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Impact of the widespread pharmaceutical pollutant fluoxetine on behaviour and sperm traits in a freshwater fish

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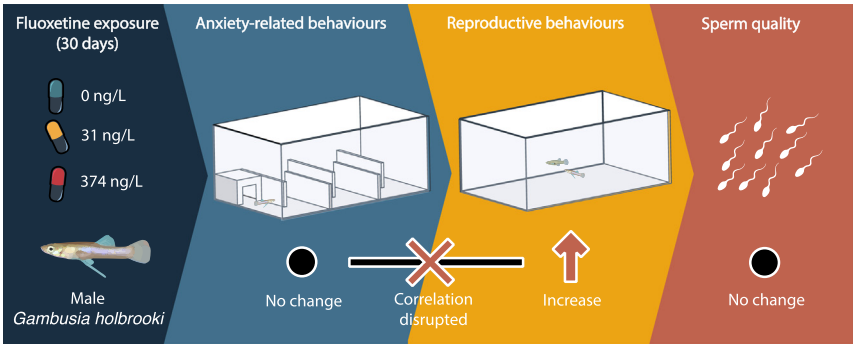
HIGHLIGHTS

- Male mosquitofish (*G. holbrooki*) exposed to fluoxetine at two realistic levels.
- Fluoxetine did not impact anxiety-related behaviours.
- Fluoxetine increased reproductive behaviour.
- Fluoxetine disrupted an across contexts correlation.
- Fluoxetine did not affect sperm quality

GRAPHICAL ABSTRACT



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ABSTRACT

Pharmaceutical pollutants are detected in aquatic habitats and wildlife tissues globally. One widespread contaminant of major concern is the antidepressant fluoxetine, which can affect behavioural and physiological processes in non-target species. Despite this, effects of fluoxetine on wildlife behaviour have seldom been investigated across multiple fitness-related contexts, especially at environmentally realistic concentrations. Accordingly, we examined impacts of 35-day fluoxetine exposure at two environmentally relevant concentrations (31 and 374 ng/L) across a suite of fitness-related contexts in wild-caught male mosquitofish (*Gambusia holbrooki*). First, we investigated anxiety-related behaviours (boldness, exploration and activity) in a novel environment (maze arena) and found no significant impacts of exposure. Second, we tested effects of fluoxetine in a reproductive context, including mating behaviour and sperm quality. We found that, relative to controls, fluoxetine exposure resulted in males spending a greater amount of time pursuing females. Further, low-exposed males were more likely to attempt copulation than unexposed males. Lastly, we investigated across-context behavioural correlations, and how fluoxetine exposure might affect such relationships. A significant positive correlation was detected in control fish between activity levels in the maze and time spent pursuing females in the reproductive assay. This relationship was disrupted by fluoxetine at both exposure levels. This is the first evidence that field-detected concentrations of a pharmaceutical pollutant can disturb across-context behavioural correlations in wildlife. Our findings provide clear evidence that fluoxetine can produce context-specific behavioural effects in fish and underscore how pharmaceutical exposure at field-detected concentrations can induce important shifts in wildlife behaviour.

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1. Introduction

Pharmaceutical pollution is a major threat to aquatic ecosystems globally (Arnold et al., 2014; Bernhardt et al., 2017; Saaristo et al., 2018). Hundreds of human and veterinary pharmaceuticals have now been detected in aquatic ecosystems and wildlife tissues around the world (Hughes et al., 2013; Küster and Adler, 2014). One pharmaceutical pollutant of environmental concern is the antidepressant fluoxetine. As with most pharmaceuticals, fluoxetine typically enters the environment via human consumption and excretion (Schultz et al., 2010). Indeed, up to 30% of administered fluoxetine can remain unmetabolised when excreted (van Harten, 1993). This incomplete metabolisation, coupled with insufficient removal by sewage treatment plants (e.g. Vasskog et al., 2006), results in fluoxetine entering aquatic environments in wastewater effluent flows. Consequently, fluoxetine (as well as its primary metabolite norfluoxetine) has been detected in surface waters worldwide at levels ranging from <1–100 ng/L, to as high as 596 ng/L in systems directly receiving wastewater discharge (Hughes et al., 2013; Schultz and Furlong, 2008; Schultz et al., 2010; Vanderford and Snyder, 2006). Once in the environment, fluoxetine can bioaccumulate in wildlife tissues (e.g. Brooks et al., 2005; David et al., 2018; Muir et al., 2017). For example, in an urban wetland receiving treated municipal wastewaters, fluoxetine—relative to 64 other pharmaceuticals present—showed the highest level of bioaccumulation in wild fish (Muir et al., 2017).

