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Widespread psychoactive pollutant augments daytime restfulness and disrupts diurnal activity rhythms in fish

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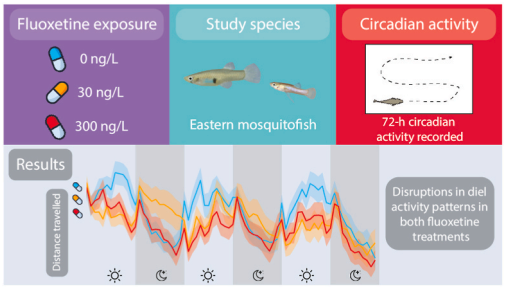
HIGHLIGHTS

- Pharmaceutical compounds are entering the environment at an unprecedented rate.
- The pharmaceutical pollutant fluoxetine disrupts circadian rhythms of wild fish.
- Disruptions to diel activity patterns was driven by increased daytime inactivity.
- Misalignments in circadian rhythms have been shown to adversely affect lifespan.

GRAPHICAL ABSTRACT



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ABSTRACT

Pharmaceutical pollution is a major driver of global change, with the capacity to alter key behavioural and physiological traits in exposed animals. Antidepressants are among the most commonly detected pharmaceuticals in the environment. Despite well-documented pharmacological effects of antidepressants on sleep in humans and other vertebrates, very little is known about their ecologically relevant impacts as pollutants on non-target wildlife. Accordingly, we investigated the effects of acute 3-day exposure of eastern mosquitofish (*Gambusia holbrooki*) to field-realistic levels (nominal concentrations: 30 and 300 ng/L) of the widespread psychoactive pollutant, fluoxetine, on diurnal activity patterns and restfulness, as indicators of disruptions to sleep. We show that exposure to fluoxetine disrupted diel activity patterns, which was driven by augmentation of daytime inactivity. Specifically, unexposed control fish were markedly diurnal, swimming farther during the day and exhibiting longer periods and more bouts of inactivity at night. However, in fluoxetine-exposed fish, this natural diel rhythm was eroded, with no differences in activity or restfulness observed between the day and night. As a misalignment in the circadian rhythm has been shown to adversely affect fecundity and lifespan in animals, our findings reveal a potentially serious threat to the survival and reproductive success of pollutant-exposed wildlife.

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1. Introduction

Contamination of the environment by pharmaceutical products is a serious global problem. Of the thousands of commercially available pharmaceutical compounds, more than 900 active compounds, and their transformation products, have been detected in the environment worldwide (Graumnitz and Jungmann, 2021; Küster and Adler, 2014). Pharmaceutical pollution is so pervasive that active pharmaceuticals have been found in the environment across all continents, including Antarctica (Wilkinson et al., 2022). Once in the environment, most compounds are 'pseudo-persistent' meaning that they are continuously discharged into the environment, resulting in chronic exposure of wildlife (Arnold et al., 2014). Typically, the target receptors of these pharmaceuticals are evolutionarily conserved across taxa (Gunnarsson et al., 2008), and it is therefore unsurprising that effects can be exerted on diverse, non-target species (Fent et al., 2006). Indeed, many studies have previously shown that exposure to environmental pharmaceutical pollutants can have adverse effects on a wide range of key fitness traits, including reproductive physiology, development, and aggressive, exploratory and social behaviours (reviewed in Brodin et al., 2014; Fent et al., 2006; Saaristo et al., 2018).

Selective serotonin reuptake inhibitors (SSRIs), a class of psychoactive compounds typically prescribed to treat depression in humans, are one of the most common groups of pharmaceutical pollutants (Gould et al., 2021; Mole and Brooks, 2019; Silva et al., 2012). SSRIs primarily inhibit the serotonin transport molecule (or SERT), preventing the re-uptake of serotonin, resulting in increased extracellular serotonin concentrations (McDonald, 2017). Fluoxetine (marketed as Prozac®), is among the most prescribed SSRIs worldwide (Gould et al., 2021; Wong et al., 2005), and has been measured in natural waterways at surface concentrations ranging from below 1–330 ng/L (reviewed in Mole and Brooks, 2019). Moreover, fluoxetine has been shown to bioaccumulate in tissues of exposed animals, including fishes (e.g., Arnnok et al., 2017; Brooks et al., 2005; David et al., 2018; Martin et al., 2019b), which are likely to be affected by fluoxetine's neuroendocrine-disrupting properties (Kreke and Dietrich, 2008). Indeed, fluoxetine has been shown to have adverse physiological effects on exposed animals, such as altered neurodevelopment (Bidel et al., 2016; Foster et al., 2010) and reproductive physiology (Campos et al., 2016; Mennigen et al., 2008). Recent research has also revealed that a wide range of behaviours, such as aggression (McCallum et al., 2017), foraging (Martin et al., 2019c), anti-predator responses (Martin et al., 2017; Peters et al., 2017), and reproductive behaviours (Bertram et al., 2018; Martin et al., 2019a) are influenced by fluoxetine exposure.

Antidepressants, including fluoxetine, also influence sleep timing, duration, composition, and continuity. For example, fluoxetine reduces the amount of rapid eye movement (REM) sleep in humans (Khawam et al., 2006; Vasar et al., 1994), decreases REM and increases non-rapid eye movement (NREM) sleep in golden hamsters (*Mesocricetus auratus*; Gao et al., 1992), and augments daytime restfulness and fragments nighttime sleep in juvenile rhesus macaques (*Macaca mulatta*; Golub and Hogrefe, 2016). While it remains unclear whether fishes have sleep states comparable to those reported in other vertebrates, they nonetheless sleep (Blumberg et al., 2020; Leung et al., 2019; Ungurean et al., 2020). More generally, evidence in taxonomic groups as diverse as humans (Dawson and Reid, 1997; Van Dongen et al., 2003; Walker, 2008), birds (Aulsebrook et al., 2021; Johnsson et al., 2022) and bees (Klein et al., 2010), shows that sleep loss can impair performance when animals are awake. As such, modifications to sleep induced by fluoxetine exposure have the potential to influence a range of fitness-related traits, such as cognition in aquatic wildlife.

