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Effects of waterborne exposure to the antidepressant fluoxetine on swimming, shoaling and anxiety behaviours of the mosquitofish *Gambusia holbrooki*

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ABSTRACT

Chemical pollution from pharmaceuticals is increasingly recognized as a major hazard to the aquatic biota. Among the wide variety of pharmaceuticals, fluoxetine (FLX) is one of the most widely prescribed antidepressants, and therefore, it is frequently identified in the aquatic environment. As FLX is designed to alter human behaviour and many physiological pathways are conserved across vertebrates, this drug may affect the behaviour of fish living in FLX-polluted environments. Here, we exposed groups of female mosquitofish *Gambusia holbrooki* to waterborne FLX for 14 days, under semi-static conditions with daily renewal of test solutions. Following exposure, we conducted a set of behavioural assays in individual fish, aimed at assessing the effects of FLX on their locomotor activity and behavioural responses. We found that FLX impaired swimming behaviour at high concentrations (25 µg/L and 50 µg/L) but not at low concentrations close to environmental levels (1 µg/L and 5 µg/L). When swimming activity was assessed 5 min after transfer of the focal fish to the testing tank, 50 µg/L FLX was the only concentration showing significant effects. However, when the same trials were performed 24 h later, 25 µg/L FLX turned out to be an effect concentration in addition to 50 µg/L. Interestingly, these concentrations would elicit fish plasma concentrations comprised within the range of human therapeutic doses. When subjected to a light/dark preference test, fish showed tendency to remain less time in the dark area at high FLX concentrations, thus suggesting an anti-anxiety response. Shoaling behaviour was not affected by FLX exposure. Our study contributes to the growing body of literature evaluating the effects of FLX on animal behaviour. Regarding the experimental design used in behavioural testing, our findings suggest that focal fish should be subjected to long habituation periods, namely of at least a few hours, in order to better assess the effects of drug exposure.

1. Introduction

Pharmaceuticals are being identified in global watersheds with increasing regularity, garnering considerable attention as emerging threats to the aquatic environment (Boxall et al., 2012; Arnold et al., 2014; Kuster and Adler, 2014). These compounds originate from a variety of sources, including the discharge of treated domestic sewage and hospital wastewater (Frédéric and Yves, 2014), runoff from aquaculture and livestock farming, and effluents from pharmaceutical manufacturing facilities (Bottoni et al., 2010). Since wastewater treatment facilities are rarely equipped to remove these compounds (Jelic et al., 2012; Blair et al., 2013), small but measureable amounts of active pharmaceuticals are commonly found in receiving watersheds (Kolpin et al., 2002; Khetan and Collins, 2007; Metcalfe et al., 2010). Most of these pharmaceuticals are designed to modulate human physiology and behaviour, eliciting their intended biological responses at relatively low doses. In addition, most of their biological targets are conserved amongst vertebrate species (Gunnarsson et al., 2008). Therefore, non-

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human vertebrates may be affected by the exposure to pharmaceuticals, raising concern over their impact on the aquatic biota (Segura et al., 2009; Corcoran et al., 2010; Boxall et al., 2012; Arnold et al., 2013).

Among the various classes of pharmaceuticals detected in wastewater effluents, antidepressants represent an extremely important class of pollutants. Environmental pollution by these drugs is increasingly recognized as a major threat for aquatic wildlife (Brodin et al., 2013). Fluoxetine (FLX) is an antidepressant commonly used for the treatment of human depression and anxiety disorders (Dulawa et al., 2004; Milea et al., 2010), and one of the world's most widely prescribed psychoactive drugs (Mennigen et al., 2011; Winder et al., 2012). Fluoxetine and its main active metabolite, norfluoxetine, are usually encountered in treated wastewater effluents and have been recorded in effluent-influenced surface waters at concentrations ranging from 0.001 μg/L up to 1.3 μg/L in Europe and North America (Kolpin et al., 2002; Christensen et al., 2009; Metcalfe et al., 2010). The physicochemical properties of FLX 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). In addition, FLX has been found to bioconcentrate in the tissues of fish sampled downstream from wastewater outfalls (Brooks et al., 2005; Ramirez et al., 2009; Schultz et al., 2010).

In target organisms, FLX elicits its therapeutic effects by acting as a selective serotonin reuptake inhibitor (SSRI), i.e. blocking the reuptake of serotonin in the synaptic cleft, which consequently increases the extracellular serotonin levels in the brain (Fuller et al., 1991; Frazer, 2001). The serotonergic system is integral to many biological processes, such as appetite and metabolism, cardiovascular functioning, reproduction and social behaviours (Winberg and Nilsson, 1993; Berger et al., 2009; Lillesaar, 2011) and is conserved across vertebrates, including fish (Mennigen et al., 2011). Fluoxetine has been reported to suppress fish appetite and reduce food intake, growth, and glucose metabolism (Gaworecki and Klaine, 2008; Mennigen et al., 2009, 2010a). FLX also altered reproductive physiology in male fish by reducing testosterone and milt production (Mennigen et al., 2010b), and increasing circulating estradiol and vitellogenin (Mennigen et al., 2010b; Schultz et al., 2011). In females, FLX exposure caused a reduction of estradiol levels, and a decrease on gene expression of aromatase and gonadotropin receptors in the ovaries, which inhibited egg production (Lister et al., 2009), or a reduction of pituitary luteinizing hormone content (Dorelle et al., 2017). However, because FLX is designed to modulate behaviour in humans, impact on behaviour is suggested to be the primary effect of FLX in wild species (Huggett et al., 2003; Rand-Weaver et al., 2013). Studies conducted so far have shown a broad range of behavioural effects in fish, such as weakened mating behaviour (Weinberger and Klaper, 2014), either decreased (Perreault et al., 2003; Dzieweczynski and Hebert, 2012; Kania et al., 2012; Kohlert et al., 2012; Barry, 2013; McCallum et al., 2017) or increased territorial aggression (McDonald et al., 2011), decreased brood defense during parental care (Forsatkar et al., 2014; Greaney et al., 2015), lowered anxiety (Egan et al., 2009; Ansai et al., 2016), decreased stress response (de Abreu et al., 2014), reduced locomotor activity (Kohlert et al., 2012; Winder et al., 2012; Barry, 2013) and lowered predator avoidance (Painter et al., 2009; Weinberger and Klaper, 2014; Pelli and Connaughton, 2015; Martin et al., 2017; Saaristo et al., 2017). Despite increasing concern surrounding the ecological effects of FLX, it remains unclear whether exposure at environmentally realistic concentrations can alter the behaviour of wild fishes. In addition, the relevance of many reported behavioural endpoints is unclear and the value of some findings has been questioned (Sumpter et al., 2014).

