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# The psychoactive pollutant fluoxetine compromises antipredator behaviour in fish☆

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ABSTRACT

Pharmaceuticals are increasingly being detected in aquatic ecosystems worldwide. Particularly concerning are pharmaceutical pollutants that can adversely impact exposed wildlife, even at extremely low concentrations. One such contaminant is the widely prescribed antidepressant fluoxetine, which can disrupt neurotransmission and behavioural pathways in wildlife. Despite this, relatively limited research has addressed the behavioural impacts of fluoxetine at ecologically realistic exposure concentrations. Here, we show that 28-day fluoxetine exposure at two ecologically relevant dosages—one representing low surface water concentrations and another representing high effluent flow concentrations—alters antipredator behaviour in Eastern mosquitofish (*Gambusia holbrooki*). We found that fluoxetine exposure at the lower dosage resulted in increased activity levels irrespective of the presence or absence of a predatory dragonfly nymph (*Hemianax papuensis*). Additionally, irrespective of exposure concentration, fluoxetine-exposed fish entered the predator 'strike zone' more rapidly. In a separate experiment, fluoxetine exposure reduced mosquitofish freezing behaviour—a common antipredator strategy—following a simulated predator strike, although, in females, this reduction in behaviour was seen only at the lower dosage. Together, our findings suggest that fluoxetine can cause both non-monotonic and sex-dependent shifts in behaviour. Further, they demonstrate that exposure to fluoxetine at environmentally realistic concentrations can alter antipredator behaviour, with important repercussions for organismal fitness.



## 1. Introduction

Pharmaceuticals are increasingly being detected in the environment, with approximately 600 of the 5000 actively manufactured pharmaceuticals having been reported in ecosystems worldwide (Küster and Adler, 2014). Indeed, pharmaceutical pollution has recently been recognised as an emerging environmental problem (Boxall et al., 2012; Arnold et al., 2014). One group of pharmaceuticals of particular concern is the selective serotonin re-uptake inhibitors (SSRIs), a class of antidepressants. These compounds (e.g., citalopram, sertraline and fluoxetine) have been repeatedly detected in the environment. In particular, fluoxetine has been detected in aquatic environments worldwide, with surface water detections typically ranging from <1 to 66 ng/L (e.g., Kolpin et al., 2002; Metcalfe et al., 2003; Glassmeyer et al., 2005; Fernández et al., 2010; González Alonso et al., 2010; Metcalfe et al., 2010; Yoon et al., 2010; Birch et al., 2015), to as high as 929 ng/L in direct sewage effluent (Bueno et al., 2007). Fluoxetine exhibits its primary pharmacological action on the serotonergic system, which is thought to play a key role in regulating a number of important behavioural and physiological functions, including, but not limited to feeding, locomotion, reproduction, aggression, fear and anxiety (Lucki, 1998; Lillesaar, 2011). Importantly, fluoxetine has the potential to impact non-target species, with its primary target molecule (serotonin transporter, 5-HTT)—along with other potential targets—being present in a wide variety of taxa (Ford and Fong, 2015), including in many fish species (e.g., Wang et al., 2006; Gould et al., 2007).

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Despite increasing concern surrounding the ecological effects of fluoxetine, it remains unclear whether exposure at environmentally realistic concentrations can alter the behaviour of wildlife (Sumpter et al., 2014). While recent studies have reported behavioural alterations in aquatic organisms resulting from acute exposure to environmentally realistic fluoxetine concentrations (e.g., De Lange et al., 2006; Painter et al., 2009; Barry, 2012; Winder et al., 2012; Bossus et al., 2014), studies employing exposure durations greater than 2 weeks are relatively uncommon. This is surprising given that the long-term therapeutic (anxiolytic-like) effects of fluoxetine are thought to be driven by adaptive changes within neurons (altered expression of 5-HT receptors), a process which can take up to 2–4 weeks (Gardier et al., 1996; Hensler, 2003). Therefore, it is possible that the anxiolytic-like effects of fluoxetine on non-target species are similarly time dependent (Stewart et al., 2014).

From an ecological perspective, understanding the potential impacts of fluoxetine and other widespread pharmaceutical pollutants on animal behaviour is crucial. Behaviour is the link between an organism's internal physiological processes and its environment, with alterations in behaviour having the potential to directly impact fitness (reviewed in Candolin and Wong, 2012; Sih, 2013; Wong and Candolin, 2014). In this regard, it is important that we address the effects of fluoxetine, as well as other pharmaceutical pollutants, from an ecological perspective, using behaviours with a direct bearing on individual and population-level fitness (Brodin et al., 2014)—such as the ability to avoid, and escape from, predators (Lima, 1998).