In animals where obtaining electrophysiological evidence for sleep is logistically or ethically challenging, sleep is typically defined as a rapidly reversible state of inactivity with increased arousal thresholds and circadian rhythmicity (Lesku et al., 2019; Siegel, 2009). Aquatic organisms such as fish, which are especially vulnerable to the effects of pharmaceutical pollution exhibit such circadian inactivity rhythms (Reebs, 1992, 2002). As sleep deprivation impairs cognitive performance, swimming behaviour, susceptibility to predation, and mortality rates in fishes (Miner et al., 2021; O'Connor et al., 2019; Pinheiro-da-Silva et al., 2018), it is important to understand if environmental exposure of fish to psychoactive drugs, such as fluoxetine, can adversely affect their circadian-controlled restful behaviours. Studies that have previously looked at the impacts of fluoxetine (and pharmaceuticals in general) on diel activity in fishes have found varied effects. For example, short-term exposure of eastern mosquitofish (*Gambusia holbrooki*) to a chemical mixture of fluoxetine, triclosan, and diazinon eroded diel activity patterns in male, but not female, fish (Melvin et al., 2016). For fluoxetine specifically, at low environmental concentrations, diurnal patterns of activity were disrupted in turquoise killifish (*Nothobranchius furzeri*; Thoré et al., 2021), whilst this was not the case in eastern mosquitofish exposed to higher concentrations of fluoxetine (Melvin, 2017). These varied effects of fluoxetine on the diel rhythms of aquatic wildlife may be due to discrepancies between previous studies, such as differences in exposure duration, exposure concentrations, sex of individuals, and species-specific differences. As the threat of exposure to these environmental contaminants is now ever-present and increasing globally, we stress the importance of testing the generality and ecological relevance of these previous findings to build a body of literature that provides insight into how fluoxetine may be affecting circadian rhythms in vulnerable organisms in an environmentally relevant scenario. Accordingly, we tested the hypothesis that exposure to fluoxetine will affect diel activity rhythms and restfulness, a behavioural correlate of sleep (Kelly et al., 2021, 2022), in wild-caught mosquitofish (*Gambusia holbrooki*), taking into account fluoxetine exposure at environmentally relevant levels on a comparatively large sample size of both male and female individuals, with activity assayed constantly across the entire exposure duration. To do this, we exposed fish to one of three nominal fluoxetine concentrations (0, 30, 300 ng/L) and recorded their activity levels over three 24-h cycles with a 12:12 h light:dark photoperiod. Based on previously established pharmacological effects of fluoxetine (Gao et al., 1992; Golub and Hogrefe, 2016; Khawam et al., 2006; Vasar et al., 1994), and effects on diel activity seen in fish exposed to environmental concentrations of fluoxetine (Thoré et al., 2021), we hypothesised that exposure would affect daytime and/or nighttime restfulness in mosquitofish.

2. Materials and methods

2.1. Animal collection and maintenance

Wild sexually mature adult eastern mosquitofish of both sexes were collected from the Science Centre Lake (37°54′28″ S, 145°08′16″ E), Monash University, Victoria, Australia in March 2021. As fish were wild-caught, the exact age of individuals could not be discerned nor controlled. Water sampling at this site over consecutive years has revealed no contamination with fluoxetine (Envirolab Services, unpublished data). Fish were housed in mixed-sex glass holding tanks (60 × 30 × 30 cm; 20 cm water depth; ~20 fish per tank) filled with reverse osmosis (RO) water and containing a 2-cm deep gravel substrate (7 mm grain size), an air bubbler, and aquatic plants (Java moss, *Taxiphyllum barbieri*). Commercially bought aquarium salts were added to housing RO water prior to introduction of fish (Aquasonic tropical water conditioner). Animals were held in a controlled-temperature room (mean ± SD: 19.1 ± 0.4 °C) maintained on a 12:12 h light:dark photoperiod, with lights-on at 0700 h, and lights-off at 1900 h. Fish were fed until satiation five days a week with a mix of commercial pellets (Aquasonic Nutra Xtreme C1 pellets; 0.8 mm) and frozen bloodworms (Hikari frozen bloodworms). Fish were maintained in the laboratory under these conditions for six months prior to experimentation.

2.2. Experimental protocol and exposure regime

For experimental trials, individual mosquitofish were removed from their housing tank and transported to an experimental tank in their respective batches ($n = 140$ fish total, housed individually; 12 males and 12 females per batch). Under 12:12 h light:dark conditions, animals were given ~22 h to acclimate before fluoxetine exposure and observational recordings commenced at lights-on the following day. Following the acclimation period, individuals were assigned to one of three fluoxetine exposure treatments: a solvent control (i.e., no fluoxetine), low-fluoxetine (nominal concentration: 30 ng/L), or high-fluoxetine (nominal concentration: 300 ng/L), with 4 male and 4 female fish assigned to each exposure treatment per batch of 24 fish. To achieve the desired nominal fluoxetine concentration in each experimental tank, stock solutions were created by dissolving 31.2 μg or 312 μg (low and high concentration, respectively) of fluoxetine hydrochloride (Sigma Aldrich; product number: F132, CAS: 56296-78-7) in 100 mL of methanol. At the start of the experimental period (0700 h), each tank (2.6 L water volume) was dosed with a 0.5 mL aliquot of stock solution. To control for solvent (methanol) effects, and to maintain consistent levels of handling across treatments, 0.5 mL of methanol was added to all control tanks at this time.

Individuals were left in their respective exposure treatments for three experimental days (i.e., three 24-h cycles) and individuals were video-recorded over the entire experimental period. At noon (1200 h) on the first and third experimental day, 40-mL water samples were drawn from each tank for analytical verification, and fish were fed a standardised amount of two frozen bloodworms at noon on the second experimental day. The animals were otherwise left undisturbed in the experimental room, with the experiment video recorded for later analysis. After completion of the three-day experimental period, fish were removed from experimental tanks and their wet body mass was measured (±0.0001 g; XS105 Analytical Balance, Mettler Toledo). Water in each trial tank was replaced to remove any chemical cues left by the mosquitofish and any remaining fluoxetine; a new batch of mosquitofish was brought in, and the experimental protocol was repeated. We ultimately obtained data for 50 control fish, 43 low-fluoxetine fish, and 47 high-fluoxetine fish.

A subset of water samples ($n = 56$) was analysed by Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025) to verify fluoxetine levels in randomly selected tanks from each treatment (1 control, 3 low-fluoxetine, and 3 high-fluoxetine tanks) on day 1 and day 3 of exposure for every second batch of experimental trials. Briefly, the concentration of fluoxetine was measured using liquid chromatography–tandem mass spectrometry (Shimadzu 8050 LCMSMS), with a quantification limit of 2 ng/L. A detailed description of this protocol is provided in Supplementary Information section 1.1, 'Analytical verification of fluoxetine'.