The Eastern mosquitofish, *Gambusia holbrooki*, is an active swimming viviparous fish of the family Poeciliidae native to freshwater basins of the east coast of the USA. This species, together with the closely related Western mosquitofish, *G. affinis*, have been introduced in many areas worldwide owing to their presumed ability to control mosquito larvae (García-Berthou et al., 2005). At present, mosquitofish inhabit a wide geographic range and often encounter and occupy polluted environments close to human habitation, where exposure to contaminants, such as FLX, is likely. Therefore, these species represent ecologically relevant organisms to study the effects of FLX on behaviour. In Argentina, *Gambusia* spp. share habitat with other native species of Cyprinodontiformes with similar ecological adaptations. As observed in other regions where they have become invasive, their distribution areas have increased in recent years and their abundance in some environments has been found to be greater than that of native species (Cabrera et al., 2017). As a result, *Gambusia* spp. have become readily accessible as experimental animals to be used in ecotoxicological studies. Here, we used a set of behavioural devices to test the effects of waterborne exposure to FLX on swimming and behavioural responses of female *G. holbrooki*.

2. Materials and methods

2.1. Fish collection and housing

Adult specimens of *G. holbrooki* (N = 112) were captured from a Río de la Plata floodplain lagoon in Buenos Aires city, Argentina (34° 32′ 26″S, 58° 26′ 40″W), located in an area with minimal human influence. Water conditions at the site of fish capture were: temperature 26.5 °C, pH 7.1, conductivity 495 μS/cm, total alkalinity 65.0 mg/L, O_2 3.4 mg/L. Fish were transferred to a 100 L aquarium where they were allowed to acclimate to laboratory conditions for two months prior to the onset of experimentation. They were maintained in dechlorinated tap water at 25 ± 2 °C, under a natural light/darkness photoperiod. Fish were fed once a day with finely ground, dried flake food (TetraMin®) and freshly hatched nauplii of *Artemia* sp.

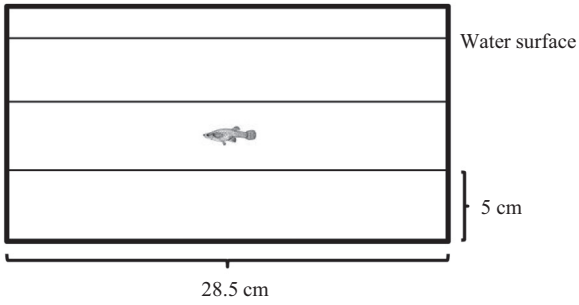
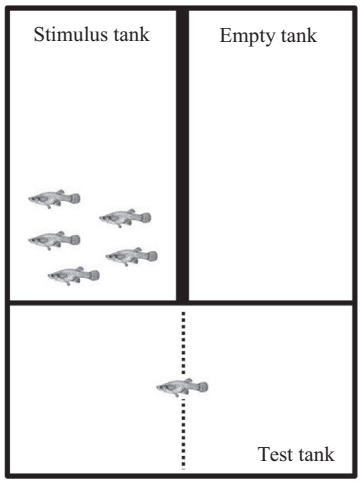
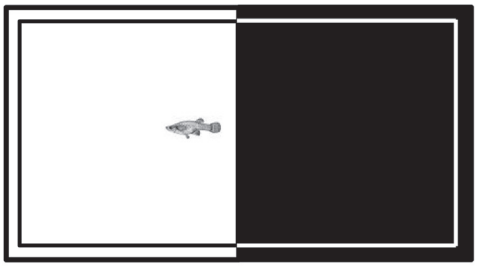
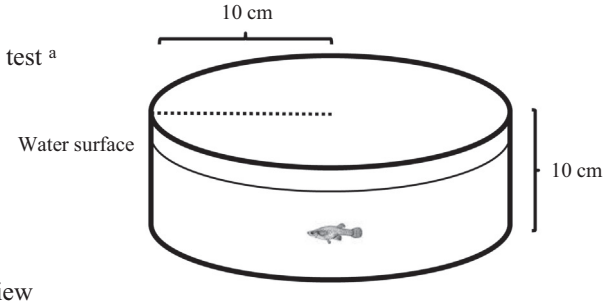
2.2. Fluoxetine exposure

Experiments were performed in a closed room at 25 ± 1 °C and a 12:12 h photoperiod. Female fish (N = 50, total length: 26.5–30.2 mm, weight: 168.2–228.4 mg) were randomly selected and transferred to bare 6 L glass aquaria containing filtered tap water (pH 7.0, conductivity 300 μS/cm, total alkalinity 44.1 mg/L, O_2 6.6 mg/L), where they were allowed to acclimate to tests conditions for 48 h before the experiment was started. Only females were used in this study in order to avoid sexual harassment by males, which might have influenced females' motor behaviour. A fresh stock solution of fluoxetine hydrochloride (99.9% of purity; Saporiti, Argentina) was prepared each week by dissolving it in Milli Q water to a 200 μg/mL concentration and stored in the dark at 4 °C. During each water renewal, the necessary volume of stock solution was added to the aquarium water in order to achieve the desired final concentrations. Fish were exposed to nominal FLX concentrations of 0 (control), 1 μg/L, 5 μg/L, 25 μg/L and 50 μg/L during 14 days. The concentrations assayed ranged from environmentally realistic levels (see Kolpin et al., 2002; Christensen et al., 2009; Metcalfe et al., 2010) to those eliciting fish plasma concentrations in the range of human therapeutic doses (see Margiotta-Casaluci et al., 2014). Treatments were done by duplicate with 5 individuals *per* aquarium. Exposure was conducted under semi-static conditions with daily renewal of whole water and FLX solutions. Fish were fed once a day with nauplii of *Artemia* sp. (100 nauplii *per* fish) during the morning whereas water and solution renewal was done during the afternoon. No mortality was recorded during the course of the experiment.