Here, using two separate experiments, we test the effects of 28-day fluoxetine exposure on antipredator behaviours of Eastern mosquitofish (*Gambusia holbrooki*) using environmentally realistic concentrations. The lower exposure treatment reflected levels typically reported in environmental surface water, whereas the higher exposure treatment reflected levels reported in and around wastewater effluent flow (see below). In the first experiment, we tested the impact of fluoxetine exposure on the performance of predator avoidance behaviour in the presence of a sympatric dragonfly nymph predator. In the second, we tested the effects of fluoxetine exposure on the predator escape behaviour of fish in response to a simulated predator strike.

## 2. Materials and methods

### 2.1. Animal collection and housing

Mosquitofish used in this study were wild-caught from the Science Centre Lake (37° 54′ 28″ S, 145° 08′ 16″ E), Monash University, Victoria, Australia. Water samples drawn from the lake revealed no fluoxetine contamination (Envirolab Services, unpublished data). Prior to experimentation, fish were acclimated to laboratory conditions (24–26 °C; 12:12 h light:dark cycle) for 3 months in mixed-sex holding tanks (80 × 45 × 45 cm, 128 L; stocking density: 100 fish per tank). Fish were fed *ad libitum* once daily with commercial fish food (Otohime Hirame larval diet; 580–910 μm).

### 2.2. Chemical exposure and monitoring

A 28-day fluoxetine exposure was performed using a flow-through system, following the design of Saaristo et al. (2013) and Bertram et al. (2015). Briefly, fish were randomly assigned to one of three exposure treatments: freshwater control, low fluoxetine and high fluoxetine. For each treatment, a large glass mixing tank (182 L) fed water into four identical separate-sex aquaria housing 30 fish (two tanks per sex; 60 × 30 × 30 cm, 54 L). During the exposure, fish were kept under a 12:12 h light:dark cycle and temperatures maintained at 24.4 ± 0.8 °C (±SD). Flow-through rates were maintained at 24 h cycling (~1.67 L/h per tank).

For the low- and high-fluoxetine treatments, a stock solution of fluoxetine was continuously added to the mixing tank (1.95 mL/min). The stock solutions (3 L) were prepared and changed daily. To achieve this, fluoxetine hydrochloride (Sigma-Aldrich; Product Number: F132, CAS: 56296-78-7) was dissolved in advance in 1 mL of methanol (32.1 μg/mL for high treatment and 321.0 μg/mL for low treatment). Then, on the day that the stock solutions were required, the methanol solvent was evaporated under a gentle nitrogen flow for 15 min before being diluted with 2999 mL of Milli-Q water. During the 28-day exposure period, 1 L water samples were periodically drawn from all exposure tanks to monitor fluoxetine concentrations (see below for measured concentrations). Specifically, following Anumol et al. (2013), the concentration of fluoxetine in each sample was analysed using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). Compound separation was achieved using an Agilent 1210 binary pump (Palo Alto, CA) equipped with a ZORBAX Eclipse Plus reverse phase column (2.1 × 50 mm). The analysis was performed using an Agilent 1210 UHPLC connected to an Agilent 6410 triple quadruple mass spectrometer (QQQ). Blank and laboratory control samples (LCS) used as quality control samples were analysed with each batch of nine samples. There was no background contamination present in blank samples and LCS recoveries were in an acceptable range (fluoxetine recovery: 70–100%; $n = 6$).

### 2.3. Experiment one: predator avoidance

To investigate the effects of fluoxetine exposure on predator avoidance behaviour, a 3 × 2 factorial design was used, incorporating exposure treatment (unexposed, low fluoxetine and high fluoxetine) and predation risk (presence versus absence of dragonfly nymph). Measured fluoxetine concentrations in the low and high treatments were 25 ± 18 ng/L (mean ± SD, $n = 12$) and 226 ± 172 ng/L ($n = 12$), respectively.

Australian emperor dragonfly nymphs (*Hemianax papuensis*) were used as a predator stimulus, having been sourced from water bodies surrounding Geelong (Victoria, Australia). All nymphs were captured from the wild 14 days before experimental trials, during which time they were not fed, in order to standardise their hunger levels. A dragonfly nymph was selected as the predator model because large nymphs (like those of *H. papuensis*) are known to predate upon small fish (Pritchard, 1964) and have been used as a predatory stimulus in similar experiments (Squires et al., 2008; Barry, 2012, 2014). Additionally, *G. holbrooki* and *H. papuensis* share similar habitat preferences (Rowe, 1987; Pyke, 2005) and have been recorded sympatrically over a significant portion of their range in Australia (ALA, 2016a,b), including the source population of mosquitofish used in this study (*pers. obs.*).