2.3. Experimental setup

Experimental trials were conducted in individual experimental tanks (25 × 15 × 15 cm; 7 cm water depth) filled with 50% water from the respective individual's housing tank and 50% RO water. Water in experimental tanks was not aerated to facilitate automated video tracking of the focal individual. There was only a gradual decrease in activity over time of individuals, possibly also due to general acclimation to experimental tanks, indicating that this would not have adversely affected the fish. The four side walls of each experimental tank were covered with an opaque film to reduce disturbance from adjacent tanks and external sources. Waterproof, infrared (IR) light-emitting diode (LED) strip lights (12 V DC, 850 nm, 120 LEDs 9.6 W per meter) were run underneath the experimental tanks, along each of the two longer walls, and were left on at all times during experimental trials. The wavelength (850 nm) of these IR lights fell outside the known visual range of the closely related western mosquitofish (*Gambusia affinis*, see Archer and Hirano, 1997; Chang et al., 2020), but provided sufficient illumination for IR-sensitive cameras to record the activity of the animal under dark conditions. An acrylic sheet (3-mm thick, white), and a corflute sheet (3-mm thick, white) was placed in between the underside of the experimental tank and the IR LED lights to provide a homogenously lit background for video recording. Experimental trials were conducted in a windowless controlled-temperature room that was kept under the same temperature conditions (mean ± SD: 20.6 ± 1.0 °C) and photoperiod as the housing room. In addition to the room ceiling lights, two tripod-mounted fluorescent lights (135 W, 5500 K, "day white") were placed within the experimental room to provide brighter daylight during the 12-h daytimes. All non-IR lights were controlled by an automated timer to produce the 0700–1900 h photoperiodic regime. A light logger (EA33 EasyView light meter with memory, Extech Instruments) was used to measure the light levels above each experimental tank within the experimental room across the light (room mean ± SD: 225.4 ± 47.4 lux) and dark (0 lux across all tanks) conditions. A downward-facing IR-sensitive camera (3.6 mm lens 2 MP USB night vision web camera, Webcamera_usb) was placed 60 cm above four experimental tanks to record four separate experimental trials simultaneously at 25 frames per second. A light sensor within the camera controlled the switching of an inbuilt 650 nm IR cut filter, providing visible spectrum colour footage during the day, and capturing near-infrared light (850 nm) during the night. This ensured that the activity of the individual mosquitofish could be recorded and tracked across both the light and dark conditions. Cameras were connected to a desktop computer, and the open-source software, OBS Studio v. 27.0.1, was used to record video footage constantly over the experimental period in batches of 24 individual trials.

2.4. Video analysis

Videos of experimental trials were analysed with the commercial video tracking software Ethovision XT v. 14.0.1326 (Noldus Information Technology bv, The Netherlands). Videos were split into 1-h bins and the frame-by-frame position of the focal individual was tracked across the three experimental days. From each 1-h video, the total distance travelled (m), the total time spent inactive, and the number of restful episodes (i.e., inactivity bouts longer than 1 min) was extracted from the tracking information for each individual. The threshold for movement was set at 0.5 cm/s over which animals were deemed to be active (Martin et al., 2017); this threshold helped account for tracking noise introduced by image-processing and any locomotion-independent movements. The video tracking efficiency (i.e., percentage of video where the subject was not successfully detected in the tracking arena, and therefore was not tracked) was also extracted from the tracking information to be included as a co-variate in the statistical analyses to account for any potential discrepancies in tracking quality.

2.5. Statistical analysis

Statistical analyses were conducted in R version 4.0.2 (R Core Team, 2020). Linear mixed effect models (LME; *lme4* package; Bates et al., 2015) were used to test for the effects of fluoxetine exposure treatment and photoperiod (i.e., 12-h light or 12-h dark) on distance travelled and amount of restfulness (i.e., time spent inactive). A negative binomial generalized linear mixed effect model with linear parameterization (GLMM; *glmmTMB* package; Brooks et al., 2017) was used to test for effects on the number of restful episodes over the three-day experiment. Due to equipment failure, approximately 1% of the total number of 1-h trial videos across all treatments were lost. Therefore, we analysed fish activity averaged per hour over the experimental period, rather than a sum of the total distance travelled or total time spent inactive per photoperiod. Each model included: (1) exposure treatment (control, low, high), (2) photoperiod (12-h light, 12-h dark), (3) the exposure by photoperiod interaction term, (4) trial day (three consecutive 24-h

experimental days), and (5) the video tracking efficiency (percentage of video where focal subject was not successfully detected) as fixed effects. Additionally, fish identity was included as a random intercept, with photoperiod modelled as a random slope, to account for variability in individual differences across photoperiodic conditions. Preliminary analyses included an exposure by photoperiod by sex interaction term as a fixed effect, but no sex by exposure interaction terms were found to be significant across any models. To achieve the most parsimonious model relevant to our hypothesis, sex was then removed from all final models. However, see Supplementary Information section 2.1, 'Sex-specific differences in natural diurnal activity' for more details. For all LMEs and GLMMs, the statistical significance of fixed effects was calculated using Type III Wald's *F*-tests with a Satterthwaite's approximation for the denominator degrees of freedom (*lmerTest* package; Kuznetsova et al., 2017), or Type III Wald's chi-squared tests, respectively. If main effects or interaction terms were statistically significant, pair-wise comparisons were performed with *p*-value adjustments made for multiple comparisons using a multivariate *t* distribution (*emmeans* package; Lenth, 2021). To ensure that there was no sampling bias of individuals between exposure groups, fish wet body mass was compared between exposure treatments and sex using a Type III analysis of variance (ANOVA) with Kenward-Roger degrees of freedom approximation. A logarithmic transformation was applied to body mass to approximate a Gaussian distribution.

3. Results

3.1. Analytical verification of fluoxetine concentrations

The mean measured concentrations (±SE) for the low- and high-fluoxetine treatments on the first day of exposure were 13.1 ± 0.9 ng/L ($n = 12$) and 116.4 ± 9.3 ng/L ($n = 12$) respectively, and on the last day of exposure were 3.1 ± 0.4 ng/L ($n = 12$) and 21.1 ± 5.0 ng/L ($n = 12$) respectively. Uptake of fluoxetine has been shown to occur within 5 h of initial exposure in Japanese medaka (*Oryzias latipes*; Paterson and Metcalfe, 2008), and maximum concentrations of fluoxetine in Japanese medaka (Paterson and Metcalfe, 2008) and bioconcentration steady states in nine-spined sticklebacks (*Pungitius pungitius*; Boström et al., 2017) were reached by the third and fourth day of exposure respectively. In general, bioaccumulation of fluoxetine has also been shown to occur in eastern mosquitofish (Martin et al., 2019b). Taken together, the initial and progressive decrease in measured fluoxetine concentrations in water is likely to be due, in part, to bioaccumulation of the compound in mosquitofish. All control treatment water samples were free from contamination (i.e., below the detection limit of 2 ng/L, $n = 8$).

3.2. Distance travelled and restfulness

We found a statistically significant interaction between exposure treatment and photoperiod on the distance travelled by mosquitofish per hour ($F_{2,149} = 3.23$, $p = 0.042$, see Table S1 for the complete results of the linear mixed effect models). Specifically, we found that, on average, control fish swam farther during the day than during the night ($t = 2.93$, df = 150, $p = 0.031$; Figs. 1a and 2). Conversely, both low- and high-fluoxetine fish did not show a preference for daytime swimming ($t = -0.72$, df = 150, $p = 0.971$; $t = 0.87$, df = 150, $p = 0.937$, respectively). For full exposure treatment by photoperiod pairwise comparisons see Table S2.