2.3. Behavioural assays

Following the exposure period, a range of behavioural responses were individually assayed in control and FLX treated fish. Diagrams of the behavioural devices and the testing parameters of behaviours are shown in Table 1. At the onset of the exposure period, each treatment

Table 1
Diagram of the devices used to test the effects of FLX on *G. holbrooki* behaviour.

Behaviour	Diagram of behavioural device	Measured parameters
Locomotor activity [a]		- Distance travelled (cm) - Average speed (cm/s) - Time moving / not moving (s) - Time spent in each third (s)
Group preference [b]		- Distance travelled (cm) - Average speed (cm/s) - Time spent in the side of conspecifics / empty side (s)
Light/dark preference [b]		- Time spent in light / dark area (s)
Novel tank test [a]		- Distance travelled (cm) - Average speed (cm/s) - Latency to free swimming (s) - Time moving / not moving (s)

[a] Lateral view
[b] Upper view

group was started on a different day of the week. Accordingly, behavioural assays following exposure were performed during five consecutive days (one treatment group *per* day) so that at all groups underwent a 14 day exposure. In addition, assays were conducted at the same time of the day to control for possible circadian variation in behaviours. The experimental room was isolated and kept quiet to minimize the interference from the outside. For measurement of testing parameters, trials were video recorded and analyzed with the Ethovision XT 12.0 video tracking software (Noldus, The Netherlands). The experimenter was not visible to the fish during video recording. The observer who analyzed the behavioural data was blind to the experimental condition of the test fish. Experiments were conducted in accordance with the Guidelines on the care and use of fish in research, teaching and testing from the Canadian Council on Animal Care (2005) as well as being in compliance with the local Ethical Committee (Protocol number 18/2014, CICUAL, FCEN, UBA).

2.3.1. Locomotor activity

To assess motor behaviour, fish from each treatment group were transferred to individual testing aquaria identical to the one used during the exposure period, containing 6 L of the same solution to which the fish had been exposed. One treatment group was assayed each day so that a total of 10 testing aquaria were used. Records were performed in order on consecutive days, starting with the control group and ending with the 50 µg/L FLX treatment. During trial recording, a glass with three horizontal lines was placed against the back wall of the testing aquarium so the water area of the tank was equally divided into three horizontal segments. Five minutes after the focal fish was introduced into the aquarium, the locomotor activity was continuously recorded by videotaping during another 5 min. After 24 h, the video recording was repeated for an additional 5 min in order to assess differences between 5 min vs 24 h after transfer of the focal fish to the testing tank.

2.3.2. Group preference

A test of group preference was conducted by placing the testing tank against two additional tanks, an empty tank and a stimulus tank, as indicated in Table 1. The stimulus tank held five mosquitofish as "stimulus fish". Videotaping was performed on the testing tank during 5 min. The amount of time that the focal fish spent on the side closer to the conspecifics was recorded and regarded as group preference or shoaling.

2.3.3. Light/dark preference

To test the light/dark preference, each testing aquarium containing a focal fish was introduced into a chamber which was divided in a light half and a dark half by coverings on the sides and the bottom with matte white and black paper, respectively. Uniform illumination was provided from 1.8 m above the aquarium. Initially, the focal fish was confined to a central compartment comprising both the black and white areas. After a 5-min habituation period, the glass panels that delimited this compartment were removed and the animal was allowed to freely explore the apparatus. From that moment, videotaping was performed on the testing tank during 5 min. The time that the fish spent in each area was recorded as an indicator of the light/dark preference.

2.3.4. Novel tank test

To assess motor behaviour in a novel environment, each focal fish was placed in a circular tank made of white plastic containing 2 L of filtered tap water. After a 5-min habituation period, the locomotor activity was video recorded during another 5 min. Latency to free swimming was defined as the time comprised between introduction of the fish and the moment at which the fish started to move freely and explore the tank. The water in the testing tank was renewed between trials.

2.4. Verification of FLX concentrations

In order to evaluate the decrease of test chemicals in the aquarium water, the actual concentration of FLX was measured by reverse-phase HPLC coupled to fluorescence detection. The column employed was a Gemini C6-phenyl, 100 × 4.6 mm, 3 µm particle size (Phenomenex, USA). The mobile phase was composed of 28% acetonitrile / 72% water with 0.4% triethylamine, adjusted to pH 4 with glacial acetic acid. Elution was performed at a flow rate of 1 mL/min. Detector was set at excitation and emission wavelengths of 230 and 310 nm, respectively. Duplicate water samples were taken upon addition of the chemicals (time 0) and after 24 h from a 50 µg/L FLX aquarium under the same conditions used in the experiment, both in presence and absence of fish. Water samples were also taken from a control aquarium lacking FLX. Samples were treated by solid phase extraction (SPE) on C18 cartridges (Thermo Scientific, USA) followed by elution with acidified methanol (1% glacial acetic acid) before injection in the HPLC. For quantification, calibration curves were constructed for peak areas, from injection of standard solutions daily prepared by adding known amounts of FLX to control water and processed in the same manner as the samples. For each set of replicate samples, mean and standard deviations were calculated after interpolation of FLX chromatographic peak area in the calibration curve (R^2 = 0.99).

