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Impacts of the antidepressant fluoxetine on the anti-predator behaviours of wild guppies (*Poecilia reticulata*)

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ABSTRACT

Chemical pollution from pharmaceuticals is increasingly recognised as a major threat to aquatic communities. One compound of great concern is fluoxetine, which is one of the most widely prescribed psychoactive drugs in the world and frequently detected in the environment. The aim of this study was to investigate the effects of 28-d fluoxetine exposure at two environmentally relevant levels (measured concentrations: 4 ng/L and 16 ng/L) on anti-predator behaviour in wild guppies (*Poecilia reticulata*). This was achieved by subjecting fluoxetine-exposed and unexposed guppies to a simulated bird strike and recording their subsequent behavioural responses. We found that exposure to fluoxetine affected the anti-predator behaviour of guppies, with exposed fish remaining stationary for longer (i.e. 'freezing' behaviour) after the simulated strike and also spending more time under plant cover. By contrast, control fish were significantly more active and explored the tank more, as indicated by the distance covered per minute over the period fish spent swimming. Furthermore, behavioural shifts were sex-dependent, with evidence of a non-monotonic dose-response among the fluoxetine-exposed fish. This is one of the first studies to show that exposure to environmentally relevant concentrations of fluoxetine can alter the anti-predator behaviour of adult fish. In addition to the obvious repercussions for survival, impaired anti-predator behaviour can have direct impacts on fitness and influence the overall population dynamics of species.

1. Introduction

Contamination of the aquatic environment by pharmaceuticals is a serious problem. Over 4000 human and veterinary pharmaceutical drugs are in use worldwide (Boxall et al., 2012), and with the booming global pharmaceutical market (US$400 billion) (WHO, 2015), the continual input of pharmaceuticals into aquatic environments is particularly concerning. Pharmaceuticals have been designed to treat disease in humans and animals by interacting with specific biological pathways and processes in target organisms (Daughton and Ternes, 1999). Because they were targeted to only modify physiology, traditionally pharmaceuticals were not perceived to pose a threat to aquatic organisms (Daughton and Ternes, 1999; Arnold et al., 2014). Hence, our current knowledge regarding the impacts of pharmaceuticals on wildlife is still rather limited and less is known about the effects on behaviour. This is surprising given that behaviour is often a more ecologically relevant end point of exposure (Brodin et al., 2013; Melvin and Wilson, 2013; Stewart et al., 2014; Wong and Candolin, 2015).

One pharmaceutical of particular concern is fluoxetine. It is one of the world's most widely prescribed pharmaceutical drugs used to treat depression and anxiety disorders in humans (Fent et al., 2006). For example, in 2013, fluoxetine was the 3rd most prescribed anti-depressant in the U.S. with 28 million prescriptions (Grohol 2013), while in 2016, over 500,000 prescriptions of fluoxetine were prescribed in England in a single month (NHS 2016). As a result of its widespread use, fluoxetine is frequently detected in aquatic environments (Metcalfe et al., 2003; Birch et al., 2015). The physicochemical properties of fluoxetine make it a potent, persistent (half-life 112–133 days: Kwon and Armbrust, 2006), and photolytically stable compound, with limited environmental degradation (Benfield et al., 1986; Gram, 1994; Hiemke and Härtter, 2000; Brooks, 2014; Silva et al., 2015). Due to its widespread usage, fluoxe-

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tine has been detected in surface waters at concentrations ranging from <1–600 ng/L around the world (Kolpin et al., 2002; Bratton et al., 2003; Metcalfe et al., 2003; Hughes et al., 2013; Birch et al., 2015; Schlusener et al., 2015). In target organisms, fluoxetine elicits its therapeutic effects by preventing the reuptake of serotonin, which subsequently increases the extracellular serotonin levels in the brain (Frazer, 2001; Fuller et al., 2006). The serotonergic system, which regulates neuroendocrine pathways related to reproduction and behaviour (Ögren et al., 1985; Cunningham et al., 2004; Jørgensen, 2007; Hood et al., 2006; Mennigen et al., 2011), is evolutionary conserved across vertebrates (Kah and Chambolle, 1983; Fuller et al., 2006; Mennigen et al., 2010). Therefore, it is not surprising that fluoxetine has been found to impact physiology and development of non-target aquatic organisms. Specifically, fluoxetine has been shown to decrease milt volume and testosterone levels in goldfish (*Carassius auratus*) (Mennigen et al., 2010), lower spermatozoa and oocyte numbers in zebra mussels (*Dreissena polymorpha*) (Lazzara et al., 2012), and reduce offspring production in mudsnails (*Potamopyrgus antipodarum*) (Pery et al., 2008). Fluoxetine clearly affects the morphology and development of fish, but what about behaviour?

Behaviour is governed by both external stimuli and internal hormonal and neural mechanisms (Bass and McKibben, 2003; Zala and Penn, 2004; Huntingford et al., 2006). Thus, behavioural changes can be immediate, sensitive and observable responses to cues in the surrounding environment (Scott and Slowman, 2004). Impact on behaviour is suggested to be the primary effect of fluoxetine in wildlife, because fluoxetine is designed to alter behaviour in humans (Huggett et al., 2003; Rand-Weaver et al., 2013). Studies conducted on fluoxetine so far have shown a broad range of behavioural effects in fish, such as decreased mating behaviour (fathead minnow, *Pimephales promelas:* Weinberger and Klaper, 2014), weakened aggression (Arabian killifish, *Aphanius dispar*: Barry, 2013), lowered anxiety (zebrafish, *Danio rerio*: Egan et al., 2009) and activity (sheepshead minnow, *Cyprinodon variegatus*: Winder et al., 2012).