In addition to fluoxetine's prevalence in aquatic habitats, its primary pharmacological target, the serotonin transporter molecule, is conserved across a variety of taxa (Gunnarsson et al., 2008; Wang and Tsai, 2006). Consequently, fluoxetine may affect wildlife through its pharmacological action at lower concentrations than are required to induce general toxicity (McDonald, 2017). Moreover, by altering the serotonin system and associated neuroendocrine pathways, fluoxetine can influence multiple fitness-related processes (Kreke and Dietrich, 2008; McDonald, 2017). For example, in fish, pharmacologically relevant dosages of fluoxetine (i.e. ≥100 μg/L) have repeatedly been shown to reduce anxiety-like behaviours (Ansai et al., 2016; Cachat et al., 2010; Wong et al., 2013). By extension, fluoxetine exposure in wildlife could result in alterations to ecologically important behaviours linked to anxiety, such as boldness (i.e. the propensity to take risks), exploration, and activity, which are directly related to fitness and are associated with a range of important processes, such as dispersal (e.g. Cote et al., 2010; Michelangeli et al., 2017) and migration (e.g. Chapman et al., 2011). Moreover, fluoxetine exposure can also disrupt reproduction (reviewed in Kreke and Dietrich, 2008; McDonald, 2017). For example, in aquatic species, fluoxetine has been shown to induce gamete release in mussels (Bringolf et al., 2010; Fong, 1998) increase ovarian growth in crayfish (Kulkarni et al., 1992), and cause shifts in the release of sex hormones in fish species (Foran et al., 2004; Khan and Thomas, 1992; Mennigen et al., 2010).

Despite fluoxetine's capacity to influence a range of biological processes, few studies have investigated the effects of environmentally realistic fluoxetine exposure on non-reproductive and reproductive behaviours concomitantly—which is also true for pharmaceutical pollutants more generally. Fewer still have considered the importance that behavioural and physiological alterations can have on individuals across multiple ecological contexts, despite growing appreciation that functionally unrelated behaviours are often correlated, whereby a shift in one trait can correspond with a shift in another (i.e. behavioural syndromes, Sih et al., 2004, 2012).

Here, we set out to test the hypothesis that 35-day fluoxetine exposure at two environmentally realistic levels (average measured concentrations: 31 and 374 ng/L) would disrupt behaviour across two ecologically important contexts in wild-caught male mosquitofish (*Gambusia holbrooki*). First, we tested the effect of fluoxetine on anxiety-related behaviours (boldness, exploration, and activity) in a novel environment (maze arena). Second, using the same males, we tested the impact of fluoxetine exposure in a reproductive context, in terms of both reproductive behaviour and sperm quality. Lastly, we tested for potential across-context behavioural correlations and the effects of fluoxetine on such relationships.

2. Methods

2.1. Animal collection and housing

The present research was approved by the Biological Sciences Animal Ethics Committee of Monash University (BSCI/2015/2). Sexually mature male (mean weight: 0.1999 ± 0.0406 g, mean length: 22.31 ± 1.26 mm; $n = 105$) and female (mean weight: 0.4036 ± 0.1990 g, mean length: 26.14 ± 3.81 mm; $n = 105$) mosquitofish were collected from a wild population at Science Centre Lake (37°54′28″ S, 145°08′16″ E), Monash University, Australia. Water samples taken from the site over consecutive years indicated no fluoxetine contamination (unpublished data). Before experimentation, fish were acclimated to laboratory conditions (24–26 °C; 12:12 h light:dark cycle) in single-sex holding tanks (80 × 45 × 45 cm, water depth: 30 cm) for 1 month. Fish were fed daily on an *ad libitum* diet of commercial fish food (Otohime Hirame). The mosquitofish was selected as a model because its life-history is well characterised, including reproductive behaviour and sperm traits (Bisazza et al., 2001; Locatello et al., 2008; McPeek, 1992). Mosquitofish have a largely coercive polyandrous mating system and internal fertilisation, with males using a modified anal fin as an intromittent organ during copulation (McPeek, 1992). Due to the species' coercive mating system (Bisazza et al., 2001), and capacity for females to store sperm (Locatello et al., 2008), sperm quality is likely to play an important role in predicting reproductive success of male mosquitofish under sperm competition (Locatello et al., 2008).