Results for restfulness were similar. We identified a marginally non-significant interaction between exposure treatment and photoperiod for the time spent restful ($F_{2,149} = 2.71$, $p = 0.069$, see Table S1). Pairwise comparisons showed that, on average, control fish were more restful at night relative to the day ($t = -3.53$, df = 150, $p = 0.005$; Figs. 1b and 3). Once again, low- and high-fluoxetine fish lacked this gross temporal organisation to their activity ($t = -0.21$, df = 150, $p > 0.999$; $t = -1.23$, df = 150, $p = 0.776$, respectively). For full exposure treatment by photoperiod pairwise comparisons see Table S3.

For the number of restful episodes (i.e., periods of inactivity longer than 1 min), we observed a statistically significant interaction between exposure treatment and photoperiod ($\chi^2 = 9.86$, df = 2, $p = 0.007$, see

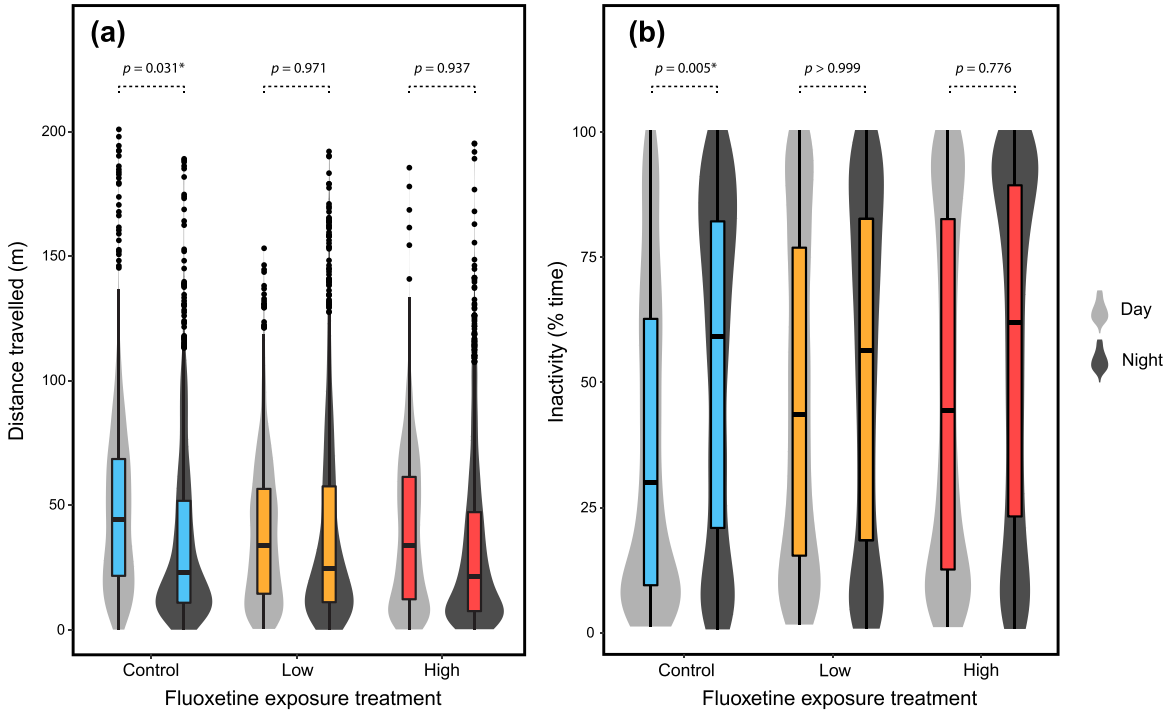

Fig. 1. The distance travelled (a) and percentage of time spent inactive (b) by mosquitofish during the day and night for each fluoxetine exposure treatment. Box plots show the median (centre line), 25th and 75th percentiles (bottom and top of each box, respectively), interquartile range multiplied by 1.5 (whiskers), and outliers (circles). * indicates statistically significant group differences.

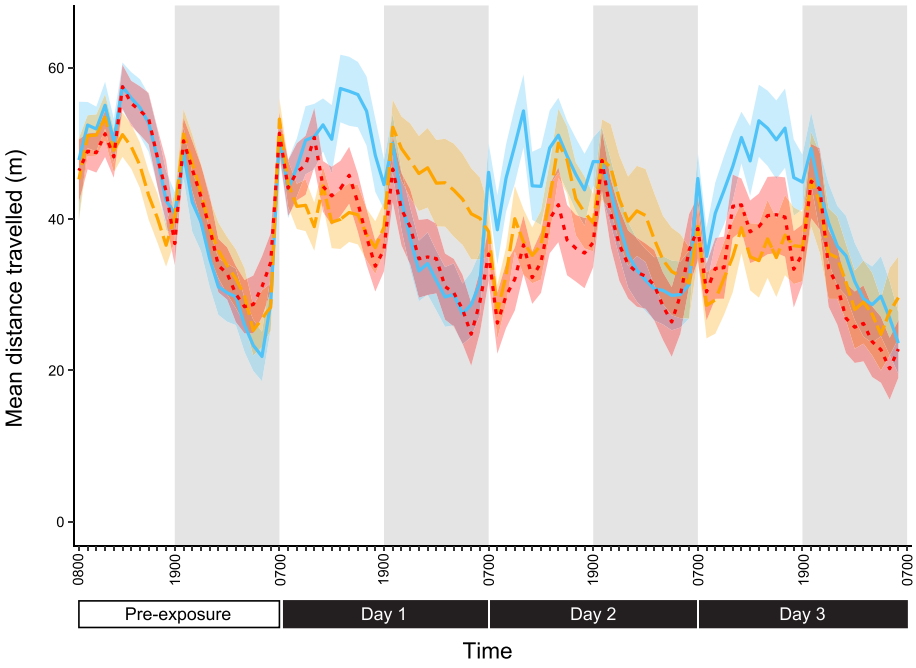

Fig. 2. Ninety-five hour time series plot of the distance travelled per hour split by fluoxetine exposure treatment. Data points are mean values (bold lines) ± SE (shaded area) of distance travelled averaged across all individuals within each exposure treatment (Control = blue solid line, Low = orange dashed line, High = red dotted line) and hourly block. The black boxes on the horizontal axis highlights the fluoxetine exposure period as the first 0800–0700 h photoperiod (white box on the horizontal axis) was an acclimation period where mosquitofish were not yet treated with fluoxetine as a pre-exposure baseline. White columns represent daytime and grey columns represent night-time. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

