharmaceutical pollutants on the behaviour of wildlife.

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1. Introduction

Pharmaceutical pollution represents a major global threat to humans and wildlife (Arnold et al., 2014; Bernhardt et al., 2017; Boxall et al., 2012; Saaristo et al., 2018). Indeed, in excess of 600 different pharmaceutical contaminants (or their transformation products) have now been detected in the environment across >71 countries spanning all continents (Aus der Beek et al., 2016; Küster and Adler, 2014). In this regard, selective serotonin reuptake inhibitors (SSRIs), a widely prescribed class of antidepressants, are among the most commonly detected pharmaceutical pollutants in the environment (Silva et al., 2012). Acting by limiting reabsorption of the neurotransmitter serotonin into the pre-synaptic nerve cell, SSRIs elevate levels of extracellular serotonin in the synaptic cleft, leading to increased activation of post-synaptic receptors (Stahl, 1998). Serotonin is ubiquitous in all animal phyla possessing nervous systems and is known to play a key role in regulating a range of physiological and behavioural processes (Fent et al., 2006; Weiger, 1997).

One SSRI of particular environmental concern is fluoxetine, which is among the most commonly prescribed antidepressants in the world (Brijnath et al., 2017; Wong et al., 2005). Fluoxetine enters and remains in the environment as a result of excretion by human patients and insufficient removal during wastewater treatment processes (Arnold et al., 2014; Mennigen et al., 2011), with many countries worldwide not presently having regulatory frameworks in place for restricting the discharge of, or monitoring, fluoxetine in drinking water and wastewater flow (e.g. Australia: Department of Agriculture and Water Resources, 2016; European Union: The Council of the European Communities, 2018; New Zealand: Ministry of Health, 2018; United States of America: Environmental Protection Agency, 2016). In this regard, fluoxetine has been detected in surface waters globally, at concentrations typically ranging from <1–100 ng/L (e.g. Batt et al., 2015; Birch et al., 2015; Hughes et al., 2013; Kolpin et al., 2002; Meador et al., 2016; Paíga et al., 2016; Wu et al., 2017), and up to 596 ng/L in systems receiving wastewater discharge (Benotti and Brownawell, 2007). Moreover, levels as high as 929 ng/L have been reported in direct effluent flow (Bueno et al., 2007; Metcalfe et al., 2010).

While levels of fluoxetine found in the environment are not sufficient to induce lethal effects (e.g. 2.89 mg/L LC_{50} for juvenile topmouth gudgeon, *Pseudorasbora parva*: Chen et al., 2018; 198 μg/L LC_{50} for fathead minnow, *Pimephales promelas*: Stanley et al., 2007), many recent studies have found that fluoxetine exposure at close to, and at, environmental concentrations can alter a range of ecologically important traits in non-target species. Reported effects include altered development (Japanese medaka, *Oryzias latipes*: Foran et al., 2004; Northern Leopard Frog, *Rana pipiens*: Foster et al., 2010; western mosquitofish, *Gambusia affinis*: Henry and Black, 2008), growth (guppy, *Poecilia reticulata*: Pelli and Connaughton, 2015; California mussel, *Mytilus californianus*: Peters and Granek, 2016) and survival (guppy: Pelli and Connaughton, 2015). Fluoxetine exposure has also been linked to alterations in various key fitness-related behaviours, including feeding rate (fathead minnow: Weinberger and Klaper, 2014), sociability (Japanese medaka: Ansai et al., 2016; Arabian killifish, *Aphanius dispar*: Barry, 2013), aggression (Arabian killifish: Barry, 2013; Siamese fighting fish, *Betta splendens*: Dzieweczynski and Hebert, 2012), phototaxis (an amphipod, *Echinogammarus marinus*: Guler and Ford, 2010; water flea, *Daphnia magna*: Rivetti et al., 2016), boldness (Siamese fighting fish: Dzieweczynski et al., 2016a, 2016b) and activity (Arabian killifish: Barry, 2013; an amphipod, *Gammarus pulex*: De Lange et al., 2006; Siamese fighting fish: Kohlert et al., 2012), as well as learning and memory retention (common cuttlefish, *Sepia officinalis*: Di Poi et al., 2013). To date, however, investigations of behavioural shifts caused by fluoxetine have focussed on testing effects of exposure independently from other stressors typically found in the environment—as is also true for pharmaceutical pollutants more generally. In nature, however, complex interactions between multiple stressors are likely to be the norm rather than the exception (Blaustein and Kiesecker, 2002; Slocum and Mendelssohn, 2008). Moreover, of the studies that have considered such interactive effects, most have focussed on other abiotic factors (e.g. mixture effects with other pharmaceuticals, see De Castro-Català et al., 2017; Painter et al., 2009), with surprisingly few having examined potential effects of pharmaceutical pollutants in combination with biotic stressors.

Predation is a ubiquitous biotic stressor that can impact fitness directly via mortality or indirectly by producing changes in prey morphology, life-history and/or behaviour (Creel and Christianson, 2008; Sih et al., 1985). Previous studies have shown that fluoxetine can alter behavioural responses of fish to visual (e.g. Martin et al., 2017; Pelli and Connaughton, 2015; Saaristo et al., 2017) and chemical (e.g. Barry, 2014) predator cues. However, to date, potential interactive effects of fluoxetine exposure and predation risk on reproductive behaviours have not been investigated. Such behaviours include conspicuous mating displays, which often communicate an individual's phenotypic and genetic quality, such as health, ability to sire young, and quality of parental care (Barber et al., 2001; Hoikkala et al., 1998; Lindström et al., 2006; Sargent, 1982). However, conspicuous sexual displays can also be costly, as they often elevate an individual's vulnerability to predators by increasing detectability and rate of predator-prey encounters (Hoefler et al., 2008; reviewed in Lima and Dill, 1990), and by limiting escape potential from would-be predators (Cooper, 1999; Killian et al., 2006). In light of such costs, individuals often adjust their reproductive behaviour according to perceived predation risk (Sih, 1994). For example, to minimise the likelihood of detection, male cross-banded tree frogs (*Smilisca sila*) reduce their calling rate—a behaviour used to attract females—when in the presence of a predator (Tuttle and Ryan, 1982). Therefore, it is important to consider potential interactions between pharmaceutical pollutant exposure and predation risk on reproductive behaviour in wildlife (reviewed in Saaristo et al., 2018).