Fluoxetine can also have a profound impact on anti-predator behaviours. In many species, behaviours, such as freezing (i.e. remaining motionless), dashing and use of shelter, form a key component of an individual's anti-predator repertoire (Lawrence and Smith, 1989; Godin, 1997; Barber et al., 2004). Apart from the obvious repercussions for survival, anti-predator behaviours can also influence other important fitness components (Abrams and Matsuda, 1993; Magurran and Nowak, 1991; Anholt and Werner, 1998; Lima, 2009). For instance, time spent foraging and time devoted to reproductive behaviours can all be affected by the presence of predators (Kotler et al., 1994; Godin, 1997; Johansson et al., 2004; Winnie and Creel, 2007). Yet, despite this, only a limited number of studies to date have examined the effects of fluoxetine and other SSRIs, on anti-predator responses. And, of those that have, the exposure concentrations used were several orders of magnitude higher than those detected in the environment (Barbosa et al., 2012; Barry 2013; Nilsson et al., 2016).

The guppy (*Poecilia reticulata*) is a small, live-bearing freshwater fish native to north-eastern South America (Houde, 1997; Jirotkul, 1999). Guppies are a well-studied model in behavioural ecology, and are an ecologically relevant species to study the effects of fluoxetine on anti-predator behaviour. This is because they inhabit a wide geographic range (Endler and Houde, 1995; Lindholm et al., 2005) and often encounter and occupy polluted environments close to human habitation, where exposure to contaminants, such as fluoxetine, is likely (Widianarko et al., 2000; Araújo et al., 2009; Willing et al., 2010). Guppies are a sexually dimorphic species, with sexually mature males being vibrantly coloured and smaller than females (Endler 1980; Houde and Endler 1990; Shohet and Watt, 2004). Males also possess a modified anal fin, the gonopodium, which is used as an intromittent organ to inseminate females. Previous research has shown that there are sex differences in the anti-predator responses of guppies. For example, in the presence of a predator, female guppies are known to devote more time to anti-predator behaviours, such as schooling and predator inspections (Magurran and Nowak, 1991; Magurran et al., 1992). Conversely, colourful males either switch from courtship to sneaky mating attempts (Magurran and Nowak, 1991), or take a risk and continue elaborate courtship displays towards females even when the predator is present (Godin and McDonough, 2002). Accordingly, the aim of our study was to investigate the effects of environmentally relevant levels of fluoxetine exposure on anti-predator behaviour in both male and female guppies. We hypothesized that fluoxetine exposure would decrease anti-predator behaviours and inhibit the reaction of exposed fish to a simulated predatory threat. As such, we expected fluoxetine-exposed fish to spend less time under plant cover and, instead, spend more time swimming and exploring their surroundings. Finally, we anticipated the behavioural responses of guppies to differ between the sexes, due to the aforementioned contrasting predator responses between male and female guppies, and because clinical studies in mammals have shown sex-specific differences to fluoxetine treatment (Dalla et al., 2010).

2. Materials and methods

2.1. Exposure setup

Wild adult guppies were collected from Alligator Creek (19°26′17.94″S, 146°57′1.09″E) in Queensland, Australia. The guppies were caught using dip nets and came from a pristine site adjacent to the Bowling Green Bay National Park. Water quality testing at this location confirmed that this guppy population had not been previously exposed to pharmaceuticals (ALS Group, Environmental Division, unpubl. data). Fish were separated by sex and acclimated to laboratory conditions (12:12 h light:dark regime, +24–26 °C) for two months prior to exposure. After acclimation, fish were randomly assigned to one of 12 separate-sex exposure tanks (60 cm × 30 cm × 24 cm; 15 fish per tank). Tanks were allocated to one of three treatments: (1) a low fluoxetine (FLX) exposure treatment (nominal concentration 50 ng/L, n = 10), (2) a high FLX exposure treatment (nominal concentration 500 ng/L, n = 9), and (3) a solvent control treatment (methanol, 0.00004%), with four tanks in total within each treatment. The level of methanol used was negligible and was therefore considered as a water control. A flow-through system following the design of Saaristo et al. (2009, 2013) was used to administer the fluoxetine or water control to the exposure tanks over a 28-d exposure period. Fish were fed *ad libitum* once daily with commercial fish granules (Otohime Hirame).

2.2. Monitoring of fluoxetine

During the exposure period, 1L water samples from each of the exposure tanks were collected weekly in glass bottles, preserved with 1 g of sodium azide, and filtered through a 0.45 μm cellulose filters (Whatman, England) before storage at 4 °C until extraction. The levels of fluoxetine in the exposure tanks were quantified with liquid chromatography-mass spectrometry (LC/MS) following the protocol of Anumol et al. (2013). Briefly, samples were spiked with isotopically labeled surrogate (100 ng/L) standard before solid-phase extraction using Agilent Plexa cartridge (200 mg). Analysis was performed with an Agilent 1210 Ultra High Performance Liquid Chromatography (UHPLC) connected to an Agilent 6410 triple quadruple mass spectrometer (QQQ). The following transitions were monitored for fluoxetine (310 > 148) and fluoxetine_d6