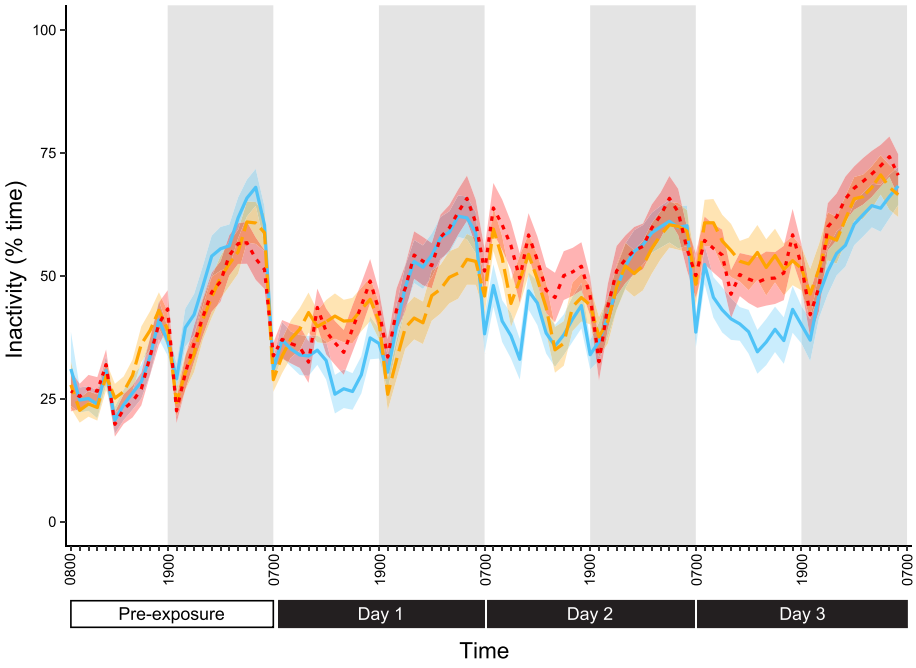

Fig. 3. Ninety-five hour time series plot of the amount of restfulness per hour split by fluoxetine exposure treatment. Data points are mean values (bold line) ± SE (shaded area) of percentage of time spent inactive averaged across all individuals within each exposure treatment (Control = blue solid line, Low = orange dashed line, High = red dotted line) and hourly block. The black boxes on the horizontal axis highlights the fluoxetine exposure period as the first 0800–0700 h photoperiod (white box on the horizontal axis) was an acclimation period where mosquitofish were not yet treated with fluoxetine as a pre-exposure baseline. White columns represent daytime and grey columns represent night-time. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table S1). Pairwise comparisons showed that control fish had a significantly higher number of restful episodes in the night compared to the day ($t = -2.95$, df = 150, $p = 0.030$; Fig. S1). However, the number of restful bouts did not significantly differ between the day and night in low- and high-fluoxetine exposed fish ($t = 1.34$, df = 150, $p = 0.708$; $t = 0.22$, df = 150, $p > 0.999$, respectively). For full exposure treatment by photoperiod pairwise comparisons see Table S4.

We also found a significant main effect of trial day on both the distance travelled and time spent restful, such that mosquitofish became progressively less active over the three days (distance travelled: $F_{3,10568.7} = 76.59$, $p < 0.001$; Fig. 2; inactivity: $F_{3,10568.7} = 211.63$, $p < 0.001$; Fig. 3).

3.3. Body mass

There was no interaction between fluoxetine exposure and sex on the wet body mass of mosquitofish ($F = 1.84$, df = 2, $p = 0.161$), nor was there any significant differences in body mass between the fluoxetine exposure groups ($F = 1.62$, df = 2, $p = 0.201$). There was, however, an effect of sex on mass ($F = 90.56$, df = 1, $p < 0.001$), with females weighing more than males (Fig. S2).

4. Discussion

We investigated whether a three-day exposure to environmentally realistic levels of the widespread psychoactive pollutant, fluoxetine,

affected diel rhythms; specifically, the normal diurnal pattern of activity in mosquitofish. We found evidence for disruption of diel swimming patterns in fluoxetine-exposed mosquitofish, which was primarily driven by augmentation of daytime inactivity. Specifically, unexposed control fish were markedly diurnal; they swam farther during the day, and, at night, were more restful, with longer periods of inactivity and more restful episodes. However, in both low- and high-fluoxetine exposed fish, this normal diel rhythm was muted, with no day/night differences in activity or restfulness observed. This disruption in the diel rhythm was primarily driven by a decrease in daytime swimming, and an increase in daytime restfulness and number of restful episodes.

Consistent with our results, Syrian hamsters (*Mesocricetus auratus*) administered with fluoxetine exhibited an increase in day-time NREM sleep (Gao et al., 1992). Similarly, juvenile male rhesus monkeys (*Macaca mulatta*) dosed with fluoxetine were less active and more restful in the day (Golub and Hogrefe, 2016). Interestingly, turquoise killifish (*Nothobranchius furzeri*) exposed to fluoxetine at a concentration similar to that employed in this study (average measured ± SD concentration: 28.2 ± 16.9 ng/L) exhibited a similar disruption in diurnal activity rhythms, where control fish travelled farther in the morning than in the evening, but this pattern was eroded in fluoxetine-exposed fish (Thoré et al., 2021). This disruption in diurnal activity seen in both the turquoise killifish (Thoré et al., 2021) and mosquitofish in the present study highlights that the effects of fluoxetine on diel activity appears to be consistent across different species. Additionally, whilst turquoise killifish were only tested for activity following 36 days of exposure to fluoxetine (Thoré et al., 2021), we show that disruptions in diurnal activity can occur almost immediately, a few hours following initial exposure. Therefore, it is likely that the disruption of the diel activity rhythms of fish caused by fluoxetine would occur at the onset of exposure and persist for weeks after. However, in contrast to our results, Melvin (2017) observed no significant change in diurnal activity patterns of male eastern mosquitofish exposed to fluoxetine over a 168-h period at concentrations many times higher than those used here (average measured range of exposure concentrations: 1570–118,560 ng/L). The significance of these divergent effects in fishes is difficult to reconcile, but could reflect a non-linear dose-dependent response of circadian rhythms to fluoxetine (Vandenberg et al., 2012).

Such non-monotonic responses are common in pharmacological and toxicological studies (Calabrese and Baldwin, 2003), and have been reported across a range of genetic, physiological and behavioural traits in fluoxetine-exposed animals (e.g., Al Shuraiqi et al., 2021; Barry, 2013; Bertram et al., 2018; Cunha et al., 2018; Fong et al., 2017; Gao et al., 1992; Guler and Ford, 2010; Martin et al., 2020; Martin et al., 2017; Rivetti et al., 2016; Tan et al., 2020; Wiles et al., 2020). The differences in effects observed at higher fluoxetine concentrations have been previously attributed to an inhibition of a finite amount of endogenous serotonin or desensitisation of serotonergic (5-HT1A) autoreceptors (Guler and Ford, 2010). More recently, a non-monotonic response to fluoxetine exposure in gene expression has been revealed as a potential mechanism of the non-linear dose responses seen at a behavioural and physiological level (Cunha et al., 2018; Rodrigues et al., 2022). As serotonergic functions and mechanisms are inter-dependent on a genomic, biochemical, physiological and behavioural level (Best et al., 2010), this introduces complexities in interpreting experimental and, thus, potential ecological effects of fluoxetine. With ever increasing evidence that fluoxetine exposure can result in non-monotonic responses, it is crucial that future studies employ exposure concentrations reflecting environmental detection levels when aiming to determine the ecological impacts of fluoxetine and other similar SSRI compounds on wildlife.