The guppy (*Poecilia reticulata*) is a small, internally fertilising poeciliid native to north-eastern South America (Rosen and Bailey, 1963) that is now found in over 69 countries around the world (Deacon et al., 2011). Guppies inhabit freshwater habitats, many of which are exposed to wastewater contaminants (Araújo et al., 2009; reviewed in Magurran, 2005), such as fluoxetine (Hughes et al., 2013). Guppies have also been the focus of extensive behavioural research examining mating tactics under predation risk (reviewed in Houde, 1997), which, in combination with their presence in polluted environments, makes them an ideal model for investigating potential effects of fluoxetine contamination and predation risk on reproductive behaviour. Indeed, guppies have recently received increasing attention as a model species in behavioural ecotoxicology (Bertram et al., 2015; Holmberg et al., 2011; Pelli and Connaughton, 2015; Saaristo et al., 2017; Tomkins et al., 2017). Male guppies engage in two alternative mating strategies, either soliciting copulations from females by performing elaborate courtship displays or engaging in surreptitious 'sneak' copulations without first courting the female (Houde, 1997). When under threat of predation, males typically favour sneaking behaviour as the conspicuous nature of courtship displays increases the likelihood of detection by predators (Endler, 1987). Moreover, sneak copulations circumvent some of the energetic costs associated with courtship displays, although sneaking also carries a relatively low probability of successful insemination, with approximately one third as many sperm being transferred during sneak copulations compared to copulations following courtship (Matthews and Magurran, 2000; Pilastro and Bisazza, 1999; Pilastro et al., 2007). Given these trade-offs, males should favour sneaking in situations where courtship displays are less effective or are relatively costly, such as in environments with high predation risk (reviewed in Houde, 1997).

Here, we examined impacts of short-term (28-day) exposure to two environmentally relevant levels of fluoxetine—nominal low and high concentrations of 40 and 400 ng/L, respectively—on male and female guppy reproductive behaviour in the presence or absence of a predatory

threat. We tested for individual effects of fluoxetine exposure and predation risk, as well as their potential interactive effects, on reproductive behaviour. Given that fluoxetine exposure at environmentally realistic levels has been shown to reduce various antipredator behaviours (e.g. Martin et al., 2017; Pelli and Connaughton, 2015), we predicted that, when subjected to an increase in perceived predation risk, exposed individuals would adjust their mating behaviour to a lesser extent than controls.

2. Materials and methods

2.1. Animal collection and housing

Guppies used in this study were laboratory-reared descendants of a wild population from Alligator Creek (19° 26′ 18″ S, 146° 57′ 01″ E), which is a rainforest-fed stream located within Bowling Green Bay National Park in Queensland, Australia (collection permit: WITK07655010). Analysis of water samples from the collection site indicated no contamination with fluoxetine (Envirolab Services, unpublished data; see Section 2.3 Chemical exposure and analyses for details of water testing). Sexually mature male and female guppies were acclimated to laboratory conditions (24 °C; 12:12 h light:dark cycle) for 5 weeks in mixed-sex holding tanks (54 L, 60 cm length × 30 cm width × 30 cm height). Guppies were fed a daily diet of commercially prepared fish pellets (Otohime Hirame larval diet; 580–910 μm).

The spangled perch (*Leiopotherapon unicolor*) and rainbowfish (*Melanotaenia splendida*) used in behavioural trials (see Section 2.2 Experimental design for details) were wild-caught specimens purchased from commercial suppliers (Australian Native Fish Enterprises in Sydney and AquaGreen in Darwin, respectively). These stimulus fish were housed for 6 weeks prior to the start of experiments, under the same laboratory conditions described above, in species-specific holding tanks (54 L, 60 cm × 30 cm × 30 cm, 5 per tank). Spangled perch and rainbowfish were fed daily with chironomid larvae (Hikari frozen bloodworms).

2.2. Experimental design

To investigate potential effects of fluoxetine exposure on male and female guppy reproductive behaviour under predation risk, a 3 × 2 factorial design was used, with 3 fluoxetine treatments (unexposed, low-fluoxetine or high-fluoxetine) and 2 levels of perceived predation risk (a predator or non-predator stimulus fish).

Stimulus fish used in behavioural trials were either predatory spangled perch (total length: mean = 7.41 cm, SD = 0.61 cm, range = 6.56–8.34 cm, $n = 7$) or non-predatory rainbowfish (total length: mean = 6.33 cm, SD = 0.91 cm, range = 5.03–7.74 cm, $n = 10$). The spangled perch is an aggressive and opportunistic omnivore, which is known to prey on crustaceans and small fish, including guppies (Davis et al., 2011). The rainbowfish, by contrast, feeds exclusively on invertebrates and plant material (Davis et al., 2011). Importantly, both species are known to co-occur with guppies from the Alligator Creek source population (Allen et al., 2002). A non-predator control treatment was included to ensure that behavioural changes caused by the predator treatment (if any) were, in fact, due to a perceived threat of predation, rather than simply the presence of a stimulus fish per se (Michelangeli and Wong, 2014). We used unexposed spangled perch and rainbowfish to exclude the possibility that fluoxetine-induced effects on guppy behaviour (if any) could have been mediated by effects on the stimulus fish, a technique employed in previous ecotoxicological experiments (e.g. Bertram et al., 2018; Tomkins et al., 2017, 2018).

2.3. Chemical exposure and analyses

Male and female guppies in this experiment were randomly allocated to one of three fluoxetine treatments—unexposed (i.e. fresh water only), low fluoxetine or high fluoxetine—and exposed using established protocols (Bertram et al., 2018; Martin et al., 2017; Saaristo et al., 2017), with some modifications (see below). Guppies were subjected to a 28-day exposure period as previous studies suggest that fluoxetine can take 2–4 weeks to exhibit its full therapeutic effects in humans (Gardier et al., 1996; Matuszcyk et al., 1998), and because recent research indicates that similar exposure periods (i.e. 21–35 days) can alter a wide range of behaviours in fish (e.g. Bertram et al., 2018; Martin et al., 2017; McCallum et al., 2017; Pelli and Connaughton, 2015; Saaristo et al., 2017). Flow-through systems were used to expose guppies from each of the three fluoxetine treatments, with two identical systems being used per treatment. Each system was comprised of a large mixing tank (81 L, 60 cm × 45 cm × 30 cm) which fed two identical sex-specific exposure tanks (54 L, 60 cm × 30 cm × 30 cm), containing 40 fish each. Exposure aquaria were equipped with 2 cm of natural gravel substrate, a large stone for refuge, a heater and an airstone. All aquaria were maintained under a 12:12 h light:dark cycle and monitored daily for temperature (mean = 24.4 °C, SD = 0.5 °C, $n = 336$), as well as being maintained at a flow-through rate of ~1.67 L/h per tank (i.e. water in each exposure tank was fully cycled once per day).

