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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