2.5. Statistical analysis

All experimental data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc comparisons between experimental groups (Statistica 7.0, StatSoft, Inc., 2004). Paired *t*-tests were performed to compare between parameters for different experimental setups or times within a single FLX concentration. When parametric assumptions were not met, a Kruskall-Wallis test followed by multiple non-parametric comparisons were performed. Significance

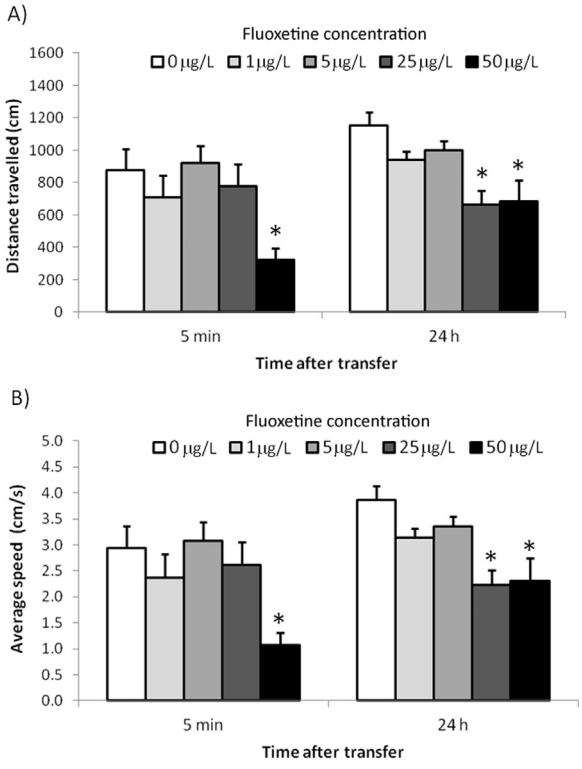


Fig. 1. Effect of FLX exposure on swimming activity of *G. holbrooki*. A) Distance travelled (cm). B) Average speed (cm/s). Asterisks indicate statistically significant differences with the control group for the corresponding time after transfer (One-way ANOVA, followed by Tukey's test).

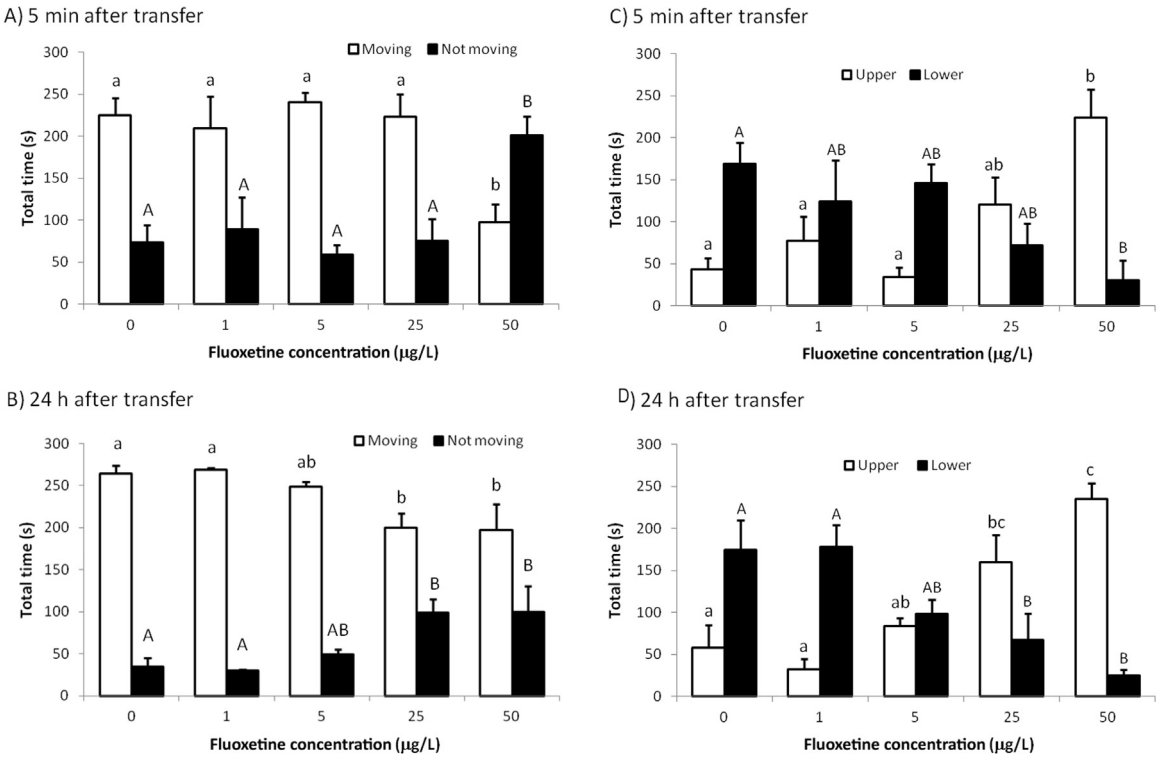


Fig. 2. Effect of FLX exposure on swimming activity of *G. holbrooki*. A, B) Total time (s) moving (white bars) and not moving (black bars) during the 5 min recording period measured 5 min after transfer (A) and 24 h after transfer (B). C, D) Total time spent (s) in the upper (white bars) and lower (black bars) thirds of the water column during the 5 min recording period measured 5 min after transfer (C) and 24 h after transfer (D). Different lowercase letters indicate statistically significant differences between treatments for the time moving in A and B, and for the time spent in the upper third in C and D; different uppercase letters indicate statistically significant differences between treatments for the time not moving in A and B, and for the time spent in the lower third in C and D (A, C, D: One-way ANOVA, followed by Tukey's test; B: Kruskall-Wallis, followed by multiple non-parametric comparisons).

was set at $p < 0.05$. Data are presented as mean ± SEM.

3. Results

3.1. Effects of FLX on locomotor activity

When the effects of FLX on the distance travelled and the average speed were evaluated 5 min after transfer of the focal fish to the testing aquarium, the only treatment showing differences with the control group was that of 50 µg/L, in which FLX elicited a slowdown effect (ANOVA, $p = 0.0292$ for both parameters). However, when the same parameters were measured 24 h after transfer, both the 25 µg/L and 50 µg/L treatments resulted significantly different from the control group (ANOVA, $p = 0.0046$ and $p = 0.0069$ for distance travelled; ANOVA, $p = 0.0044$ and $p = 0.0071$ for average speed) (Fig. 1A, B). For control animals, the distance travelled and average speed were higher 24 h after transfer than 5 min after transfer although differences were not significant (paired *t*- test, $p = 0.224$ for both parameters) (Fig. 1A, B).