To achieve the nominal low- and high-fluoxetine treatment concentrations—40 and 400 ng/L, respectively—stock solutions were prepared daily. This involved firstly dissolving fluoxetine hydrochloride (Sigma Aldrich; Product Number: F132, CAS: 56296-78-7) in methanol (low: 18 μg/mL, high: 180 μg/mL). Then, for each exposure system, a 1 mL aliquot of this solution was evaporated to dryness under a gentle nitrogen gas stream, before being diluted to a 3 L stock solution using reverse osmosis water. Unexposed stock solutions contained 3 L of reverse osmosis water only. For each exposure system, stock solutions were continuously fed into each of the mixing tanks (1.95 mL/min) using a peristaltic pump (Watson Marlow 323 U/MC).

Fluoxetine concentrations were measured weekly in all exposure tanks within the low and high treatments, as well as in half of the unexposed aquaria to ensure the absence of contamination. This involved water samples (40 mL) being drawn from each tank using a serological pipette (Macroman, Gilson), which were then stored at 4 °C in amber glass bottles, and analysed within 4 days of collection. Samples were analysed by Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025), where 39 mL of each sample was acidified to pH 6, with 20 μL of 1 μg/mL norfluoxetine (CAS: 56161-73-0; Novachem, Germany) in methanol being added to each sample to serve as a surrogate standard. The fluoxetine and norfluoxetine surrogate were then eluted using dichloromethane: isopropanol:ammonium hydroxide (78:20:2, *v*/*v*/*v*; 3 mL) and evaporated to dryness under a gentle stream of argon. The fluoxetine was reconstituted in 100 μL of ethyl acetate, and 50 μL of heptafluorobutyric anhydride derivatising agent (United Chemical Technologies, purchased from PM Separations, QLD, Australia) was added and evaporated. The samples were then analysed using gas chromatography-tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware, USA) with a limit of quantification of 2 ng/L. A detailed description of the chemical analysis protocol is provided in Bertram et al. (2018).

The measured average fluoxetine concentrations in the low- and high-fluoxetine treatments were 60.51 ng/L (SD = 21.54 ng/L, $n = 16$) and 349.85 ng/L (SD = 158.64 ng/L, $n = 16$), respectively. Both of these exposure treatments are environmentally realistic, with the lower level falling within the range of concentrations detected in surface waters (e.g. Hughes et al., 2013; Kolpin et al., 2002; Wu et al., 2017), while the higher level is within the upper ranges reported in aquatic systems heavily impacted by wastewater discharge (Benotti and Brownawell, 2007; Lara-Martín et al., 2015). No contamination with fluoxetine was detected in any of the unexposed aquaria across the 28-day exposure ($n = 8$ measurements).

2.4. Behavioural trials

Guppy behaviour was recorded in 54 L observation tanks (60 cm × 30 cm × 30 cm; water depth: 20 cm), which were separated into two compartments (40 cm × 30 cm × 30 cm and 20 cm × 30 cm × 30 cm) using a transparent perforated divider (see electronic supplementary material, Fig. S1 for a schematic diagram of the tank set-up). For each trial, the transparent partition was randomly allocated to the left or right of the observation tank to account for any potential side bias. Fifteen minutes before the trial, a randomly selected stimulus fish (i.e. a predator or non-predator of known identity) was placed into the smaller compartment. In addition to this visual stimulus, chemical cues of the stimulus fish (250 mL of holding-tank water) were added to the larger compartment—where guppies would be located—as guppies can use both visual and chemical cues to detect predators (Bleakley et al., 2006). A 5 cm zone abutting the transparent partition was used to measure the total time male and female guppies spent in the area closest to the stimulus fish. This 5 cm zone represented the spangled perch's striking range (i.e. 'strike zone') and was based on previous literature investigating fast-start performance and strike distance in closely related teleost fish (Domenici and Blake, 1997; Webb, 1978).

Each behavioural trial involved one male and one female guppy being randomly collected from the same exposure treatment (unexposed, low-fluoxetine or high-fluoxetine). This was done because wild guppies that inhabit the same body of water are likely to experience similar levels of fluoxetine contamination, especially due to their strong schooling preference (Houde, 1997). Pairs consisting of one male and one female were used to disentangle effects caused by fluoxetine exposure and predation risk on reproductive behaviour (if any) from additional interacting stressors, such as audience effects (Makowicz et al., 2010) and male-male competition (Jirotkul, 1999). The two guppies were placed in separate opaque containers (300 mL) filled with trial tank water, and floated on the water's surface within the larger trial tank compartment for a 5 min acclimation period. Opaque containers were used to prevent the guppies from interacting with visual and chemical cues of the stimulus fish before the trial. After acclimation, both guppies were simultaneously released into the larger compartment, with their behaviour then being video-recorded for 15 min (Canon PowerShot S120). After each trial, observation tanks were drained and refilled. Each stimulus fish was also returned to its holding tank in preparation for subsequent trials.

Behaviours performed by guppies were quantified from trial videos using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007). Each video was watched four times to measure male and female reproductive behaviour, as well as zone use in either sex. Specifically, to examine possible effects of fluoxetine on male mating strategy, we measured the number of male courtship bouts (i.e. male orienting towards the female and performing stereotyped 'sigmoid' courtship displays before attempting a copulation) and coercive sneak copulation attempts (i.e. male surreptitiously approaching the female from behind to copulate) directed towards females (Houde, 1997). In addition, to test the potential for fluoxetine exposure to alter reproductive interest in guppies under predation risk, we measured the total time spent by males and females actively following their potential mate. Male guppies frequently and persistently follow females in their pursuit of mating opportunities and, in female guppies, actively following potential suitors is a strong predictor of actual mating intent (Houde, 1997). Lastly, we monitored the total time spent by male and female guppies within 5 cm (i.e. the strike zone) of the stimulus fish (Kramer and Bonenfant, 1997). This was also used to determine whether guppies perceived the difference in threat posed by the predatory spangled perch and non-predatory rainbowfish.