The circadian rhythm is directly controlled by the neuroendocrine system, and any alterations by neuroactive compounds can result in dysregulation of the circadian clock, and consequential disruptions to circadian activity patterns (Urbanski, 2011). At the genetic level, the circadian rhythm is composed of multiple positive and negative regulators interacting to maintain transcription and translation feedback loops (Vatine et al., 2011). Gene *per2* is a key clock gene whose expression is induced by light and has been shown to control the circadian rhythm in mammals and other vertebrates, including in zebrafish (Ben-Moshe et al., 2014; Delaunay et al., 2003). Meanwhile, the product of gene *nr1d1* is a negative transcription inhibitor that prevents the transcription levels of several clock genes including *per2*, thus regulating the circadian rhythm (Ueda et al., 2005). In larval zebrafish, exposure to fluoxetine at a concentration of 100 ng/L significantly modulated the expression of *per2* and, subsequently, regulated the expression of *nr1d1*, thus establishing an underlying mechanistic pathway by which fluoxetine can affect circadian rhythms and circadian activity patterns in non-target species (Wu et al., 2017). Interestingly, adult zebrafish exposed to another psychotherapeutic drug, diazepam, demonstrated an alteration in the transcription of multiple genes involved in rhythmic processes including *per2*, and also reported an associated change in locomotory behaviour, indicating that the dysregulation of circadian rhythm gene expression directly mediates behavioural responses to pollutant exposure (Oggier et al., 2010). Therefore, it is likely that these mechanistic changes in circadian-related gene expression caused by fluoxetine leads to alterations in circadian patterns of behaviour and physiology, which may have adverse effects on an organism's fitness.

Circadian rhythms exist ubiquitously across animals, plants and fungi, and have been shown to confer an adaptive advantage (Yerushalmi and Green, 2009). An internal clock allows an organism to anticipate recurring environmental and ecological changes, such as temperature, daylight, presence of predators and availability of resources, and to synchronize physiological processes accordingly (Patke et al., 2020; Yerushalmi and Green, 2009). A misalignment of the internal clock with predictable daily environmental conditions can hinder fecundity, survivorship, and longevity (DeCoursey et al., 2000; Emerson et al., 2008; Klarsfeld and Rouyer, 1998; Wyse et al., 2010). With this in mind, the fluoxetine-induced alteration in the diurnal activity patterns observed in this study could be an indicator of a more widespread issue of circadian clock disruption in wildlife.

4.1. Conclusions

The prevalence of psychoactive pollutants, such as fluoxetine, in the environment has greatly increased over the past few decades (Bernhardt et al., 2017; Graumnitz and Jungmann, 2021; Wilkinson et al., 2022). Although many studies have documented the broad anxiolytic effects of these compounds (reviewed in Brodin et al., 2014; Gould et al., 2021), this study is one of the first to show that a psychoactive drug, at environmentally-relevant concentrations, disrupts diurnal activity patterns in fish over successive day/night cycles, with mosquitofish showing unnatural restfulness during the day. As natural biological rhythms are crucial to the maintenance of key behavioural and physiological traits that facilitate survival and reproduction, a dysregulation in the circadian clock may be detrimental to an organism's fitness. Therefore, it is important to understand how exposure to pharmaceutical pollutants may be affecting circadian rhythms, and whether there are any adverse impacts on survival and reproductive success.

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Ethics

Experiments complied with Australian law and were approved by the Monash University Animal Ethics Committee (Project ID 27830).

Authors contributions

Conceptualization: H.T., J.M.M., L.A.A., J.A.L., B.B.M.W.; Data curation: H.T.; Formal analysis: H.T.; Funding acquisition: H.T., J.A.L., B.B.M.W.; Investigation: H.T.; Methodology: H.T., J.M.M., L.A.A., J.A.L., B.B.M.W.; Project administration: H.T., B.B.M.W.; Resources: J.A.L., B. B.M.W.; Visualization: H.T., J.M.M., L.A.A., J.A.L., B.B.M.W.; Writing – original draft: H.T., J.A.L., B.B.M.W.; Writing – review & editing: H.T., J. M.M., L.A.A., J.A.L., B.B.M.W.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.138446.

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SUPPLEMENTARY INFORMATION

Widespread psychoactive pollutant augments daytime restfulness and disrupts diurnal activity rhythms in fish

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Supplementary Information

1. Materials and methods

1.1 Analytical verification of fluoxetine

Water samples (40 mL) drawn from experimental tanks were stored in 50 mL conical centrifuge tubes. For verification of fluoxetine concentrations and to confirm the absence of contamination of control samples, 3.6 mL of sample was aliquoted into 4 mL vial followed by 400 µL of 1% formic acid (LCMSMS grade, ≥99.5%). To act as a surrogate standard, 20 µL of 9 ng/mL 17α-trenbolone (17α-hydroxyestra-4,9,11-trien-3-one; CAS: 80657-17-6; Novachem, Germany) in methanol (LCMSMS grade, ≥99.9%) was then added to each sample, as well as to all calibration standards. To act as an internal standard, 20 µL of 90 ng/mL Fluoxetine-D_6 (Cerilliant, USA) in methanol (LCMSMS grade, ≥99.9%) was then added to each sample, and to all calibration standards. Each sample was then transferred to liquid-chromatography vials using 4 mm RC syringe filter (0.2 µm), before samples were analysed using LC-MS/MS. Injection of 20 µL was performed in electrospray ionization (ESI) mode onto Shim-pack XR-ODS III (1.6 µm) 50 mm length × 2.0 mm inner diameter. Two transitions were monitored each for fluoxetine (quantification ion: 310.3 → 44.3, collision energy –14 V; confirmatory ion: 310.3 → 148.3, collision energy: –10 V) and the 17α-trenbolone surrogate standard (quantification ion: 271.2 → 253.1, collision energy –22 V; confirmatory ion: 271.2 → 107.1, collision energy: –34 V). The gradient started at an initial 90% of 0.1% formic acid in water and 10% of 5% water in acetonitrile for 0.5 min before decreasing to 60% of 0.1% formic acid in water at 1.5 min until 3.75 min, then decreased to 0% of 0.1% formic acid in water at 4 min until 8 min, to a final of 90% of 0.1% formic acid in water and was kept at this for 4 min.

An eight-point calibration curve was used, and calibration standards were analysed alongside experimental samples. This involved an intermediate solution of 1 µg/mL of fluoxetine being prepared in methanol (LCMSMS grade, ≥99.9%). A working standard was

then prepared in methanol with 1% formic acid (LCMSMS grade, ≥99.5%) at 0.09 and 9 ng/mL. Calibration standards were made in unexposed aquaria water and were treated identically to experimental samples. No fluoxetine contaminations were detected in any control unexposed tank throughout the exposure period. With this method, for fluoxetine, a limit of quantification (LOQ) of 2 ng/L was obtained.