2.2. Chemical exposure and monitoring

Male mosquitofish were randomly allocated to three treatment groups for 35 days: unexposed (i.e. fresh water), low fluoxetine and high fluoxetine. A 35-day exposure duration was selected because the full therapeutic effects of fluoxetine typically take 2–4 weeks to manifest in humans (Gardier et al., 1996; Hensler, 2003), and the spermatogenic cycle of *G. holbrooki* takes 30 days (Koya and Iwase, 2004). The nominal fluoxetine exposure concentration of the low treatment (40 ng/L) was selected to represent levels repeatedly detected in surface waters, while the nominal high concentration (400 ng/L) was selected to represent the higher end of surface water detections (reviewed in Hughes et al., 2013). The design of the chemical exposure followed previously published protocols (Bertram et al., 2018a; Martin et al., 2017). Briefly, exposure involved three identical flow-through systems (24 h cycling), one per treatment, with each system comprising 4 aquaria (60 × 30 × 30 cm, water depth: 25 cm), housing 30 fish each. The low- and high-fluoxetine exposure systems both received a constant supply of fluoxetine stock solution (replaced daily) and fresh water, whereas the unexposed system received fresh water only. The low- and high-fluoxetine stock solutions (6 and 60 μg/L, respectively) were prepared following methods described in Bertram et al. (2018a). Weekly water samples (200 mL) were taken from all of the low and high exposure tanks to measure fluoxetine concentrations. Additionally, water samples were collected from each unexposed tank fortnightly to ensure the absence of fluoxetine. Water samples were analysed by Envirolab Services using gas chromatography coupled to tandem mass spectrometry (7000C Triple Quadrupole GC–MS/MS, Agilent Technologies, Delaware, USA), based on methods described in Bertram et al. (2018a). Mean measured concentrations for the low- and high-fluoxetine treatments were 30.61 ng/L (SD = 6.28, $n = 24$) and 374.50 ng/L (SD = 62.91, $n = 24$). No fluoxetine contamination was detected in the unexposed system ($n = 12$), with the limit of quantification for fluoxetine being 2 ng/L.

2.3. Behavioural assays

A total of 105 males were used in maze and mating behaviour assays (unexposed: $n = 37$, low-fluoxetine: $n = 32$, high-fluoxetine: $n = 36$). Each male was first tested in the maze assay, which was followed by a 1 h rest period, after which each fish was tested in the reproductive assay. This design minimised any potential carryover effects that may have influenced behaviour in the maze trials (Bell, 2013). Behavioural assays were filmed with a digital camera, with behavioural endpoints quantified from the footage using JWatcher v1.0 (Blumstein and Daniel, 2007). During video quantification, observers were blind to treatment. All trials were conducted in aged fresh water (i.e. no fluoxetine) and, after each trial, tanks were drained and refilled to avoid any potential influence of conspecific chemical cues on the behaviour of focal fish.

The maze assay employed was adapted from Ward (2012) and followed the design of Bertram et al. (2018b). Specifically, each maze arena (60 × 30 × 30 cm, water depth: 10 cm) had a refuge box (10 × 10 × 10 cm) at its beginning, as well as five internal opaque walls that obscured the swimming path of fish and delineated six maze arms (Fig. 1a). The floor of the maze was divided by 5 cm gridlines used to measure activity levels (see below). At the beginning of each trial, a focal fish was first introduced into the refuge and allowed to acclimate for 5 min. After acclimation, a door to the refuge was remotely opened, allowing the fish to exit and explore the maze. Over a 20 min trial, we quantified three behavioural traits: (1) boldness, measured as the time taken to emerge from the refuge (emergence test), (2) exploration, measured as the time taken to complete the maze after the fish had exited the refuge (novel environment test), and (3) activity, measured as the total number of gridlines crossed (novel environment test). Trials concluded after 20 min, irrespective of whether or not the fish had completed the maze. After the trial, males were transported to individual temporary holding tanks (30 × 15 × 15 cm, water depth: 10 cm) where they were rested for 1 h.

Following this rest period, males were tested in a reproductive behaviour assay. In these trials, males were paired randomly with a novel unexposed stimulus female in an observation tank (60 × 30 × 30 cm, water depth: 10 cm; Fig. 1b). Stimulus females were unexposed to disentangle the indirect effects that fluoxetine-induced behavioural changes in one sex might have on the other, a technique employed in previous ecotoxicological studies (e.g. Saaristo et al., 2013; Tomkins et al., 2017, 2018). In addition, each stimulus female was only used in a single trial to avoid any potential carryover effects on behaviour. Before the commencement of the reproductive assay, the focal male and stimulus female were acclimated to trial water for 5 min in separate transparent containers (6 × 5 × 3 cm) within the observation tank. At the beginning of the trial, both fish were released and allowed to freely interact for 20 min, during which time we quantified the total time spent by males actively following the female within 5 cm (i.e. association behaviour), and the total number of male copulation attempts.

2.4. Sperm quality

To test for potential effects of fluoxetine on sperm quality, we measured both sperm performance and viability (i.e. the proportion of live sperm) immediately after behavioural trials. Both traits are important predictors of fertilisation success, especially under sperm competition (reviewed in Snook, 2005).