Fish behaviour in the presence or absence of a dragonfly nymph was recorded in an observation tank (60 × 30 × 30 cm, 54 L), with 5 cm grid lines dividing the bottom of the arena. For each trial, focal fish were selected at random from exposure tanks and allocated to one of three observation tanks. Observation tanks were filled to a depth of 5 cm with aged water, with all tanks being emptied and dried between trials to control for any potential cross-contamination of chemical cues. In the predator-exposure trials, unexposed (male: $n = 19$, female: $n = 19$), low-fluoxetine exposed (male: $n = 16$, female: $n = 20$) and high-fluoxetine exposed (male: $n = 20$, female: $n = 19$) fish were individually presented with the visual and chemical cues of dragonfly nymphs. This was achieved by confining a nymph to one side of the observation tank in a small glass cage (6 × 2 × 2 cm) with a mesh net opening at one end

(2 × 2 cm) that allowed predator chemical cues to enter the tank environment. Trials in the absence of a predator were also conducted, where unexposed (male: $n = 19$, female: $n = 20$), low-fluoxetine exposed (male: $n = 16$, female: $n = 20$) and high-fluoxetine exposed (male: $n = 20$, female: $n = 20$) fish were presented with an identical, but empty, glass cage. Before the beginning of each trial, fish were acclimated for 5 min behind a clear partition positioned 10 cm from the edge of the tank on the opposite side to the predator. The partition was remotely lifted after acclimation, allowing the focal fish to freely explore the tank while its behaviour was recorded from above (Canon PowerShot S120).

Over the 5 min trial, we measured three behaviours: activity level, latency to enter the 'strike zone' and the total number of entries into the 'strike zone'. Activity level was measured by counting the total number of 5 cm grid lines crossed throughout the trial. Latency to enter the 'strike zone' and number of entries into the 'strike zone' were measured by observing the time taken for the fish to first enter the 1 cm zone around the perimeter of the predator cage and, subsequently, the number of times the fish ventured into this zone during the course of the trial. This zone represented the predator's striking range (i.e., 'strike zone') and was based on previously reported hunting tactics of *H. papuensis* nymphs (Rowe, 1987). Specifically, this strike zone was based on the length of the nymphs' striking mouth appendage or labium (mean length ± SD = 1.01 ± 0.14 cm, $n = 30$). All behaviours were quantified from video recordings using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007).

### 2.4. Experiment two: predator escape

To investigate the impacts of fluoxetine exposure on predator escape behaviour, mosquitofish were subjected to a simulated predator strike. In this experiment, the measured concentrations of fluoxetine in the low and high exposures were 8 ± 7 ng/L (mean ± SD, $n = 10$) and 97 ± 45 ng/L ($n = 12$), respectively. As with Experiment 1, behavioural trials were performed using randomly selected fish from unexposed (male: $n = 35$, female: $n = 34$), low-fluoxetine (male: $n = 33$, female: $n = 33$) and high-fluoxetine (male: $n = 34$, female: $n = 34$) exposure tanks.

Trials were conducted in an observation tank (12 × 12 × 5 cm, 0.72 L) filled to 3 cm with aged water. Trials took place in shallow water to limit the vertical displacement of fish during the escape response, to more accurately measure horizontal escape velocity (Langerhans et al., 2004). Prior to behavioural recordings, fish were acclimated for 5 min in the observation tank, before a cylindrical metal probe with a rubber stopper (5 mm in diameter) was dropped into the tank to elicit an escape response. The probe was dropped within 3 cm of the fish, following Langerhans et al. (2004). To ensure the probe was dropped in a consistent manner, an automated lever system was used (see Fig. S1). The escape response of the focal fish was recorded from above (Canon PowerShot S120). Between trials, observation tanks were emptied and dried.

For each fish, two predator escape behaviours were measured after the simulated predator strike was delivered: the C-start escape response and the freezing response. Firstly, C-start escape response is a reflexive escape behaviour, initiated in response to a fear stimulus (e.g., a predator strike). The C-start escape is common among many species of fish (as well as amphibians) and is characterised by three distinct stages: rest, C-bend and propulsion (Hale et al., 2002). Each of these three stages were represented by a single camera frame captured at 30 frames per second, following Langerhans et al. (2004) and Grigaltchik et al. (2012). Specifically, frame one represented the rest stage prior to the probe being dropped. Frame two captured the C-bend, immediately after the probe was dropped. Lastly, frame three captured the propulsion stage, where the focal fish moved rapidly away from the fear stimulus. The C-start velocity (cm/sec) was calculated as the distance travelled between frame two and frame three using a point of mass tracking software (TrackerV8; Open Source Physics, USA). Not all fish exhibited C-start behaviour, with the performance of the C-start therefore being recorded as a binary response. Fish that did not perform a C-bend by frame two were given a score of zero.