Assessment of the time that mosquitofish were moving or static 5 min after transfer showed that fish exposed to 50 µg/L FLX spent significantly more time motionless than fish from the remaining treatment groups (ANOVA,$0.0113 < p < 0.0481$) (Fig. 2A). When measurements were repeated 24 h later, both the 25 µg/L and 50 µg/L treatments showed significant differences with the control group (Kruskall-Wallis, $p = 0.0036$ and $p = 0.0051$, respectively), as well as with the 1 µg/L treatment (Kruskall-Wallis, $p = 0.0034$ and $p = 0.0049$, respectively) (Fig. 2B).

Assessment of fish distribution over the water column 5 min after transfer showed that 50 µg/L treated fish remained more time in the upper third than fish from the 0 µg/L (control), 1 µg/L and 5 µg/L treatments (ANOVA, $p = 0.0008$, $p = 0.0058$ and $p = 0.0007$, respectively), and less time in the lower third than control fish (ANOVA, $p = 0.0456$) (Fig. 2C). Noticeably, when testing was performed 24 h later, both the 25 µg/L and 50 µg/L treatments showed effects on fish vertical distribution. Fish exposed to 25 µg/L FLX spent more time in the upper third and less time in the lower third than fish from the control and 1 µg/L treatments (ANOVA, $p = 0.0247$ and $p = 0.0038$ for upper third; ANOVA, $p = 0.0487$ and $p = 0.0393$ for lower third). Fish exposed to 50 µg/L FLX remained more time in the upper third than fish from the control, 1 µg/L and 5 µg/L treatments (ANOVA, $p = 0.0002$, $p = 0.0001$ and $p = 0.0010$, respectively) and less time in the lower third than control and 1 µg/L treated fish (ANOVA, $p = 0.0034$ and $p = 0.0027$, respectively) (Fig. 2D).

A representative videotape showing the effects of waterborne FLX on swimming activity of mosquitofish during the group-exposure period can be found as Supplementary Material (S1).

Supplementary material related to this article can be found online at 10.1016/j.ecoenv.2018.07.085.

When mosquitofish were exposed to a novel environment in a white circular tank, a significant decrease in the distance travelled and the average speed of control fish was observed as compared to values recorded in glass testing aquaria (paired *t*-test, $p = 0.0012$ and $p = 0.0001$, respectively) (Fig. 3A, B; compare to Fig. 1A, B). In addition, control fish exposed to a novel environment spent less time moving and more time static than previously recorded in glass testing tanks (paired *t*-test, $p = 0.0030$ and $p = 0.0019$, respectively) (Fig. 3C; compare to Fig. 2A). Upon comparisons between treatments, fish exposed to 50 µg/L FLX showed lower values of distance travelled and average speed than fish from the remaining treatment groups, although differences were not significant (ANOVA, $p = 0.1639$ and $p = 0.1542$, respectively) (Fig. 3A, B). In addition, comparisons of either the time

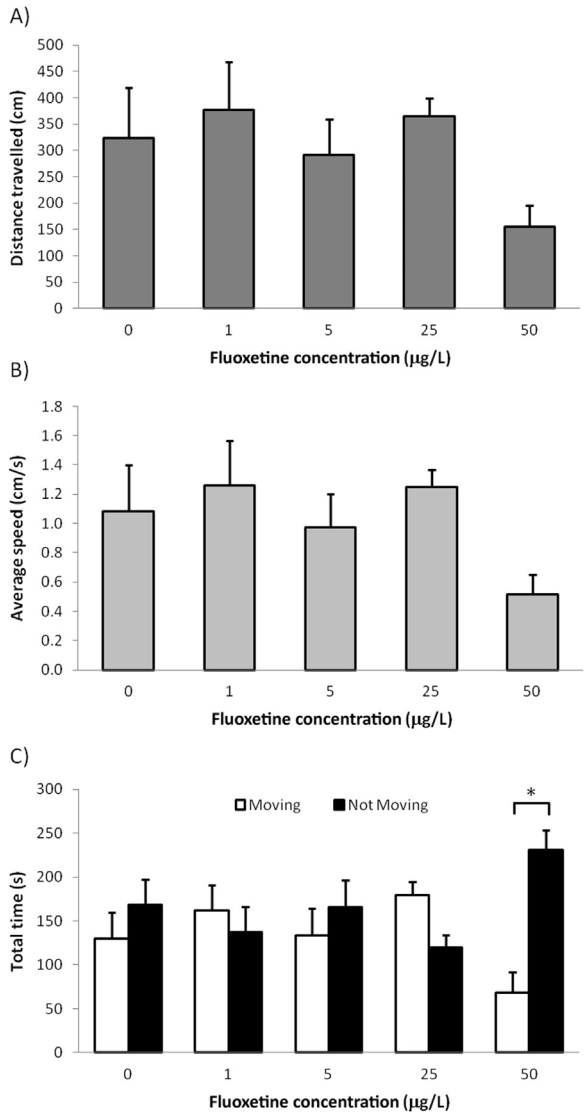


Fig. 3. Effect of FLX exposure on swimming activity of *G. holbrooki* in a novel environment. A) Distance travelled (cm). B) Average speed (cm/s). C) Total time (s) spent moving (white bars) and not moving (black bars) during the 5 min recording period following habituation. Asterisk indicates statistically significant differences between time spent moving and not moving for a given FLX concentration (paired *t*-test).