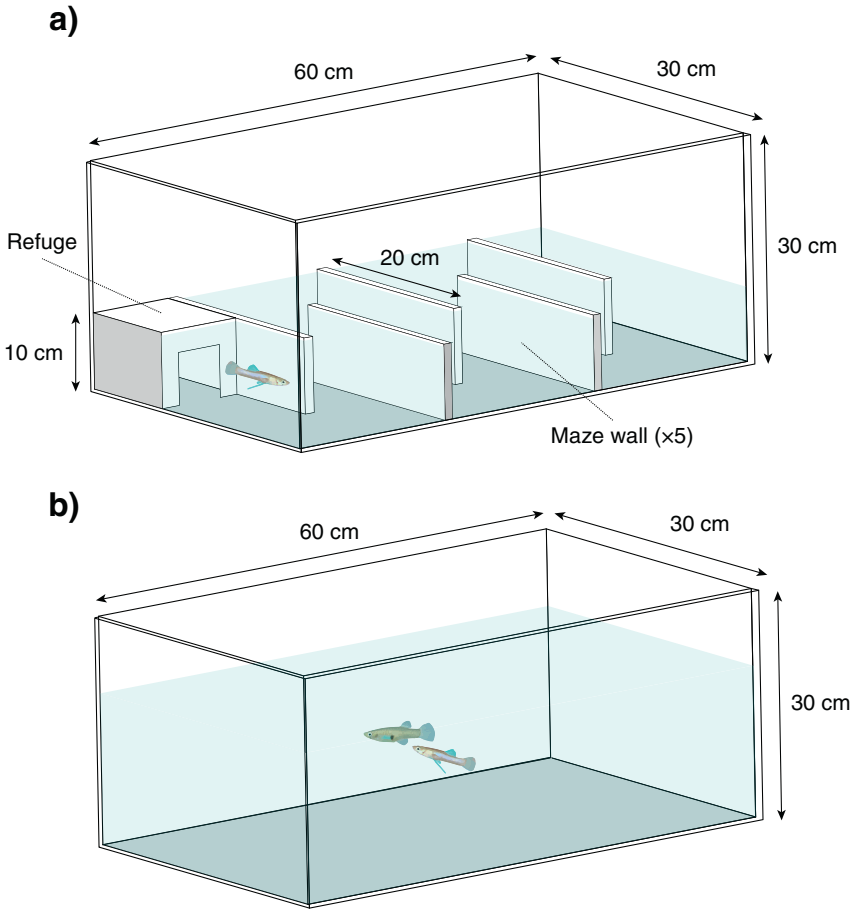

Fig. 1. (a) Maze assay, in which males were assessed for boldness, exploration, and activity. (b) Reproductive behaviour assay, in which males were paired with a single unexposed stimulus female and assessed for association behaviour and copulation attempts.

All sperm analyses were conducted blind to treatment and followed the protocols of Bertram et al. (2018a). Firstly, fish were euthanised (clove oil, 40 mg/L) and a sample of their ejaculate collected. Sperm performance was then measured using computer-assisted sperm analysis (CASA) software (v.14, CEROS, Hamilton-Thorne Biosciences, Beverly, MA, USA) for 96 males (unexposed: $n = 33$, low fluoxetine: $n = 28$, high fluoxetine: $n = 35$). A total of 9 males (4 unexposed, 4 low fluoxetine, 1 high fluoxetine) tested in behavioural trials did not provide sperm and were hence excluded from analyses. A minimum of 1000 sperm were tracked per male (mean = 1136.17, SE = 7.40) using a video camera (XC-ST50, Sony, Japan) coupled to a negative phase-contrast microscope (CX41, Olympus, 10× objective). These sperm tracks were used to obtain five measures of sperm performance (Table S1 for definitions): (1) average velocity of sperm along its average path (VAP, μm/s), (2) straight-line velocity from the first detection to the last detection (VSL, μm/s), (3) average point-to-point velocity along its path (VCL, μm/s), (4) linearity of the sperm path (LIN, %), and (5) percentage of motile sperm (MOT).

A second aliquot of sperm was collected from each male and analysed for the proportion of live sperm using a live/dead sperm viability kit (L-7011; Molecular Probes Inc., USA). For 2 males (1 unexposed, 1 high-fluoxetine), an insufficient volume of ejaculate was extracted to adequately perform viability counts in conjunction with CASA, with these males being excluded from further analyses. In total, sperm viability was assessed for 89 males (unexposed: $n = 32$, low-fluoxetine: $n = 27$, high-fluoxetine: $n = 30$). Sperm samples were first stained with a fluorescent membrane-permeant nucleic acid stain (SYBR-14), which stains live sperm green under fluorescent light. The sample was then counter-stained with propidium iodide, which stains dead sperm red. Using a fluorescence microscope (Leica DFC425C, Leica Microsystems, Germany), 12 non-overlapping fields were then photographed. Subsequently, the proportion of live sperm was calculated by counting a minimum of 150 sperm per male (mean = 320.73, SE = 13.28).

2.5. Morphological analysis

Immediately after sperm analysis, euthanised males were measured (standard length; ±0.01 mm) and weighed (±0.0001 g), and condition index calculated following previously published protocols (Bertram et al., 2015; Martin et al., 2017). These morphological traits were also recorded for stimulus females. The relative size of the male to the stimulus female (i.e. male size minus female size) was not statically different across treatment groups (ANOVA: $F_{2,102} = 1.87$, $p = 0.159$).