Secondly, the amount of time spent by fish exhibiting freezing behaviour was recorded for 1 min following the deployment of the simulated predator strike. Freezing behaviour is another common antipredator behaviour in fish, in which the fish ceases all movement (except for those involved in respiration) after a fear-inducing stimulus (Godin, 2002; Brown and Magnavacca, 2003). Freezing behaviour is a strategy of crypsis, used to avoid detection when a predator is within close proximity or has already launched a strike (Godin, 2002). The total time spent exhibiting freezing behaviour was calculated using velocity data from each frame over 1 min (frame duration: $^1/_3$ sec). Specifically, total freezing time reflected the total amount of time that a fish spent moving less than 0.5 cm/s.

### 2.5. Morphological analysis

In both experiments, immediately after behavioural trials, fish were euthanised with an overdose of anaesthetic clove oil (40 mg/L) and morphological measurements were taken. Fish were weighed (±0.0001 g) and their standard length measured (±0.01 mm). An index of fish condition was then derived from a regression of fish mass (g) against standard length (mm). This condition index was calculated as the residuals from the least squares regression line and was calculated for males and females separately. Condition index was used as a proxy for health and was compared to investigate any possible shifts in fish heath as a result of fluoxetine exposure.

### 2.6. Statistical analysis

Data were analysed in R version 3.2.2 (R Development Core Team, 2015) and were checked for normality (Shapiro-Wilk test; *shapiro.test* function; Royston, 1995) and homogeneity of variance (Fligner-Killeen test; *fligner.test* function; Conover et al., 1981) as appropriate. Additionally, exposure tank number—as a measure of tank effects—was initially included in all models, but did not significantly affect any of the response variables. Therefore, exposure tank was excluded from all final models to increase the predictive power of our analyses.

For experiment one, models testing the impacts of fluoxetine exposure on predator avoidance behaviours included three predictors (fluoxetine treatment, predator presence or absence and fish sex), as well as one covariate (condition index). Activity (number of 5 cm grid lines crossed) was analysed using a three-way Analysis of Covariance (ANCOVA) (*aov* function; Chambers and Hastie, 1992). Tukey's Post Hoc testing (*glht* function, *multcomp* package; Hothorn et al., 2008) was used to further investigate relationships between treatment groups. A Cox Proportional Hazards Survival Analysis (*survreg* function, *survival* package; Therneau and Grambsch, 2000) was used to compare the latency of fish to enter the strike zone. A *Weibull* hazard function was selected as the most appropriate distribution for the model, determined by a comparative analysis of multiple hazard distributions using an ANOVA. The model met the assumption of proportionality, as tested by examining the interaction between Schoenfeld residuals and log time (*coxph* and *cox.zph* functions, *survival* package; Grambsch and Therneau, 1994). Lastly, the number of entries by fish into the strike zone was analysed using a Generalised Linear Model (GLM). Vuong tests (*vuong* function, *pscl* package; Vuong, 1989) indicated

zero-inflation of count data. To address this, a zero-inflated Poisson GLM (ZIP GLM) was used (*zeroinfl* function, *pscl* package; Zeileis et al., 2008).

In experiment two, the number of C-starts performed was compared among treatments using a chi-square test (*chisq.test* function; Patefield, 1981). C-start velocity data were square root transformed and compared among treatments using a two-way ANCOVA. The ANCOVA included two predictors (treatment and fish sex), as well as one covariate (condition index). Total time spent performing freezing behaviour following the fear stimulus (sec) was rank-transformed and tested using a two-way ANCOVA. This model revealed a significant interaction between treatment and sex. As a result, the sexes were subsequently analysed separately to identify the main effect of fluoxetine. Tukey's Post Hoc testing was used to further examine the relationships between treatments.

For each experiment, the condition index of fish was also compared among treatments using three-way ANOVAs. To meet assumptions of normality, condition index was transformed using a rank normal function.