2. Supplementary results

2.1 Sex-specific differences in natural diurnal activity

Preliminary analyses included an exposure by photoperiod by sex interaction term as a fixed effect to test if fluoxetine affected any sex-specific differences in diel activity. There were no significant three-way interactions between exposure treatment, photoperiod and sex for both distance travelled and time spent restful ($F_{2,146.1} = 0.02$, $p = 0.979$; $F_{2,146.1} = 0.89$, $p = 0.413$, respectively). There were also no significant two-way interactions between exposure treatment and sex for both distance travelled and time spent restful ($F_{2,146} = 0.24$, $p = 0.789$; $F_{2,145.9} = 0.02$, $p = 0.978$, respectively). There were, however, significant two-way interactions between sex and photoperiod for both distance travelled and time spent restful ($F_{1,146.2} = 8.34$, $p = 0.004$; $F_{1,146.4} = 24.46$, $p < 0.001$, respectively), with a more pronounced natural diurnal activity rhythm seen in males compared to females, regardless of exposure treatment. As fluoxetine affected the diel activity of individuals regardless of sex, sex was removed from final analytical models to achieve a more parsimonious model. Importantly, we also note here that qualitatively, the exposure treatment specific effects do not change between models with or without the inclusion of sex.

Table S1. Results of linear mixed effect (LME) models for a) distance travelled and b) percentage of inactivity, and of a generalized linear mixed effect model (GLMM) for c) number of restful episodes. Significance for LME models calculated from Type III Wald's *F*-tests using Satterthwaite's approximation for the denominator degrees of freedom, and for GLMM models calculated from Type III Wald's Chi-square tests. * indicates statistically significant *p*-values.

a) LME model for distance travelled per hour			
Fixed effects	Numerator, denominator df	*F*	*p*
Video tracking efficiency	1,10764.5	224.41	<0.001*
Trial day	3,10567.8	76.59	<0.001*
Exposure treatment	2,149.0	1.01	0.367
Photoperiod	1,151.1	2.90	0.091
Exposure treatment:photoperiod	2,149.0	3.23	0.042*
b) LME model for percentage of inactivity per hour			
Fixed effects	Numerator, denominator df	*F*	*p*
Video tracking efficiency	1,10820.2	368.07	<0.001*
Trial day	3,10568.7	211.63	<0.001*
Exposure treatment	2,148.9	1.49	0.229
Photoperiod	1,152.2	7.80	0.006*
Exposure treatment:photoperiod	2,149.0	2.71	0.069
c) GLMM model for number of restful episodes (i.e. inactive periods longer than 1 min)			
Fixed effects	df	χ^2	*p*
Video tracking efficiency	1	4.96	0.026*
Trial day	3	416.18	<0.001*
Exposure treatment	2	7.03	0.030*
Photoperiod	1	8.68	0.003*
Exposure treatment:photoperiod	2	9.86	0.007*

Table S2. Results for the pairwise comparisons examining the effects of the interaction between fluoxetine exposure and photoperiod on the distance travelled per hour (cm), averaged over experimental days. The Kenward-Roger method was used to calculate degrees of freedom and *p*-values were adjusted with a multivariate *t* adjustment. 'C' = control treatment, 'L' = low-fluoxetine treatment and 'H' = high-fluoxetine treatment. * indicates statistically significant *p*-values.

Exposure treatment by photoperiod pairwise contrasts	Estimate	SE	df	*t* ratio	*p*
C_day - L_day	979	410	149	2.39	0.126
C_day - H_day	860	401	149	2.14	0.215
L_day - H_day	–119	416	149	–0.29	0.999
C_day - C_night	1059	361	150	2.93	0.031*
L_day - L_night	–278	387	150	–0.72	0.971
H_day - H_night	322	371	150	0.87	0.937
C_night - L_night	–357	477	149	–0.75	0.097
C_night - H_night	123	467	149	0.26	0.999
L_night - H_night	480	484	149	0.99	0.892

Table S3. Results for the pairwise comparisons examining the effects of the interaction between fluoxetine exposure and photoperiod on the time spent restful, averaged over experimental days. The Kenward-Roger method was used to calculate degrees of freedom and *p*-values were adjusted with a multivariate *t* adjustment. 'C' = control treatment, 'L' = low-fluoxetine treatment and 'H' = high-fluoxetine treatment. * indicates statistically significant *p*-values.

Exposure treatment by photoperiod pairwise contrasts	Estimate	SE	df	*t* ratio	*p*
C_day - L_day	–322.2	140	149	–2.30	0.155
C_day - H_day	–312.9	137	149	–2.28	0.161
L_day - H_day	9.3	142	149	0.07	0.999
C_day - C_night	–389	110	150	–3.53	0.005*
L_day - L_night	–25	118	150	–0.21	0.999
H_day - H_night	–139.1	113	150	–1.23	0.776
C_night - L_night	41.8	142	149	0.29	0.999
C_night - H_night	–63	139	149	–0.45	0.997
L_night - H_night	–104.8	144	149	–0.73	0.970

Table S4. Results for the pairwise comparisons examining the effects of the interaction between fluoxetine exposure and photoperiod on the number of restful episodes longer than 1 min in duration, averaged over experimental days. *p*-values were adjusted with a multivariate *t* adjustment. Results are given on the log scale. 'C' = control treatment, 'L' = low-fluoxetine treatment and 'H' = high-fluoxetine treatment. * indicates statistically significant *p*-values.

Exposure treatment by photoperiod pairwise contrasts	Estimate	SE	df	*t* ratio	*p*
C_day - L_day	–0.511	0.232	150	–2.20	0.192
C_day - H_day	–0.537	0.228	150	–2.36	0.136
L_day - H_day	–0.026	0.234	150	–0.11	0.999
C_day - C_night	–0.470	0.159	150	–2.95	0.030*
L_day - L_night	0.226	0.169	150	1.34	0.708
H_day - H_night	0.035	0.161	150	0.22	0.999
C_night - L_night	0.184	0.222	150	0.83	0.947
C_night - H_night	–0.032	0.217	150	–0.15	0.999
L_night - H_night	–0.216	0.225	150	–0.96	0.906