2.6. Statistical analysis

Data were analysed in R v3.2.2 (R Development Core Team, 2015) and checked for normality (Shapiro-Wilk test) and homogeneity of variance (Fligner-Killeen test), where appropriate. All models included treatment (unexposed, low-fluoxetine, and high-fluoxetine) as a predictor, and male length as a covariate. Models used to assess behavioural parameters in the reproductive assay also included female length as a covariate. Across all models, continuous covariates were centred to improve the interpretability of main effects. In addition, fish ID and exposure tank number were treated as random effects in all models.

Time taken to exit the refuge at the start of the maze arena, and time taken to complete the maze, were each compared across treatments using Cox mixed-effect proportional hazards models (*coxme* function, *survival* package). For all models, fish were right-censored (i.e. scored as incomplete) if they did not perform the event during the 20 min assay. Both models met the assumption of proportionality, as tested by examining the interaction between Schoenfeld residuals and log time (*coxph* and cox.*zph* functions, *survival* package). A linear mixed-effect model (LME; *lme* function, *nlme* package) was used to compare the total number of 5 cm gridlines crossed in the maze across treatments.

Total time spent by males associating with females in the reproductive assay was compared across treatments using an LME. Copulation attempts were compared across treatments using a generalised mixed-effect model (GLMM; *glmer* function, *lme4* package) with a binomial distribution (i.e. 'attempted' or 'did not attempt'). This was done because an insufficient number of fish conducted the behaviour (<15% across all groups) for it to be analysed as a count variable.

Within each treatment group, a series of Spearman's rank-order correlation tests were used to investigate potential across-context relationships between behaviours in the maze assay (boldness, exploration, and activity) and reproductive behaviour (total time males spent following females).

Sperm performance measures (VAP, VSL, VCL, LIN, MOT), sperm viability, and male morphological traits (length, weight, and condition index) were compared across treatments using LME models. To meet assumptions of normality, a square root folded transformation was applied to sperm motility, a rank-normal transformation was applied to sperm viability, and both male length and weight were cube-root transformed.

3. Results

3.1. Behavioural assays

No significant effect of treatment was detected on the time taken for fish to exit the refuge (boldness) in the maze arena, the time taken to complete the maze (exploration) or activity levels in the maze (coxme: all $p > 0.05$; Table S2–S3). In addition, male length did not significantly affect any of the measured behaviours (coxme: all $p > 0.05$; Table S2).

Total time spent by males associating with females in the reproductive assay was affected by fluoxetine treatment (LME: $F_{2,100} = 4.30$, $p = 0.016$; Fig. 2). Specifically, males in the low- and high-fluoxetine treatments spent significantly longer associating with females than did unexposed males ($t = 2.64$, $df = 100$, $p = 0.001$, and $t = 2.42$, $df = 100$, $p = 0.018$, respectively). There was, however, no significant difference in association behaviour between low- and high-fluoxetine exposed males ($t = -0.31$, $df = 100$, $p = 0.753$). More generally, a marginally

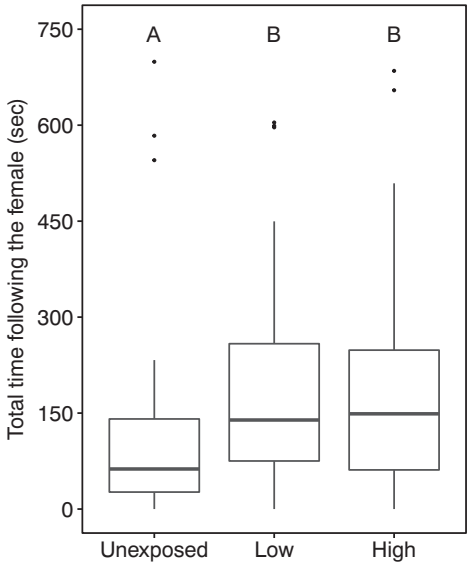

Fig. 2. Total time males spent actively following a female (i.e. associating) across unexposed ($n = 37$), low-fluoxetine ($n = 32$) and high-fluoxetine ($n = 36$) treatments. Box plots show 25th, 50th (median) and 75th percentiles. Groups that share a capital letter are not significantly different from one another.

non-significant effect of stimulus female length was detected on the total time spent by males associating with females ($t = 1.94$, $df = 100$, $p = 0.054$). Male length did not affect the total time spent associating with females ($t = 1.00$, $df = 100$, $p = 0.319$).

Fluoxetine exposure also impacted the likelihood of males to perform a copulation attempt. Specifically, a greater proportion of males from the low treatment attempted to copulate than did unexposed males (GLMM: $z = 2.38$, $df = 100$, $p = 0.017$), with 22% of low-exposed males attempting at least one copulation, as opposed to 3% of unexposed males. There was a similar, but marginally non-significant difference in the proportion of males that attempted to copulate between the high-fluoxetine treatment (13%) and the unexposed treatment ($z = 1.85$, $p = 0.065$), while no significant difference was observed between the low- and high-fluoxetine treatments ($z = -0.47$, $p = 0.882$). More generally, female length positively associated with the likelihood of males performing copulatory behaviour ($z = 2.35$, $p = 0.047$). Male length, however, did not significantly affect the likelihood of copulation ($z = -0.51$, $p = 0.611$).