## 3. Results

### 3.1. Experiment one: predator avoidance

In the predator avoidance experiment, fluoxetine exposure significantly impacted fish activity levels (ANOVA: $F_{2,222} = 3.78$, $p = 0.025$), though no difference was detected in activity levels in the presence versus absence of a predator (ANOVA: $F_{1,222} = 0.34$, $p = 0.560$; Table S1), or between the sexes (ANOVA: $F_{1,222} = 0.90$, $p = 0.344$; Table S1). Fluoxetine exposure caused a non-monotonic shift in activity. Specifically, fish in the low-fluoxetine treatment were significantly more active than unexposed fish (Tukey's HSD: $t = 2.66$, $p = 0.023$; Fig. 1), while there was no difference in activity between high versus unexposed, or low versus high, fluoxetine treatments (Tukey's HSD: $t = 1.90$, $p = 0.144$ and $t = 0.82$, $p = 0.692$, respectively; Fig. 1). Latency to enter the strike zone was significantly reduced in both fluoxetine treatments compared to unexposed fish (Cox Regression Survival Analysis: low treatment, $z = -2.20$, $p = 0.028$; high treatment, $z = -2.94$, $p = 0.003$; Fig. 2). However, the latency to enter the strike zone was consistent between trials with and without a predator (Cox Regression Survival Analysis: $z = 0.70$, $p = 0.482$; Table S1). Additionally, across all treatments (regardless of exposure), male fish more rapidly entered the strike zone compared to females (Cox Regression Survival Analysis: $z = -4.06$, $p < 0.001$; Table S1). There was no difference in the number of strike zone entries between fluoxetine-exposed and unexposed fish, with an average of 3.31 ± 0.30 (mean ± SE) for unexposed, 3.61 ± 0.34 for low-exposed and 3.80 ± 0.43 for high-exposed fish (ZIP GLM: comparison between control and low, $z = 0.20$, $p = 0.839$; control and high, $z = -0.26$, $p = 0.795$; low and high, $z = -1.29$, $p = 0.199$). Similarly, there was no difference in strike zone entries for trials with and without a predator (ZIP GLM: $z = -0.02$, $p = 0.982$; Table S1), although, across trials, males were found to enter the strike zone significantly more often than females (ZIP GLM: $z = 4.72$, $p < 0.001$; Table S1).

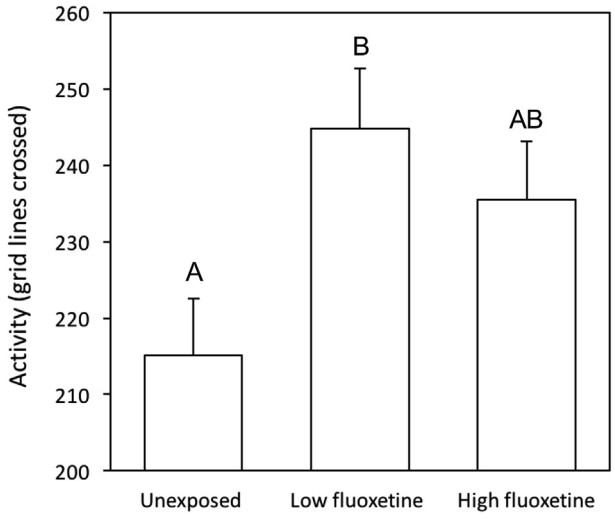


**Fig. 1.** Mean (+SE) activity levels (i.e., number of 5 cm grid lines crossed) for unexposed ($n = 77$), low-fluoxetine ($n = 72$) and high-fluoxetine ($n = 79$) treatments. Treatments without letters in common are significantly different.

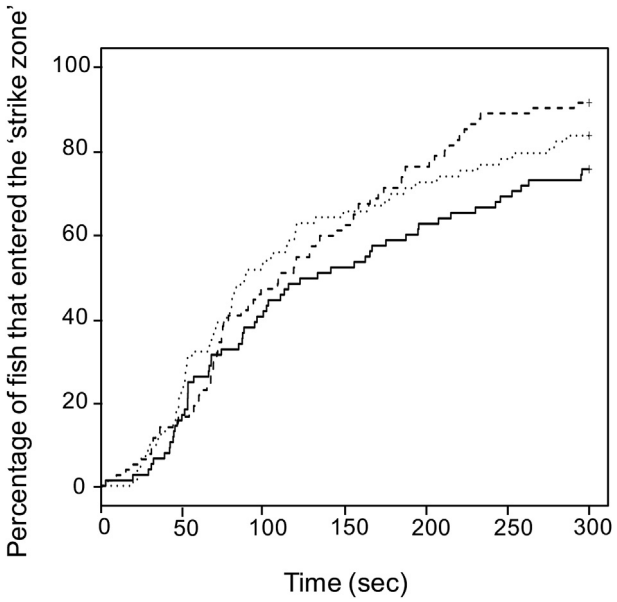


**Fig. 2.** Percentage of fish that entered the 'strike zone' over time, with the unexposed treatment represented by a solid line ($n = 77$), low-fluoxetine treatment by a long dash ($n = 72$) and high-fluoxetine treatment by a short dash ($n = 79$).