2.5. Morphology and colouration analysis

Subsequent to behavioural trials, males ($n = 156$) and females ($n = 156$) were euthanised with an overdose of anaesthetic clove oil (40 mg/L) and dabbed dry, before being measured for standard length (i.e. snout to caudal peduncle; Kincrome digital calipers, ±0.01 mm). The wet weights of males and females were also measured using an electronic balance (Scientech ZSA-210 digital analytical scale, ± 0.0001 g). A sex-specific index of body condition was calculated by plotting weight (g) against standard length (mm) to produce a least-squares regression line. Sex-specific condition index was calculated as the residuals of this regression line (i.e. males: weight = −0.128 + 0.012 × length; females: weight = −0.644 + 0.041 × length). Additionally, the percentage area of orange pigmentation on the body of each male was analysed using photographic colouration analysis, following Bertram et al. (2015). Briefly, this involved photographing each male's right side in a standard position (Nikon D90, shutter speed = 1/250, Nikon AF Micro-Nikkor 60 mm f/2.8D). Photoshop (version 2017.0.1) was then used to isolate the body surface (excluding fins) of each fish and calculate percentage area of orange pigmentation using the colour range tool. Orange colouration was initially included in all models of guppy behaviour as female guppies show a strong preference for males that possess greater orange pigmentation (Houde, 1987), including fish from the population used in the present study (Bertram et al., 2015; Gamble et al., 2003).

2.6. Statistical analysis

Data were analysed in R version 3.2.3 (R Development Core Team, 2015). Where appropriate, response variables were checked for normality (Shapiro-Wilk test, *shapiro.test* function; Royston, 1995) and homogeneity of variance (Bartlett test, *bartlett.test* function; Bartlett, 1937). In analysing behavioural responses, a small suite of biologically meaningful predictors (based on previously established relationships) were initially included in all models, consisting of male standard length (mm), male condition index, male orange pigmentation (%), female standard length (mm) and female condition index. For each of these models, covariates were selected by performing backward stepwise elimination (i.e. covariates were sequentially excluded based on their impact on the Akaike Information Criterion [AIC]; see electronic supplementary material, Table S1 for model summaries). Where appropriate, a rank-normal transformation (*rntransform* function, *GenABEL* package; Aulchenko et al., 2007) was applied to approximate normality of the residuals. All models analysing behavioural responses included stimulus fish ID as a random effect to account for potential variation in guppy behaviour caused by the presence of specific stimulus individuals. Additionally, exposure system ID—as a measure of tank effects—was initially included in all behavioural models as a random effect but did not significantly affect any of the response variables, explaining <1% of the variation in the data, and was, therefore, excluded in each case to increase predictive power. In all behavioural models detailed below, where relevant, third- and second-order interactions between fixed effects were removed using reverse stepwise elimination, leaving only those that were statistically significant (at $\alpha = 0.05$).

To both address zero-inflation and incorporate a random effect, separate zero-inflated generalised linear mixed-effect models (*glmmADMB* function, *glmmADMB* package; Fournier et al., 2012) were used to compare the number of courtship events, as well as the number of sneak attempts, performed by males towards females. The model generated to investigate the number of courtship displays had two fixed predictors (fluoxetine treatment and stimulus fish type), one continuous covariate (female condition index) and one random effect (stimulus fish identity). The model used to examine the number of male sneaking events included two fixed predictors (fluoxetine treatment and stimulus fish type), three continuous covariates (male standard length, male condition index and female condition index) and one random effect

(stimulus fish identity). Zero-inflated models were used, as Vuong tests (*vuong* function, *pscl* package; Vuong, 1989) indicated that the data were zero-inflated in both cases. A negative binomial distribution was selected as the most appropriate family for both models, as it accounted for over-dispersion of the count component (Zuur et al., 2009). For both courting and sneaking behaviours, general linear hypothesis tests (GLHTs; *glht* function, *multcomp* package; Hothorn et al., 2008) were used to compare mean responses across treatment levels.

Two separate linear mixed-effects models (LME; *lme* function, *nlme* package; Pinheiro et al., 2018), one per sex, were used to analyse male and female guppy reproductive interest (i.e. total time males and females spent following each other). Each of these models had two fixed effects (fluoxetine treatment and stimulus fish type), two continuous covariates (female model: male orange pigmentation and female standard length; male model: male standard length and female condition index) and one random effect (stimulus fish identity). Males and females were investigated using separate models because male guppies are known to perform more intense following behaviour than females (Houde, 1997).

A LME was used to test the total time spent by guppies within 5 cm of the stimulus fish and included three fixed effects (fluoxetine treatment, stimulus fish type, and sex), one continuous covariate (standard length) and one random effect (stimulus fish identity). Sex was included as a fixed effect to investigate whether males and females spent different amounts of time within the vicinity of the predator or non-predator.

Finally, impacts of fluoxetine on male and female morphology (i.e. standard length, weight, and condition index in both sexes, as well as male area of orange pigmentation) were assessed using separate sex-specific ANOVA models. For males, weight was square root transformed before analysis, in order to approximate normality of the residuals. Likewise, for females, data for each morphological trait were rank-normal transformed before analysis.

3. Results

3.1. Reproductive behaviour

Regarding the number of courtship displays performed by males towards females, interactions between fluoxetine exposure treatment and stimulus fish type were non-significant (glmmADMB; $p \geq 0.213$) and were removed. Further, number of courtship events was not significantly affected by fluoxetine treatment (glmmADMB; all $p \geq 0.199$; Fig. 1a) or stimulus fish type (glmmADMB; $z = 0.76$, $p = 0.445$; Fig. 2a). However, a non-significant positive trend was detected between female condition index and the number of courtship displays performed by males (glmmADMB; $z = 1.89$, $p = 0.058$).

For the total number of sneak attempts carried out by males towards females, no significant interactions between fluoxetine exposure treatment and stimulus fish type were found (glmmADMB; all $p \geq 0.180$), with the interaction terms therefore being removed. Fluoxetine treatment significantly affected the number of male sneak attempts performed towards females, with high-fluoxetine males engaging more frequently in sneak attempts than unexposed males (glmmADMB; $z = 2.08$, $p = 0.038$; Fig. 1b). However, the number of sneaks did not differ significantly between unexposed and low-fluoxetine males (glmmADMB; $z = 0.96$, $p = 0.339$; Fig. 1b) or low- and high-fluoxetine males (glmmADMB; $z = 0.84$, $p = 0.400$; Fig. 1b). Stimulus fish type did not significantly affect the number of sneak attempts performed by males towards females (glmmADMB; $z = 1.64$, $p = 0.101$; Fig. 2b). Additionally, the number of sneaks performed by males did not associate significantly with male standard length (glmmADMB; $z = 1.08$, $p = 0.281$), male condition index (glmmADMB; $z = 0.74$, $p = 0.457$) or female condition index (glmmADMB; $z = 1.45$, $p = 0.147$).