For unexposed fish, there was a significant positive correlation between total time spent by males actively following females and male activity levels in the maze (Spearman's correlation: $r_s = 0.34$, $p = 0.040$; Fig. 3). However, this relationship was not seen in either low- or high-fluoxetine exposed males ($r_s = 0.16$, $p = 0.377$, and $r_s = 0.06$, $p = 0.740$, respectively; Fig. 3). Further, no significant correlation was detected between the total time males spent associating with females and boldness, or exploration, for any of the treatment groups (all $p > 0.05$; Table S4).

3.2. Sperm quality

Fluoxetine, irrespective of exposure level, did not significantly affect any measure of sperm performance or viability (LME: all $p > 0.05$; Table S5–S6). More generally, male length was positively associated with sperm motility ($F_{1,95} = 4.90$, $p = 0.029$) but did not associate significantly with any other measured sperm traits (Table S5).

3.3. Morphology

Fluoxetine exposure had no significant effect on male length (LME: $F_{2,102} = 0.37$, $p = 0.689$) or weight (LME: $F_{2,102} = 1.51$, $p = 0.225$). Additionally, a marginally non-significant effect of fluoxetine exposure was detected on condition index ($F_{2,102} = 2.95$, $p = 0.057$), with low-fluoxetine exposed males having a significantly lower condition than high-fluoxetine exposed males ($t = -2.38$, $df = 102$, $p = 0.019$). By contrast, control males showed an intermediate condition that was not significantly different from either low- or high-fluoxetine exposed males ($t = -1.67$, $df = 102$, $p = 0.099$ and $t = 0.45$, $df = 102$, $p = 0.752$, respectively).

4. Discussion

We found that fluoxetine did not significantly impact latency to emerge from a refuge (i.e. boldness), time to complete a maze after exiting the refuge (i.e. exploration), or the number of 5 cm gridlines crossed (i.e. activity). To date, only three other studies have employed environmentally realistic dosages (<1–596 ng/L) to investigate impacts of fluoxetine on anxiety-related behaviour in fish (Dzieweczynski et al., 2016a, 2016b; Margiotta-Casaluci et al., 2014). In concordance with our study, Margiotta-Casaluci et al. (2014) reported no effect of fluoxetine on boldness and exploration in fathead minnow (*Pimephales promelas*) at environmentally realistic exposure levels, although they did see an increase in boldness and exploration at concentrations exceeding those detected in the environment (72,000 ng/L for 28 days). By contrast, Dzieweczynski et al. (2016a, 2016b) reported that exposure to an environmentally relevant level of fluoxetine (500 ng/L for 1–15 days) significantly reduced boldness in Siamese fighting fish (*Betta splendens*). Such differences between studies could be due to different exposure durations and/or species-specific sensitivities. We suggest that further investigations at multiple time points in a range of species are warranted to elucidate the impacts of environmentally realistic fluoxetine exposure on anxiety-related behaviour.

In the reproductive behaviour assay, both low- and high-fluoxetine males spent more time associating with a female than did controls. For mosquitofish—and poeciliid fish more generally—the propensity of males to associate closely with females is a reliable and biologically meaningful estimate of male mating intent (Dosen and Montgomerie, 2004; Wong et al., 2005). Furthermore, since mosquitofish have internal fertilisation, actively following (i.e. being in close proximity to) females is essential for males to successfully mate (Bisazza et al., 2001). Low-fluoxetine exposure also increased the likelihood of males attempting copulation in comparison to controls. Further, a similar, but marginally non-significant, trend was seen towards high-fluoxetine-exposed males being more likely to copulate than unexposed males. An increase in copulatory behaviour is likely to result in increased mating success. For example, Evans et al. (2003) reported that male mosquitofish that perform more frequent copulation attempts are more likely to successfully transfer sperm. Moreover, in male western mosquitofish (*Gambusia affinis*), number of copulation attempts associates positively with proportion of offspring sired (Deaton, 2008). Taken together, the behavioural changes seen here are expected to result in increased