### 3.2. Experiment two: predator escape

The number of fish that performed a C-start response did not differ significantly between treatments (unexposed: 79.71%, low-fluoxetine exposed: 78.79%, high-fluoxetine exposed: 78.26%; Chi-squared test: $\chi^2 = 0.040$, df = 2, $p = 0.980$). Likewise, C-start escape velocity (cm/sec) did not differ across treatments, with a mean ± SE escape velocity of 32.19 ± 1.68 for unexposed fish, 32.75 ± 1.94 for low-exposed fish and 30.36 ± 1.80 for high-exposed fish (ANOVA: $F_{2,156} = 0.48$ $p = 0.620$). In addition, there was no effect of sex on C-start velocity (ANOVA: $F_{1,156} = 0.23$, $p = 0.635$), with the average C-start velocity of males and females being 32.68 ± 1.49 and 30.96 ± 1.44, respectively. There was, however, a significant interaction between treatment and sex on freezing behaviour (sec) following the simulated strike (ANOVA: $F_{2,192} = 5.16$, $p = 0.006$). Freezing behaviour of both male and female fish was altered by fluoxetine exposure (ANOVA: males $F_{2,99} = 6.92$ $p = 0.001$ and females $F_{2,97} = 9.10$, $p < 0.001$), although the effects of fluoxetine were sex-dependent. Specifically, males exposed to both low and high treatments exhibited significantly reduced freezing behaviour compared to unexposed fish (Tukey's HSD: $t = -2.52$, $p = 0.035$ and $t = -3.63$, $p = 0.001$, respectively; Fig. 3). For females, fluoxetine exposure had a non-monotonic effect

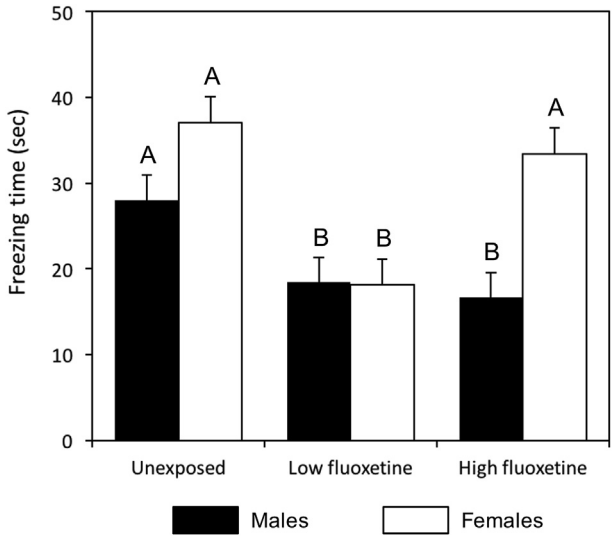


**Fig. 3.** Mean (+SE) freezing time (sec) over 1 min following a simulated predator strike for unexposed (males: $n = 35$; females: $n = 34$), low-fluoxetine (males: $n = 33$; females: $n = 33$) and high-fluoxetine (males: $n = 35$; females: $n = 34$) treatments. Treatments without letters in common are significantly different.

on freezing behaviour, with low-fluoxetine exposure resulting in a reduction of freezing behaviour compared to both unexposed and high-exposed fish (Tukey's HSD: $t = -4.02$, $p < 0.001$ and $t = -3.36$. $p = 0.003$, respectively; Fig. 3), while no difference was detected between unexposed and high-exposed fish (Tukey's HSD: $t = -0.66$, $p = 0.786$; Fig. 3). Additionally, males (regardless of exposure) generally spent less time exhibiting freezing behaviour, with a mean ± SE of 29.60 ± 1.90 s, compared to females at 21.12 ± 1.73 s ($F_{1,192} = 14.28$, $p < 0.001$).

### 3.3. Morphology

No significant differences were detected in condition index between unexposed, low-exposed and high-exposed treatments for fish used in the predator avoidance experiment (ANOVA: $F_{2,225} = 0.06$, $p = 0.940$; Table S2). Similarly, there were no differences in condition index across unexposed, low-exposed and high-exposed treatments for fish used in the predator escape experiment (ANOVA: $F_{2,201} = 0.13$, $p = 0.880$; Table S2).