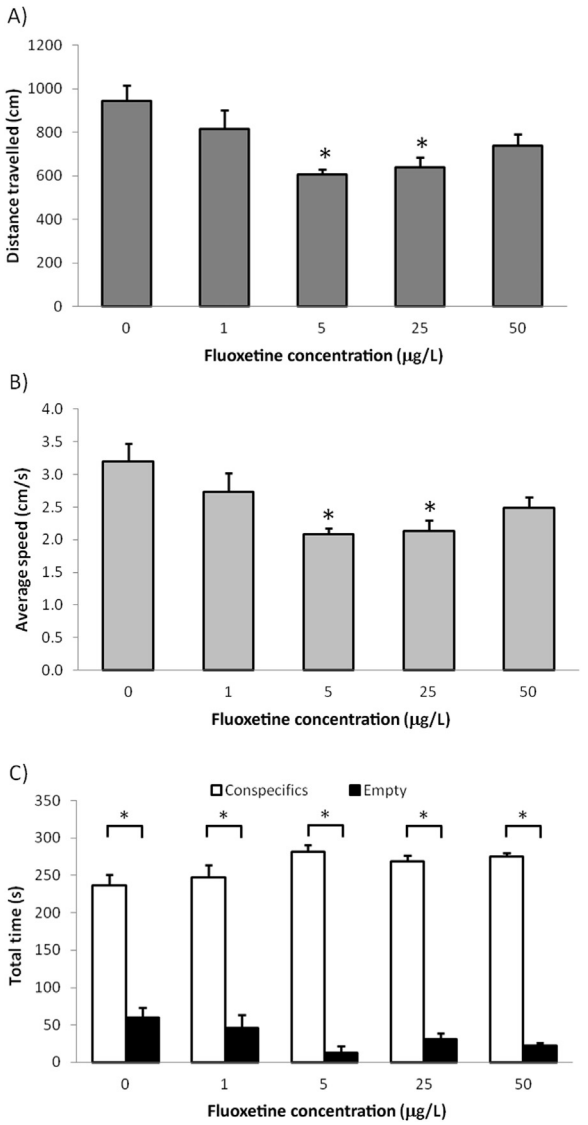


Fig. 4. Effect of FLX exposure on group-preference behaviour of *G. holbrooki*. A) Distance travelled (cm). B) Average speed (cm/s). C) Total time (s) spent in the side of the tank with conspecifics (white bars) or in the side of an empty tank (black bars) during the 5 min recording period. Asterisks indicate statistically significant differences with the control group in A and B (One-way ANOVA, followed by Tukey's test), and differences between time spent in the side of conspecifics and in the empty side for a given FLX concentration in C (paired *t*-test).

moving or not moving revealed no differences between treatments (ANOVA, $p = 0.0896$). However, fish exposed to 50 µg/L FLX remained significantly more time motionless than moving (paired *t*-test, $p = 0.0178$) (Fig. 3C). No differences between treatments were observed for the latency to free swimming (data not shown).

3.2. Effects of FLX on group preference and light/dark preference behaviours

Mosquitofish subjected to a group preference test showed comparable values of distance travelled and average speed to those recorded during assessment of motor behaviour (Fig. 4A, B; compare to Fig. 1A, B). Comparisons of FLX treatments with the control group showed that these parameters were lower in the 5 µg/L and 25 µg/L treatments (ANOVA, $p = 0.0099$ and $p = 0.0151$ for distance travelled; ANOVA, $p = 0.0138$ and $p = 0.0144$ for average speed) (Fig. 4A, B). On the other hand, fish spent more time in the side of the aquarium with conspecifics than in the empty side at all tested concentrations (paired *t*-test, $0.0001 < p < 0.0033$), with no differences between treatments (ANOVA, $p = 0.0788$) (Fig. 4C).

When subjected to a light/dark preference test, control fish showed a preference for the dark area, spending more time on this side of the aquarium (paired *t*-test, $p = 0.0487$). This preference for the dark area was lost in all FLX treatments (paired *t*-test, $p > 0.1799$). However, when comparing the time spent in the dark area between control and FLX-treated fish, no significant differences were evidenced (ANOVA, $p = 0.6748$) (Fig. 5).

3.3. Concentration of FLX in the water

The measured concentrations of FLX in replicate water samples taken at 0 h and 24 h from an aquarium containing 50 µg/L FLX are indicated in Table 2. The actual concentration at time 0 was above 90% of the nominal concentration both in the presence and absence of fish. A

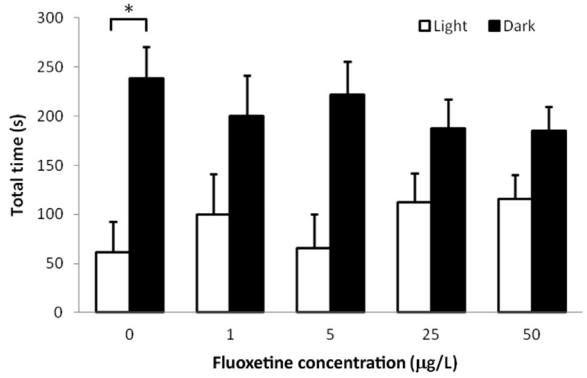


Fig. 5. Effect of FLX exposure on light/dark preference behaviour of *G. holbrooki*. Total time spent (*s*) in the light (white bars) and dark (black bars) areas of the aquarium during the 5 min recording period. Asterisk indicates statistically significant differences between time spent in the light and dark areas for a given FLX concentration (paired *t*-test).

Table 2
Actual concentrations of FLX measured in the aquarium water at 0 h and 24 h after addition of the chemical.

Time (h)	Nominal concentration Fish absent		50 µg/L Fish present	
	Actual concentration (µg/L)	%	Actual concentration (µg/L)	%
0	45.21 ± 2.96	90.4 ± 5.9	48.21 ± 1.75	96.4 ± 3.5
24	43.51 ± 3.22	87.0 ± 6.4	46.77 ± 3.48	93.5 ± 7.0

Values are means ± standard deviations of the concentrations recorded in two samples taken from an aquarium containing 50 µg/L FLX, in presence and absence of fish. % are values for the measured concentrations expressed as percentage of the nominal concentration.

minimal decline of the actual concentration was observed over the 24 h period of solution renewal, measured levels of FLX decreasing only 3% with respect to the initial concentration, both in the presence and absence of fish. FLX was not detected in samples from the control aquarium.

4. Discussion

Behavioural assays are being increasingly incorporated into studies of aquatic toxicology, and there is a need for reliable and repeatable assays, with improved experimental designs. Ideally, these assays will be suited to test behavioural effects in a standardized manner across a wide variety of fish species, specifically non-model species that inhabit affected waterways (Brooks, 2014; McCallum et al., 2017).