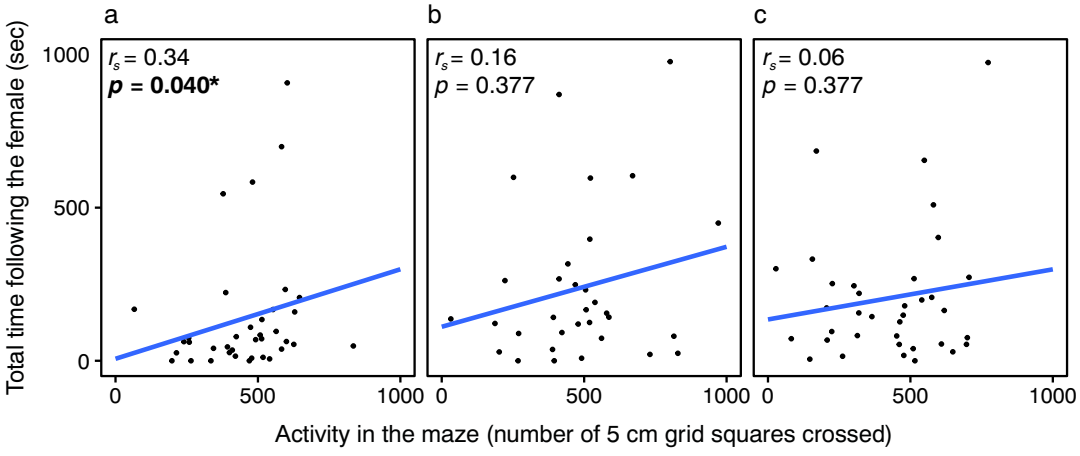

Fig. 3. Across-context behavioural correlations between activity in the maze assay and total time spent following a female in the reproductive assay for males in the unexposed (a; $n = 37$), low-fluoxetine (b; $n = 32$) and high-fluoxetine (c; $n = 36$) treatments.

male mating success in exposed fish. However, from the perspective of females, an increase in mating effort by exposed males could also be costly, with male sexual harassment previously shown to impinge on female foraging efficiency (Pilastro et al., 2003). The resulting increase in sexual conflict could ultimately lead to shifts in the strength and direction of sexual selection, which, in turn, can influence population demography by affecting the quality and quantity of offspring produced (Wong and Candolin, 2014).

The increase in reproductive behaviour observed in fluoxetine exposed fish could be caused by an increase in serotonin concentrations and, consequently, shifts in the hypothalamic–pituitary–gonadal (HGP) axis (reviewed in Kreke and Dietrich, 2008; McDonald, 2017). In fish, increases in extracellular serotonin concentrations have been shown to stimulate the release of gonadotropin-releasing hormones (GnRHs) and gonadotropic hormones (GTHs; reviewed in Kreke and Dietrich, 2008; McDonald, 2017), as well as androgens, which are known to regulate sexual behaviours (Borg, 1994; Munakata and Kobayashi, 2010). Thus, it is possible that the increased reproductive behaviour of male mosquitofish is due to a serotonin-induced increase in hormones responsible for mediating sexual behaviours. In humans, chronic fluoxetine exposure should ultimately lead to a return to pre-treatment serotonin levels, driven by compensatory responses of the brain to perturbed serotonin concentrations, which can take several weeks (reviewed in Andrews et al., 2015). Therefore, it is interesting that, after a 35-day exposure, we saw an increase in reproductive behaviour, which would be expected with increased serotonin concentrations. Perhaps, at fluoxetine concentrations as low as those used here, compensatory responses to perturbed serotonin concentrations are less pronounced or rapid. Such a possibility could be addressed by directly measuring serotonin levels in the brain. It is also worth highlighting that, even for humans, the tolerability, efficacy, and mechanism of action of SSRIs have all been the subject of controversy and debate (reviewed in Walker, 2013).

To date, only a handful of studies have addressed impacts of environmentally realistic fluoxetine exposure on reproductive behaviour in fish (Bertram et al., 2018a; Dzieweczynski and Hebert, 2012; Forsatkar et al., 2014; Fursdon et al., 2018; Schultz et al., 2011; Weinberger and Klaper, 2014). In concordance with the present study, both Bertram et al. (2018a) and Fursdon et al. (2018) reported an increase in copulatory behaviour in male poeciliid fish (*Gambusia holbrooki* and *Poecilia reticulata*, respectively) following ecologically relevant fluoxetine exposure (479 ng/L for 30 days, and 350 ng/L for 28 days, respectively). Similarly, Weinberger and Klaper (2014) reported an increase in reproductive behaviour (i.e. nest tending) in male fathead minnows after 28-day fluoxetine exposure, although this was only seen at 1000 ng/L and not at the lower concentration tested (100 ng/L). In contrast, no effect of fluoxetine exposure was detected on the reproductive behaviour of fathead minnows (2.3 and 28 ng/L for 21 days; Schultz et al., 2011) or Siamese fighting fish (540 ng/L for 6 days; Dzieweczynski and Hebert, 2012). In a separate study using Siamese fighting fish, however, a decrease in reproductive behaviour has also been reported (540 ng/L for 3 days; Forsatkar et al., 2014). Differences in fluoxetine-induced effects across these studies may be a result of different exposure durations and species-specific sensitivities. Indeed, the modulatory function of serotonin on the HPG axis seems to vary considerably across fish species (Kreke and Dietrich, 2008). This disparity may also be a result of the different reproductive behaviours assessed. For example, some of the studies incorporated male-male competition in their measure of reproductive behaviour while others did not. Reproductive behaviours in the presence of male-male competition, may, therefore, not be impacted by fluoxetine exposure to the same degree as reproductive behaviour performed in the absence of such aggression. Indeed, as mentioned above, Bertram et al. (2018a) reported an increase in copulatory behaviour in male mosquitofish in the absence of male-male competition, however, in a separate assay under direct male-male competition, this effect was not evident.