## 4. Discussion

We found that exposure to environmentally relevant concentrations of fluoxetine increased mosquitofish locomotor activity regardless of the presence or absence of a predator, as well as decreasing the time taken to enter a predator's 'strike zone'. Fluoxetine exposure, however, did not affect the number of times fish entered the striking range of a predator. Additionally, while there was no effect of fluoxetine on C-start escape performance, fluoxetine-exposed fish showed a reduction in freezing behaviour following a simulated predator strike. Further, the behavioural shifts seen in both the predator avoidance and predator escape experiments occurred at comparatively lower fluoxetine concentrations (25 and 8 ng/L, respectively) than have previously been reported to have induced behavioural effects in vertebrates.

The general increase in activity levels—in both the presence and absence of the predator—seen at the low-fluoxetine dosage (i.e., 25 ng/L) contrasts with previous studies that have tested the effects of environmentally relevant fluoxetine exposure in other taxa (Barry, 2012, 2014; Winder et al., 2012). Specifically, Barry (2012) found that Arabian killifish (*Aphanius dispar*) showed a decrease in activity after exposure to fluoxetine at 300 ng/L and in the presence of conspecific alarm cues, with no difference between the lower dosage and control treatment (30 ng/L). Additionally, Barry (2014) found that tadpoles of the Arabian toad (*Bufo arabicus*) reduced their activity when exposed to 300 and 3000 ng/L of fluoxetine, though, again, no effect was reported at a lower dosage (30 ng/L). The differences between these findings could potentially be explained by differences in exposure scenarios, with Barry (2012, 2014) employing a shorter duration of exposure (7 and 14 days, respectively) in comparison to the present study (28 days). Acute and chronic fluoxetine exposures have been shown to produce different, and even conflicting, behavioural effects. Specifically, acute SSRI exposure seems to increase anxiety-like behaviour, while chronic exposure reduces the occurrence of such behaviour (Herculano and Maximino, 2014). The acute effects of fluoxetine are predominately the result of enhanced serotonergic neurotransmission, while fluoxetine's therapeutic (anxiolytic-like) effects—which can take 2–4 weeks to manifest—are driven by altered expression of serotonin receptors (Gardier et al., 1996; Hensler, 2003; Stewart et al., 2014). Therefore, it is possible that the increased activity levels seen in the present study are a result of the chronic effects of fluoxetine through neuronal adaptation (i.e., therapeutic effects), whereas the reduced activity observed by Barry (2012, 2014) may have been a result of the anxiety-inducing effects of acute exposure. More broadly, our findings are concordant with the recent study of Kellner et al. (2016), where 21-day exposure to another SSRI, citalopram, increased swimming activity in three-spined sticklebacks (*Gasterosteus aculeatus*) (Kellner et al., 2016).

In addition, we found evidence of a non-monotonic dose-response relationship, a phenomenon that has increasingly been detected as a result of low level antidepressant exposure (reviewed in Ford and Fong, 2015). Specifically, only fish exposed to the lower fluoxetine dosage (and not the higher dosage) showed a significant increase in activity levels (number of grid lines crossed) compared to unexposed fish. Broadly, these results are concordant with a number of studies that have similarly reported non-monotonic dose-response relationships to fluoxetine in various freshwater species (e.g., De Lange et al., 2006; Painter et al., 2009; Sánchez-Argüello et al., 2009; Guler and Ford, 2010; Di Poi et al., 2013; Bossus et al., 2014). These non-linear behavioural shifts resulting from low-dosage fluoxetine exposures seem to parallel those reported in endocrine disrupting chemicals (Vandenberg et al., 2012; Ford and Fong, 2015). Such findings underscore the fact that many of the potential causes for non-monotonic responses described for endocrine disrupting chemicals (reviewed in Vandenberg et al., 2012) may also be relevant to neurotransmission and neuroendocrine disruptors, such as fluoxetine.

Fluoxetine exposure reduced the average time taken for fish to enter the predator 'strike zone', as was evident in both fluoxetine treatments. In this respect, we rule out the possibility that reduced latency to enter the strike zone was merely a result of heightened activity, given that fish exposed to the higher treatment did not significantly differ in activity levels from control fish and yet, despite this, ventured into the strike zone more rapidly. Instead, it would appear that both low- and high-exposed fish are actively investigating and approaching the predator strike zone, irrespective of whether the predator was present or absent. Given that mosquitofish responded similarly to both the dragonfly predator and the empty glass cage (predatory control), it is possible that they did not perceive the dragonfly nymphs as a predatory threat. In this regard, a lack of dietary cues (i.e., alarm cues from recently consumed prey)—with nymphs having not been fed for a week to