In terms of the total time spent by males and females following their potential partner, interactions between fluoxetine exposure treatment and stimulus fish type were non-significant (LME, males: $p = 0.343$; females: $p = 0.401$) and were removed from the model. The refitted models indicated no significant main effects of fluoxetine treatment (LME; males: $F = 0.47$, $p = 0.629$, Fig. 3a; females: $F = 0.40$, $p = 0.669$, Fig. 3b). Further, the total time spent by males following females did not differ significantly across stimulus fish type (LME; $F = 0.39$, $p = 0.539$; Fig. 4a). However, females spent significantly less time following males when in the presence of a predatory spangled perch than a non-predatory rainbowfish (LME; $F = 6.52$, $p = 0.022$; Fig. 4b). In addition, while area of orange pigmentation in males had no significant effect on the total time spent by females performing following behaviour (LME; $F = 2.18$, $p = 0.143$), female standard length associated negatively with total time spent following males (LME; $F = 18.51$, $p < 0.001$). Lastly, male standard length and female condition index were not significant indicators of the total time males followed

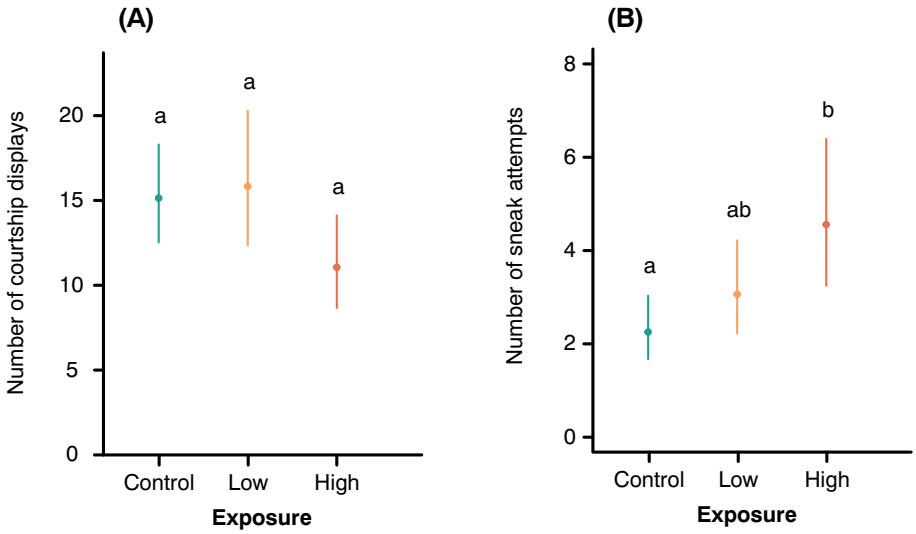

Fig. 1. Mean (±SE) number of (a) courtship displays, and (b) sneak attempts, performed by males towards females when both guppies were subject to either unexposed (0 ng/L, $n = 51$), low-fluoxetine (61 ng/L, $n = 52$) or high-fluoxetine (350 ng/L, $n = 53$) treatment, with continuous predictors being held at their means. Treatments without lower case letters in common are significantly different.

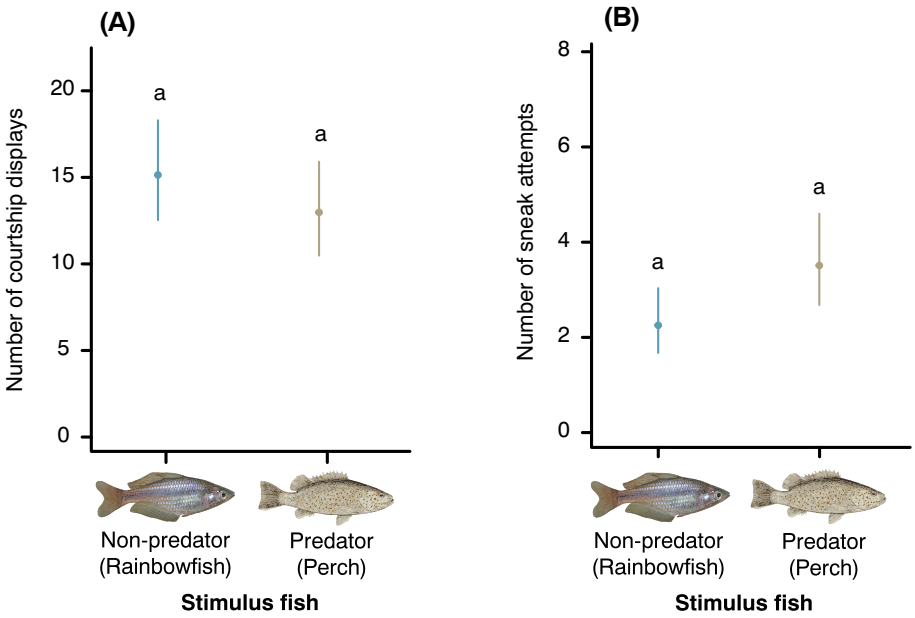

Fig. 2. Mean (±SE) number of (a) courtship displays, and (b) sneak attempts, performed by males towards females when in the presence of a non-predatory ($n = 66$) or predatory ($n = 90$) stimulus fish, with continuous predictors being held at their means. Treatments without lower case letters in common are significantly different.

females (LME; $F = 0.06$, $p = 0.812$ and $F = 2.55$, $p = 0.113$, respectively).

3.2. Predator avoidance behaviour

In analysing the total time spent by males and females within 5 cm of the stimulus fish, no significant interactions were detected between the categorical predictors (i.e. fluoxetine exposure treatment, stimulus fish type, and sex) (LME; three-way interaction, $p = 0.332$; refitted model two-way interactions, $p \geq 0.245$), and were thus removed. Main effects of fluoxetine exposure (LME; $F = 1.13$, $p = 0.324$, Fig. 5a) and sex (LME; $F = 0.01$, $p = 0.921$) were also non-significant. However, guppies spent significantly less time within the 5 cm zone when in the presence of a predatory spangled perch compared to a non-predatory rainbowfish (LME; $F = 8.01$, $p = 0.013$, Fig. 5b). Total time spent within the 5 cm zone was also positively associated with guppy standard length, with larger individuals spending more time in this area (LME; $F = 27.56$, $p < 0.001$).