In our study, the experimental setup was designed to minimize fish manipulation during trials in order to obtain results as realistic as possible regarding their behavioural responses. Manipulation of test specimens, e.g. transfer of the focal fish into a recording aquarium, may induce stress that transitorily affects their behaviour, so that records performed shortly after transfer may be not fully representative. In fact, we had observed that the locomotor activity of the groups of mosquitofish exposed to 25 µg/L FLX was clearly affected during the experiment, fish appearing motionless and remaining close to the water surface. However, in trials performed 5 min after transfer of the focal fish to the testing tank, measured values of the distance travelled, average speed, time moving/not moving and time spent in the upper/lower thirds in the 25 µg/L treatment group did not differ significantly from those of the control group. Thus, 25 µg/L resulted a no-effect concentration when individual swimming activity was assessed 5 min after transfer, whereas 50 µg/L was the only concentration showing significant effects. However, when the same parameters were measured 24 h after transfer, 25 µg/L turned out to be an effective concentration in addition to 50 µg/L, i.e. significant differences with the control group were recorded in both treatments. Under these circumstances, it is possible that a sort of "novel tank" effect, i.e. fish being less active in an unfamiliar environment, might have affected the assessment of locomotor activity when measurements were done few minutes after transfer, as it is usually performed and reported in the literature. In fact, when comparing the distance travelled, average speed and time moving of control fish between 5 min and 24 h after transfer, lower values of these parameters were observed for the 5-min habituation period, suggesting that a partial attenuation of fish locomotion could be caused by the exposure to the novel testing tank. This effect would be lost after a 24-h habituation interval, thus enabling that the effects of intermediate FLX concentrations, namely 25 µg/L in our study, become apparent. Therefore, in order to obtain more realistic results of the effects of drug exposure on fish locomotion, we suggest that behavioural assays be done after specimens undergo longer habituation periods, of a few hours at least. In our study, we decided to repeat the recordings 24 h after fish transfer in order to be sure that the aforementioned effect would be lost, and also to exclude the time of the day as a source of difference in fish behavioural responses, given that circadian rhythmicity has been reported for mosquitofish locomotor activity (Melvin, 2017). It should be noted that the water in each testing tank was spiked with the appropriate volume of FLX stock solution in order to reach a FLX concentration equal to the one present in the group-exposure tank, thus avoiding potential loss of drug exposure during behavioural trials. Therefore, fish tested 24 h after transfer were exposed to FLX for one day longer than those tested 5 min after transfer. Nonetheless, we consider that the differences observed at the 25 µg/L treatment were not the result of just a 24-h longer treatment within a 14-day exposure protocol.

It has been shown that FLX exposure may affect multiple physiological and behavioural processes in fish through its role in modulating the serotonergic system (Gaworecki and Klaine, 2008; Mennigen et al., 2009, 2010a, 2010b, 2011; Schultz et al., 2011; de Abreu et al., 2014; Paula et al., 2015). In our study, we conducted a 14-day exposure to FLX and assessed the impacts on a range of behaviours important for mosquitofish fitness. For instance, decreased locomotor activity as related to FLX exposure could potentially result in lower survival in fish populations in natural ecosystems. Warner (1966) was among the first to establish that swimming behaviour can be a sensitive indicator of chemical stress. Impairment of swimming capacity may reduce a fish's ability to feed, avoid predators and reproduce, and is therefore considered an ecologically relevant parameter (Little and Finger, 1990). In our study, fish motor activity was not affected by FLX exposure at a low, environmentally relevant concentration (1 µg/L). However, when exposed to high concentrations (25 and 50 µg/L) mosquitofish showed impaired swimming behaviour, slowing down their movement and remaining closer to the surface. This pattern of results is consistent with other studies reporting behavioural effects in fish locomotion following exposure to FLX concentrations above environmental levels. Winder et al. (2012) reported that sheepshead minnow (*Cyprinodon variegatus*) treated with 300 µg/L FLX exhibited reduced locomotor activity after a few hours following exposure. Siamese fighting fish (*Betta splendens*) exposed to 350 µg/L and 705 µg/L FLX showed a significant decrease in locomotion on days 19 and 11 of drug exposure, respectively, and the effects persisted for at least 13 days after FLX removal (Kohlert et al., 2012). More recently, Eisenreich et al. (2017) reported that a 30-min exposure to 3 mg/L FLX decreased aggression and normal swimming behaviour in *B. splendens* and provided evidence for a motor inhibition as the main behavioural mechanism of action for fluoxetine's attenuation of aggression. A few studies have reported effects of exposure to environmentally relevant concentrations of FLX on swimming responses. Barry (2013) reported that Arabian killifish (*Aphanius dispar*) exposed to 300 ng/L FLX for 7 days reduced their swimming speed by

38% after addition of a predator alarm substance. Contrarily, Martin (2017) observed a general increase in activity levels of *G. hoolbroki* exposed to 25 ng/L FLX for 28 days, both in the presence and absence of a predator. The differences between these findings could potentially be explained by differences in exposure scenarios, as acute and chronic FLX exposures have been shown to produce different, and even conflicting, behavioural effects.

Following trials evaluating the effects of FLX exposure on mosquitofish locomotion, we performed an assay aimed at assessing the shoaling behaviour of FLX-exposed individuals. The values of the distance travelled and average speed recorded in this assay (Fig. 4) were comparable to those registered previously in the swimming response assay (Fig. 1). This was an expected result as these trials were performed in the same testing tanks, without fish manipulation that might have affected their motor behaviour. The treatments showing significant differences with the control group were 5 µg/L and 25 µg/L FLX. Fish exposed to 50 µg/L FLX showed lower values for both parameters, although differences with the control group were not significant. Regarding the effects on group preference, mosquitofish remained close to the stimulus fish over 80% of the time at all tested concentrations, with no difference between treatments, i.e. FLX exposure did not affect their shoaling behaviour.