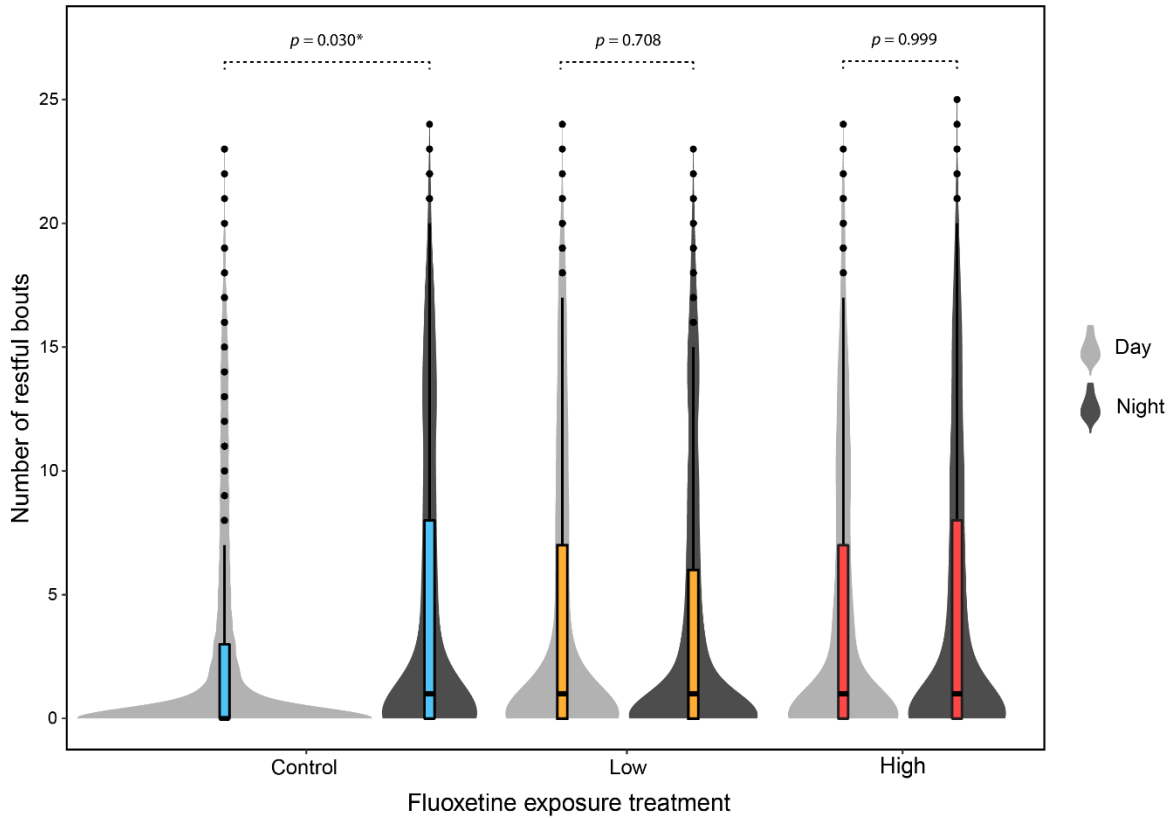

Figure S1. The number of restful episodes (i.e., periods of inactivity >1 min) each day and night plotted by fluoxetine exposure treatment. Box plots show the median (centre line), 25th and 75th percentiles (bottom and top of each box, respectively), interquartile range multiplied by 1.5 (whiskers), and outliers (circles). * indicates statistically significant group differences.

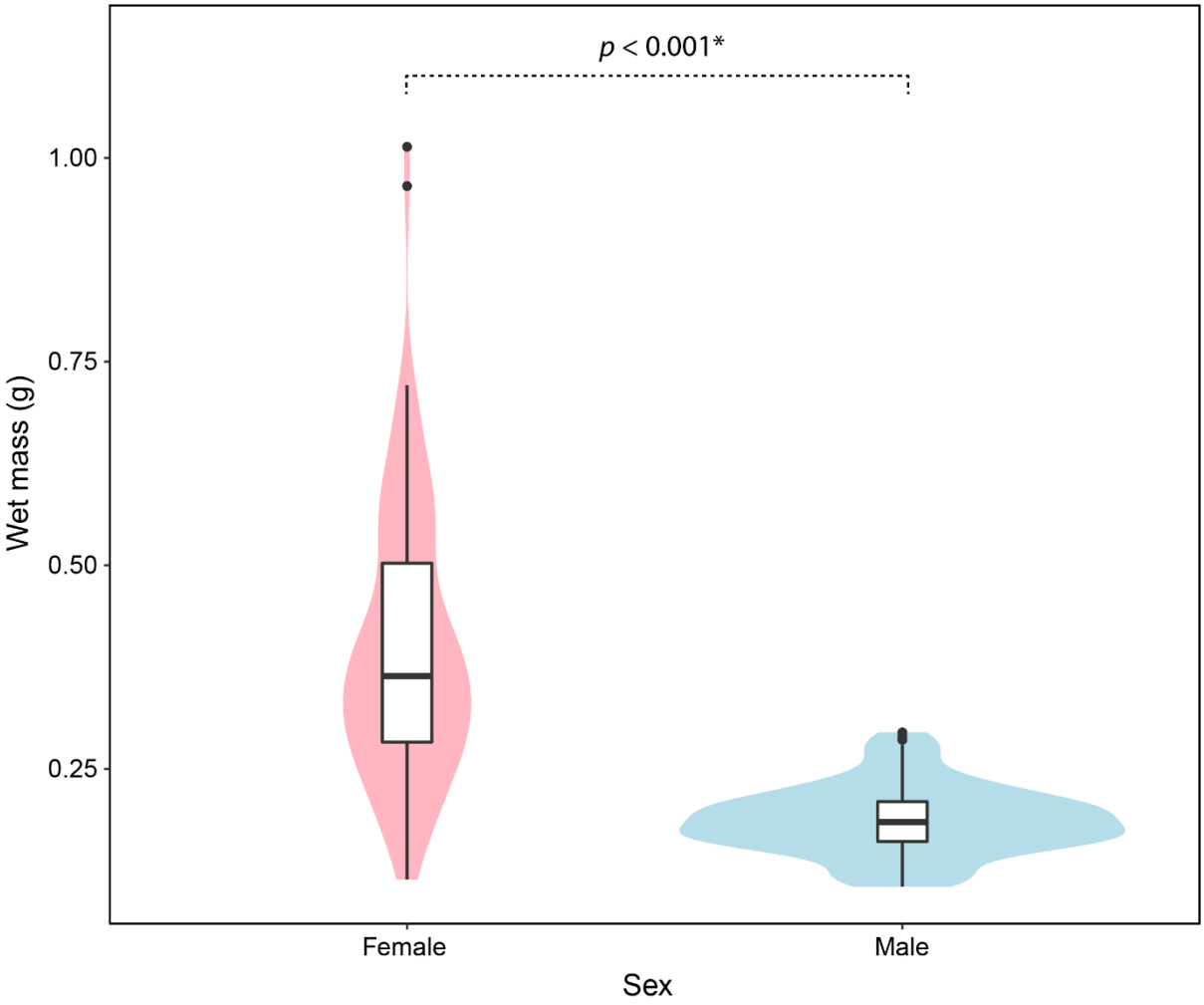

Figure S2. Wet body mass of mosquitofish split by sex. Box plots show the median (centre line), 25th and 75th percentiles (bottom and top of each box, respectively), interquartile range multiplied by 1.5 (whiskers), and outliers (circles). * indicates statistically significant group differences.