3.3. Morphology and colouration analysis

Fluoxetine treatment did not significantly impact male standard length (ANOVA; $F_{2,153} = 0.70$, $p = 0.497$), weight (ANOVA; $F_{2,153} = 0.28$, $p = 0.760$) or condition index (ANOVA; $F_{2,153} = 0.60$, $p = 0.550$). Additionally, area of orange pigmentation in males did not differ significantly between fluoxetine treatments (ANOVA; $F_{2,153} = 0.60$, $p = 0.551$). Similarly, in females, fluoxetine exposure levels did not significantly impact standard length (ANOVA; $F_{2,153} = 0.24$, $p = 0.787$), weight (ANOVA; $F_{2,153} = 0.31$, $p = 0.732$) or condition index (ANOVA; $F_{2,153} = 1.69$, $p = 0.188$).

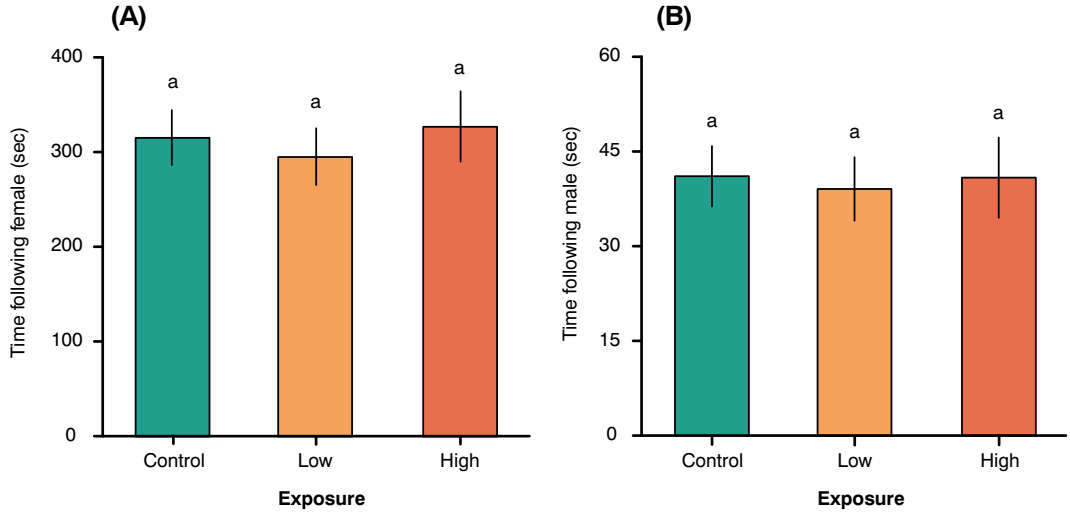

Fig. 3. Mean (±SE) amount of time spent by (a) male, and (b) female, guppies performing following behaviour towards their potential partner, in unexposed (0 ng/L, $n = 51$ per sex), low-fluoxetine (61 ng/L, $n = 52$ per sex) and high-fluoxetine (350 ng/L, $n = 53$ per sex) treatments. Treatments without lower case letters in common are significantly different.

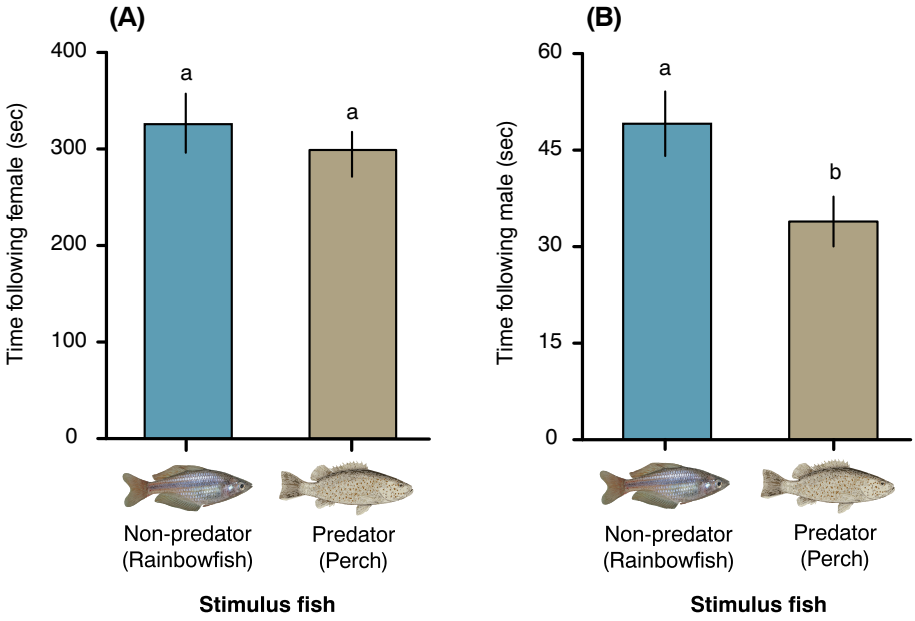

Fig. 4. Mean (±SE) amount of time spent by (a) male, and (b) female, guppies performing following behaviour towards their potential partner when in the presence of a non-predatory ($n = 66$ per sex) or predatory ($n = 90$ per sex) stimulus fish. Treatments without lower case letters in common are significantly different.

4. Discussion

In this study, exposure to an environmentally realistic level of fluoxetine altered the mating strategy of male guppies, which was true independent of the presence of a predator. Specifically, fluoxetine increased male coercive 'sneak' copulations in the high-exposed treatment, relative to unexposed males, while courtship displays and following behaviour were not significantly affected. The total time spent by females following males was also unaffected by fluoxetine treatment. Moreover, fluoxetine exposure did not significantly affect male or female predator avoidance behaviour. This was the case despite guppies in this study demonstrating a capacity to perceive differences in predation risk posed by stimulus predatory spangled perch and non-predatory rainbowfish, with both males and females spending less time in the vicinity of the predatory stimulus, and with females also following males less in the predator treatment.