standardise hunger levels—may have contributed to them having been perceived as less threatening (Smith and Belk, 2001; Ward and Mehner, 2010). Despite this, the nymphs used in this experiment would be expected to pose a predatory threat, particularly given that starved predators are likely to have the highest motivation to feed (e.g., Altwegg, 2003). Conversely, it is possible that the presence of both the predator and the empty glass cage resulted in similar levels of avoidance or, alternatively, inspection-like behaviour. In many fish species, including mosquitofish, individuals will actively approach novel objects or predators to gain information about potential resources and threats ('inspection behaviour') (Godin and Davis, 1995; Smith and Belk, 2001). Active inspection behaviour would explain why both low- and high-exposed fish demonstrated reduced latency to enter the 'strike zone', regardless of activity levels. Further, citalopram exposure (1.5 and 15 μg/L for 21 days) has previously been shown to increase inspection-like behaviour of a novel object in three-spined sticklebacks (Kellner et al., 2016). Critically, irrespective of why the fish responded similarly across trials with and without the predator, the fact that fluoxetine-exposed fish were more active and more readily entered the 'strike zone', could, in turn, increase their vulnerability to predation (Skelly, 1994; Johansson, 1995).

For the predator escape experiment, we found that fluoxetine exposure did not affect the C-start escape ability of fish. Exposed fish performed a similar number of C-start escapes, and did so at a similar velocity, compared with unexposed fish. This was surprising given that serotonin has been reported to have an inhibitory effect on Mauthner cells, neurons thought to be responsible for initiating C-start behaviour (Korn and Faber, 2005). Despite this, our results are comparable to those of Painter et al. (2009), where no difference was detected in the C-start ability of the adult fathead minnow (*Pimephales promelas*) following a 2-week fluoxetine exposure (at 25, 125 and 250 ng/L). The similar results reported in the present study and by Painter et al. (2009) suggest that 12–28 day exposure to environmentally relevant levels of fluoxetine (9–250 ng/L) may not be sufficient to significantly impact C-start escape ability in adult fish.

Fluoxetine exposure decreased the amount of freezing behaviour performed by fish subsequent to a simulated predator strike. This result may again be mediated by the anxiolytic effects of fluoxetine, causing a reduction in fear-like responsiveness to a potentially threatening situation. More generally, other SSRIs have also been shown to reduce fear-like responsiveness. Specifically, Endler guppies (*Poecilia wingei*) exposed to citalopram (2.3 and 15 μg/L for 21 days) reduced their freezing behaviour (among other anxiety-related behaviours) in a novel diving test (Olsén et al., 2014). In the present study, the effect of fluoxetine on freezing behaviour also appears to be sex-dependent. Females, but not males, demonstrated a non-monotonic dose-response relationship in freezing behaviour, exhibiting a reduction in freezing behaviour in the low-fluoxetine treatment only (i.e, 8 ng/L). By contrast, males showed a significant decrease in freezing behaviour at both low and high dosages (i.e, 8 and 97 ng/L). Sex-specific fluoxetine sensitivities have been reported previously in clinical studies using human and animal models (Dalla et al., 2010; Kercmar and Majdic, 2014). The sex differences observed in this study emphasise the importance of testing the ecological impacts of fluoxetine exposure on both males and females. Intriguingly, in both experiments, we also saw evidence of sex differences in behaviours regardless of exposure. Although this was not the focus of our study, these findings suggest that male and female mosquitofish differ in their behavioural responses to predatory stimuli.

In summary, we report that 28-day exposure to fluoxetine at environmentally relevant concentrations resulted in altered antipredator behaviour of adult fish. Further, some of these behavioural shifts seem to reflect non-monotonic and sex-dependent dose-responses. The behavioural shifts seen here could affect prey vulnerability, since active individuals that readily approach potential predators are more likely to be detected and captured (Skelly, 1994; Johansson, 1995). Moreover, the observed reduction in freezing behaviour in fluoxetine-exposed fish may increase the likelihood of detection (or re-detection) and capture by a predator following an initial strike. Indeed, our results suggest that fluoxetine exposure at environmentally realistic concentrations can alter antipredator behaviour and, in doing so, compromise the fitness of exposed wildlife. More broadly, such findings highlight the potential for pharmaceutical contaminants to affect ecosystem function and stability by altering the behavioural dynamics of predator-prey interactions (Brodin et al., 2014; Wong and Candolin, 2014).

## Ethics

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

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## Conflict of interest statement

The authors declare the inexistence of any conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.10.010.

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