Interestingly, we found a positive across-context correlation in unexposed fish between reproductive behaviour (i.e. time spent by males following females) and activity in the maze, although this was not present in fluoxetine-exposed fish. Evidence of a behavioural correlation between reproduction and activity levels in unexposed fish suggests that these two traits are either directly coupled through some kind of causal (e.g. a gene or hormone that affects both behaviours) and/or an indirect link (e.g. shaped by individual experience and learning feedback loops; reviewed Sih et al., 2004). Since this relationship was absent in fluoxetine-exposed fish, we suggest that fluoxetine-induced effects on neuroendocrine pathways like the HPG and hypothalamic-pituitary-adrenal axes disrupted this behavioural correlation. Given that fluoxetine exposure also impacted reproductive behaviour in this study, we hypothesise that the absence of across-context behavioural correlation is a result of shifts in the HPG axis of exposed fish. To the best of our knowledge, this is the first evidence that field-detected concentrations of a pharmaceutical pollutant may cause a breakdown in across-context behavioural correlations (i.e. behavioural syndromes). In light of this, future research may wish to employ pre- and post-exposure behavioural tests across contexts, in combination with endocrine measures (e.g. plasma hormone levels), to identify the extent to which fluoxetine exposure may disrupt the presence of behavioural syndromes in wildlife. Given that behavioural syndromes have been linked with the ability of species to respond to environmental change and invasive potential (reviewed in Sih et al., 2012), pollution-induced disruption of behavioural correlations could have significant, yet overlooked, consequences for fitness.

We did not find evidence of fluoxetine-induced effects on any measured sperm traits. To date, only the present study and that of Bertram et al. (2018a) have examined effects of environmentally realistic concentrations of fluoxetine on sperm quality in fish, both of which reported no effect. However, Bertram et al. (2018a) did report an increase in the total sperm count of fluoxetine-exposed fish, an endpoint not measured in the present study. Other studies have addressed impacts of exposure on different gonad-related endpoints. For example, in adult Japanese medaka (*Oryzias latipes*), gonadal somatic index and gonadal steroidogenesis were unaffected by 4 weeks of fluoxetine treatment at a range of concentrations (i.e. 0–5000 ng/L; Foran et al., 2004). In addition, Mennigen et al. (2010) reported that 14-day exposure to fluoxetine at 540 ng/L did not affect basal milt volume in male goldfish, although a reduction was observed at 54,000 ng/L. More broadly, in humans and rodents, sexual dysfunction and decreased sperm motility has been reported as a side effect of fluoxetine treatment (reviewed in Nørr et al., 2016). Given that the dosages used in these studies are much higher than were used in the present study, it is possible that spermicidal effects of fluoxetine might only be seen at higher dosages than those used here.

While neither male length nor weight was affected by fluoxetine exposure, a marginally non-significant impact of exposure was detected on condition index, which was driven by a decrease in the condition of low-exposed fish relative to those in the high-exposed treatment. Previous studies have reported a decrease in condition index as a result of fluoxetine exposure (Bertram et al., 2018a; Gaworecki and Klaine, 2008; Latifi et al., 2015), although, with the exception of Bertram et al. (2018a), these effects were seen at higher concentrations than those found in the environment. Given the marginal nature of our results, we suggest that the impacts of environmentally realistic fluoxetine exposure on morphological traits, like condition index, warrant further investigation.

5. Conclusions

In summary, fluoxetine exposure for 35 days at 31 and 374 ng/L impacted male reproductive behaviour, while sperm traits and anxiety-related behaviour in the same individuals were unaffected. Additionally, fluoxetine at both dosages disrupted the presence of an across-context

behavioural correlation (i.e. behavioural syndrome), with a positive correlation being detected between reproductive behaviour and boldness in unexposed fish only. Taken together, these findings suggest that fluoxetine exposure can induce context-specific effects, thus highlighting the need to address the impacts of pharmaceutical exposure over multiple ecologically important contexts. More broadly, shifts in reproductive behaviour support a growing body of evidence that psychoactive pharmaceuticals at field-detected concentrations can induce subtle—but important—changes in wildlife behaviour. The next step in identifying the risk posed by fluoxetine (and other psychoactive pollutants) is to address the potential for synergistic or antagonistic effects during exposure to pharmaceutical mixtures, using a combination of pollutants that are both readily detected in the environment and act via similar mechanisms.

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Authors' contributions

JMM, MGB, MS and BBMW conceived and designed the experiments, which JMM, MGB and TEE carried out. Video and statistical analysis were performed by JMM, MGB, JLT and MM. All sperm analysis was coordinated by MKOB, which JMM, MGB and SLH conducted. The manuscript was drafted by JMM. All authors contributed critically to the drafts and gave final approval for publication.

Data accessibility

Data deposited in the Dryad Digital Repository.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.09.294.

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