Fluoxetine exposure affected the frequency of male sneaking behaviour performed towards females, with high-fluoxetine (350 ng/L) males performing a greater number of sneak attempts than males in the unexposed treatment. This shift in the use of male alternative mating strategies towards coercive sneaking behaviour is likely to have implications for male fitness. Specifically, although an increase in sneak copulations could potentially improve male reproductive success due to a general increase in mating attempts, sneaking is associated with reduced insemination efficiency given that successful sneaks deliver approximately one third as many sperm as copulations preceded by courtship

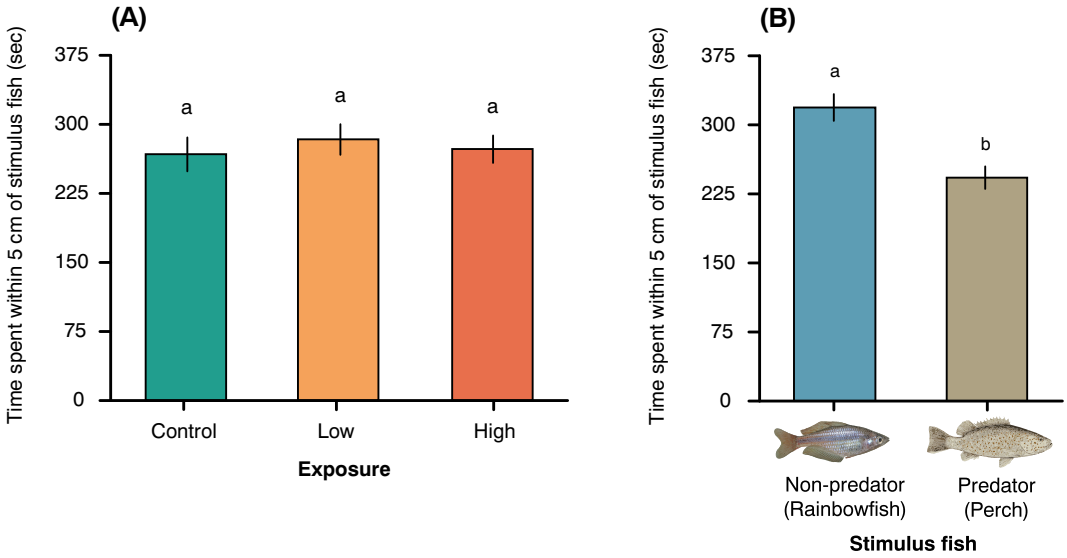

Fig. 5. Mean (±SE) amount of time spent by male and female guppies within 5 cm of the stimulus fish when (a) from the unexposed (0 ng/L, $n = 51$ pairs), low-fluoxetine (61 ng/L, $n = 52$ pairs) and high-fluoxetine (350 ng/L, $n = 53$ pairs) treatments, and (b) in the presence of a non-predatory ($n = 66$ pairs) or predatory ($n = 90$ pairs) stimulus fish. Treatments without lower case letters in common are significantly different.

displays (Pilastro and Bisazza, 1999). Females may also avoid males performing excessive sneak copulations (i.e. harassment) (Houde, 1997; Magurran and Seghers, 1994), thereby disadvantaging fluoxetine-exposed males. In addition, increased male sexual harassment could have indirect implications for female fitness by, for example, increasing predator exposure (Pocklington and Dill, 1995) and/or reducing foraging efficiency (Magurran and Seghers, 1994; Pilastro et al., 2003). More broadly, a fluoxetine-induced shift towards male mating strategies that circumvent female mate choice could have population-level consequences by impacting the quality and quantity of offspring produced (Candolin and Heuschele, 2008; Candolin and Wong, 2012; Wong and Candolin, 2015).

Consistent with our results, several studies have similarly reported fluoxetine-induced changes to male reproductive behaviours in fish. In particular, Weinberger and Klaper (2014) found that fluoxetine exposure increased nest-tending behaviour in male fathead minnows (1000 ng/L for 28 days). Likewise, Bertram et al. (2018) found an increase in male copulatory behaviour in eastern mosquitofish (*Gambusia holbrooki*) following a 30-day exposure at 479 ng/L. However, in contrast to those studies, Schultz et al. (2011) and Dzieweczynski and Hebert (2012) reported no impact of fluoxetine exposure on reproductive behaviour in fathead minnows (2.5–28 ng/L for 21 days) or Siamese fighting fish (540 ng/L for 3 days), respectively. One possible reason for the differences observed across studies is social context. For example, in the study by Bertram et al. (2018), the increase in male copulatory behaviour was only observed in the absence of male-male competition while no such effect was seen when a rival was present. Thus, reproductive responses could be affected by whether males are allowed to directly interact with each other (e.g. Dzieweczynski and Hebert, 2012; Schultz et al., 2011) or whether males are tested in the absence of competitors (e.g. Weinberger and Klaper, 2014). In this regard, an important avenue for future research will be examining potential effects of fluoxetine on increasingly complex behavioural interactions and across different social contexts (e.g. audience effects: Makowicz et al., 2010; male-male competition: Jirotkul, 1999).

Certain reproductive behaviours may also be more sensitive to disruption by fluoxetine exposure than others. Indeed, evidence of endpoint-specific sensitivity was apparent in the current study, with a fluoxetine-induced increase in male sneak copulations but no significant change in courtship displays or following behaviour. In this respect, the physiological mechanisms by which fluoxetine affects male mating strategies warrants further investigation. Indeed, in general, the mechanisms through which SSRI exposure can alter male sexual behaviours are not fully understood (Fent et al., 2006; Prasad et al., 2015). Fluoxetine has the ability to bioaccumulate in fish tissues, including the brain (e.g. Brooks et al., 2005; David et al., 2018; Ramirez et al., 2009), reaching a steady state at approximately 4 days, and possessing a bioconcentration rate of 20–240 L/kg depending on the species (Boström et al., 2017; Silva et al., 2016). Once in the body, fluoxetine can influence extracellular levels of serotonin, which is known to play a key role in regulating reproductive function in fish via both central (i.e. preoptic-hypothalamic area and pituitary) and peripheral (i.e. gonadal) pathways (Dorelle et al., 2017; Prasad et al., 2015). Specifically, through the central pathway, serotonin interacts with the hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-interrenal (HPI) axes (Kreke and Dietrich, 2008), affecting the production of gonadotropin-releasing hormone and luteinising hormone (Kreke and Dietrich, 2008; McDonald, 2017; Yaron and Sivan, 2006). Fluoxetine affects peripheral pathways by influencing the production of testosterone and other androgens (Fernandes et al., 2011; Mennigen et al., 2010a, 2011), which are known to mediate reproductive behaviour (Munakata and Kobayashi, 2010).