In our study, two assays were performed to assess a putative anxiolytic effect of FLX exposure on *G. holbrooki*. One of the tests that have been developed for the measurement of anxiety in fish is the scototaxis (dark/light preference) protocol (see Maximino et al., 2010). In this behavioural model, the focal fish is placed in a central compartment of a half-black, half-white tank; following habituation, the fish is allowed to explore the tank and the number and duration of entries in each area are recorded. Most fish species demonstrate a preference for the dark area; an increase in the time spent in the white compartment is interpreted as an anti-anxiety behaviour. In our study, we established a habituation interval of 5 min, whereas fish were allowed to explore the apparatus freely for an additional 5 min period. The duration of the recording session was limited to 5 min, rather than using longer periods of 10 or 15 min, because the time course of exploration in our study did not reveal significant changes for the time spent in the dark/light compartment at different time periods. We observed that under no FLX exposure, mosquitofish spent more time in the dark area, as evidenced in other telesots. This preference for the dark compartment was lost at all tested FLX concentrations. However, upon comparison of the time spent in the dark half of the aquarium, no differences between treatments were observed. Even so, a tendency towards a decrease of the time spent in the dark area was evidenced in the 25 µg/L and 50 µg/L treatments, indicating that we may have lacked power to identify differences at high FLX concentrations. Thus, exposure to the high 'therapeutic' concentrations of FLX could have had an anxiolytic effect on mosquitofish, showing a similar pattern of response with the effects exerted on swimming activity.

Another assay commonly used for the assessment of anxiety responses in fish is the novel tank test. It is based on the instinctive behaviour of fish to seek protection when they are transferred to a novel and unfamiliar environment (i.e. observation tank) by diving to the bottom and remaining in an alert motionless status until the environmental conditions are perceived as safe enough to initiate exploration of the new environment. In our study, the effect of the novel environment was evidenced in the reduction of the average speed, distance travelled and time moving of control and FLX-exposed fish (Fig. 3), as compared to values of these parameters recorded in glass aquaria which were perceived as a familiar environment (Figs. 1; 2A, B). An anti-anxiety response would have been expressed as an increase in locomotor activity at increasing FLX concentrations. However, fish exposed to 1–25 µg/L FLX remained as motionless as control fish, whereas fish treated with 50 µg/L FLX were significantly less active, suggesting that the slowdown effect of FLX at the higher concentration was prevalent to the putative anxiolytic effect.

According to the read-across hypothesis (Rand-Weaver et al., 2013), the mode-of-action of a drug will 'read-across' from humans to other organisms, leading to similar effects in the different organisms (e.g. in humans and fish) and, further, these similar effects will occur at similar blood concentrations. Interestingly, FLX effects on mosquitofish swimming behaviour were evidenced at water concentrations that would elicit plasma concentrations comprised within the range of human therapeutic doses, thus endorsing this theory. In humans, behavioural changes during FLX treatment occur when plasma concentrations reach between 90 µg/L and 300 µg/L (Amsterdam et al., 1997; de Freitas et al., 2010; Margiotta-Casaluci et al., 2014). Recently, Margiotta-Casaluci et al. (2014) showed that fathead minnow were more exploratory in a novel tank when their plasma FLX concentrations reached levels similar to those needed to elicit therapeutic responses in humans. In their study, this 'therapeutic' effect in the fish occurred at water concentrations equal or greater than 38 µg/L for a 28-d exposure, and of 72 µg/L for a 14-d exposure. Using calculations from the Fish Plasma Model (Huggett et al., 2003 modified for FLX by Margiotta-Casaluci et al., 2014), the range of FLX concentrations in water eliciting fish plasma concentrations of FLX within the human therapeutic range would be 24 µg/L–80 µg/L. Using this model, the water concentrations of FLX used in our study would result in steady state concentrations of FLX in mosquitofish plasma of 3.7 µg/L, 18.7 µg/L, 93.5 µg/L and 186.9 µg/L for those fish exposed to 1 µg/L, 5 µg/L, 25 µg/L and 50 µg/L, respectively. Therefore, mosquitofish exposed to 25 µg/L and 50 µg/L may have experienced a plasma concentration of FLX within the range of human therapeutic doses, as noted for fathead minnow by Margiotta-Casaluci et al. (2014). Changes in fish behaviour at FLX concentrations of 1 µg/L or even lower have been reported by some authors (Pelli and Connaughton, 2015; Dzieweczynski and Hebert, 2012; Greaney et al., 2015; Weinberger and Klaper, 2014). However, in a current review of FLX and its effects in fish, Sumpter et al. (2014) noted that most of the documented behavioural effects occur at water concentrations of 30 µg/L to 100 µg/L. Then, our findings add to the evidence that behavioural effects are elicited at concentrations at least 10-fold greater than environmental levels.

Lastly, under the experimental conditions used in our study, FLX proved to be stable over the 24 h period of solution renewal. Based on the actual concentrations measured from an aquarium containing 50 µg/L FLX, we assume that mosquitofish were exposed to stable FLX concentrations that were close to the nominal values. Kwon and Armbrust (2006) reported that the half-life of FLX in water at pH 7 is 277 days. Then, our results add evidence to the stability of FLX in aqueous solution. It should be noted that although measurements were done only in samples from the 50 µg/L treatment due to detection constrains of the technique employed, comparable results are expected with the other assayed concentrations.

5. Conclusions

The results of our study indicate that FLX exposure caused adverse effects on mosquitofish locomotor activity at high concentrations, namely those eliciting human therapeutic doses, but not at low concentrations next to environmental levels. Our research can be added to a growing body of literature indicating that FLX has little notable impact on fish behaviour at environmentally relevant concentrations (Sumpter et al., 2014). However, it should be noted that although the concentrations of single pharmaceuticals may be below the thresholds for which adverse behavioural effects are reported, different compounds may coexist in the aquatic environment and additive responses may induce behavioural impairment in fish chronically exposed to this class of pollutants. Such evidences suggest that further assessment of the effects of pharmaceuticals, including exposure to mixtures and field studies are required.

From a methodological point of view, our study shows that the significance of the results of behavioural experiments evaluating the

effects of FLX exposure on locomotor activity was higher when individual responses in focal fish were assessed 24 h after transfer to the testing tank, instead of a few minutes after, as it is usually performed and reported in the literature. Thus, in order to obtain more realistic results, we recommend that focal fish remain in testing tanks for at least a few hours before behavioural trials are performed.

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