Fluoxetine exposure did not significantly affect the total time spent by male or female guppies within 5 cm of the stimulus fish (predatory or non-predatory). This is surprising given that fluoxetine-induced shifts in serotonin could—through its effects on the HPI axis—alter the synthesis of adrenocorticotropic hormone and, accordingly, cortisol, which is important for mediating stress response in fish (reviewed in McDonald, 2017). Indeed, a number of earlier studies have reported altered antipredator behaviours (i.e. predator-related stress behaviour) as a result of fluoxetine exposure (e.g. Martin et al., 2017; Pelli and Connaughton, 2015; Saaristo et al., 2017; Weinberger and Klaper, 2014). However, to date, the effects of environmentally relevant fluoxetine exposure on antipredator behaviour have been mixed. For example, Martin et al. (2017), using eastern mosquitofish, and Pelli and Connaughton (2015), using guppies, both reported a decrease in antipredator behaviour as a result of environmentally realistic fluoxetine exposure (8 ng/L for 28 days and 30 ng/L for 21 days, respectively). By contrast, Weinberger and Klaper (2014) reported no effect of fluoxetine on antipredator behaviour of fathead minnows at environmentally relevant concentrations but did observe a decrease at concentrations exceeding those detected in the environment (>1000 ng/L for 28 days). In addition, Saaristo et al. (2017) observed an increase in antipredator behaviour in guppies (16 ng/L for 28 days). In this regard, differences in reported effects could be due to differences in the types of predatory stimulus used (and, thus, the perceived level of threat), species-specific differences in sensitivity to fluoxetine exposure, or a combination of both. Future research could, therefore, investigate how different predatory threats may mediate behavioural responses of prey to fluoxetine exposure.

While the total time males and females spent within 5 cm of the stimulus fish was not affected by fluoxetine, both sexes spent less time within this zone in the presence of the predatory spangled perch than that of the non-predatory rainbowfish. This indicates that males and females did, in fact, recognise the chemical and/or visual cues from the spangled perch as a threat (Sih et al., 1985; Swaney et al., 2015). It is worth noting, however, that the similarity of responses across the two sexes may also have been driven by male following behaviour, with males often remaining in close proximity to females for the majority of the trial and, hence, matching the females' use of the 5 cm zone. In addition, guppy following behaviour was altered by the presence of a predator, independent of fluoxetine exposure. Specifically, female guppies followed males significantly less in the presence of a predator than a non-predator, while males were unaffected by stimulus fish type. These results are supported by earlier investigations of wild guppy populations, where females have been shown to have reduced sexual interest and follow males less often when under threat of predation (Godin and Briggs, 1996), while males remain risk insensitive (Magurran and Nowak, 1991; Magurran and Seghers, 1990). One reason for this pattern is the considerable sexual size dimorphism seen in guppies, which affects predator strategy (Dill et al., 1999). Indeed, some predators have been found to preferentially target females over males, which are smaller (Pocklington and Dill, 1995). Hence, females could potentially decrease their own risk by reducing interactions with males (Dill et al., 1999), whereas males may be more likely to disregard predation risk in favour of increased potential mating opportunities (Dill et al., 1999; Magurran and Nowak, 1991).

In the present study, we found no interactive effects of fluoxetine exposure and predation threat, with each stressor acting on different behaviours independently. To date, only two studies have explicitly investigated interactions between natural environmental stressors and fluoxetine exposure in freshwater biota. In estuarine crabs (*Cancer productus*), Peters et al. (2017) found an antagonist interaction between predation threat and fluoxetine exposure, with unexposed crabs reducing foraging behaviour under risk of predation and fluoxetine-exposed crabs increasing foraging behaviour regardless of predation risk. In contrast, Barbosa et al. (2017) reported a synergistic effect of water temperature and fluoxetine exposure in water fleas, with the interaction of both stressors resulting in higher fitness costs than when in isolation. Hence, while previous studies have shown that fluoxetine exposure in combination with other environmental stressors does have the potential to induce interactive effects on behaviour, our findings suggest

that this is not always inevitable. In this regard, sertraline (another SSRI) and predation stressors have been shown to have no interactive effect on activity or boldness in freshwater snails (*Radix balthica*), although this was due to sertraline having no effect on either behaviour (Hedgespeth et al., 2018), making uncovering potential interactions challenging. Further research is therefore necessary to determine how fluoxetine and other antidepressants may interact with additional stressors such as predation risk, and how these potential interactions may affect wild populations.

We found no significant morphological effects of fluoxetine on either male or female guppies, in terms of standard length, weight or condition index. In contrast, previous studies in other fish species have reported a decline in body condition after fluoxetine exposure (e.g. convict cichlid, *Amatitlania nigrofasciata*: Latifi et al., 2015; goldfish, *Carassius auratus*: Mennigen et al., 2010b), including at field-detected concentrations (e.g. eastern mosquitofish: Bertram et al., 2018). In goldfish, fluoxetine exposure (5 μg/g body weight for 13 days) resulted in both reduced food intake and weight gain by increasing the expression of potent inhibitory feeding neuropeptides in the brain (Mennigen et al., 2009). Whether this is also the case in guppies remains to be tested. Clearly, further research is needed to elucidate the mechanisms underpinning morphological changes (if any) induced by fluoxetine, both within and between species.

5. Conclusion

We report that short-term (28-day) exposure to an environmentally relevant concentration of the widespread pharmaceutical contaminant fluoxetine altered reproductive behaviour in male, but not female, guppies. More specifically, males in the high-fluoxetine treatment (350 ng/L) exhibited an altered mating strategy, performing a higher number of coercive sneaking copulations than unexposed males, regardless of perceived predation risk (i.e. in the presence of both a predator and non-predator). Contamination of the environment with pharmaceuticals, such as fluoxetine, that are capable of disrupting key fitness-related behaviours, such as mating strategy, is a major concern. Therefore, although we found no interactive effects between fluoxetine and perceived predation risk, further research on co-effects between pharmaceuticals and other environmental stressors is certainly needed to better understand potential impacts of these contaminants on ecological and evolutionary processes in wildlife.

Ethics

The research detailed in this paper was approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2016/21) and complied with all relevant State and Federal laws of Australia.

Authors' contributions

All authors conceived and designed the experiments, which JBF and JMM conducted. JBF, JMM and MGB carried out statistical analysis and wrote the manuscript. All authors contributed to manuscript preparation and gave final approval for publication.

Competing interests

The authors declare that we have no competing interests.

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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.2018.09.046.

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