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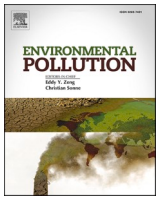

Fluoxetine enhances reproductive output without affecting spawning-site selection in turquoise killifish

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ABSTRACT

Pharmaceutical contamination of aquatic ecosystems is widespread, with mounting evidence that pollutants such as the antidepressant fluoxetine can boost fish offspring production. But such increases may come at a hidden cost if pharmaceuticals also disrupt key reproductive behaviors—like where fish choose to spawn—that determine whether those offspring ultimately survive. To test this possibility, we exposed turquoise killifish (*Nothobranchius furzeri*) to a field-realistic concentration of fluoxetine (317 ng/L) in a controlled laboratory experiment. Fish could choose among spawning sites of white, orange, or black sand, both in single pairs and in groups. Fluoxetine-exposed fish produced about 50% more eggs in single pairs, but this effect was not observed in groups. Regardless of social context, fish deposited roughly four times more eggs on black sand than on orange or white sand, and fluoxetine did not alter spawning-site preference. In addition, fluoxetine did not affect habitat preference in a non-mating context, with both control and fluoxetine-treated fish spending more time over darker backgrounds. These results show that environmentally relevant fluoxetine concentrations can enhance reproductive output without necessarily affecting spawning-site selection. The broader ecological significance of such changes will depend on how pharmaceutical-induced shifts in reproduction interact with natural social and habitat dynamics in the wild.

1. Introduction

Pharmaceuticals continuously contaminate aquatic ecosystems, where they can exert far-reaching but still poorly understood effects on wildlife (Brand et al., 2025; Wilkinson et al., 2022). Among them, antidepressants such as fluoxetine have attracted particular concern (Martin et al., 2025), because they can alter the behavior and reproduction of aquatic animals even at very low concentrations. Environmentally relevant levels of fluoxetine, typically in the mid-to-high ng/L range (Mole & Brooks, 2019), have been shown to affect activity (Martin et al., 2017; Thoré, Brendonck, et al., 2021a), social interactions (Mason et al., 2021; Thoré et al., 2020) and aggression (Barry, 2013; Perreault et al., 2003), risk-taking (Martin, Bertram, Saaristo, Fursdon, et al., 2019b; Polverino et al., 2021) and exploratory behavior (Orozco-Hernández et al., 2022; Vera-Chang et al., 2018), and reproduction (Fursdon et al., 2019; Martin, Bertram, Saaristo, Ecker, et al., 2019a) in a broad range of aquatic species, including stickleback (Sebire et al., 2015), mosquitofish (Martin, Bertram, Saaristo, Ecker, et al., 2019a), medaka (Foran et al., 2004), Trinidadian guppy (Saaristo et al., 2017), fathead minnow (Weinberger II and Klaper, 2014), zebrafish (Al Shuraiqi et al., 2021), Siamese fighting fish (Forsatkar et al., 2014), and turquoise killifish (Thoré, Philippe, et al., 2021b). In several of these species, fluoxetine exposure has even been found to increase reproductive output, with exposed individuals engaging in more than twice as many mating attempts (Bertram et al., 2018; Martin, Bertram, Saaristo, Ecker, et al., 2019a) or producing over twice as many eggs or offspring

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as unexposed controls (Thoré et al., 2020).

Despite this growing body of work, the ecological risks posed by pharmaceuticals remain difficult to predict (Bertram et al., 2024; Thoré et al., 2023). An increase in reproductive output does not necessarily translate into higher fitness if exposure simultaneously alters reproductive decision-making, such as spawning-site selection, in ways that reduce offspring survival. More broadly, chemical exposure can also modify how aquatic organisms perceive and use their environment, including through altered attraction to or avoidance of particular habitats or environmental cues (Islam et al., 2025; Sandoval Herrera et al., 2025; Stremmel et al., 2023). Such behavioral shifts may lead individuals into suboptimal environments or alter their exposure to ecological risks, yet they are rarely considered in standard ecotoxicological tests, which are typically conducted under highly simplified laboratory conditions (Bertram et al., 2022; Mason et al., 2021). These constraints mean that laboratory studies cannot capture the full ecological complexity of natural habitats. Still, controlled experiments remain essential for identifying behavioral pathways through which pollutants may influence fitness-related outcomes, thereby providing a mechanistic foundation for future ecological risk assessments.

For many substrate-spawning fish, reproductive success depends not only on egg production but also on the selection of suitable spawning sites (Taylor et al., 2019). This selection can arise from multiple motivational systems, including both reproductive decision-making and broader attraction–avoidance responses, such as risk-related or anxiety-driven background preferences (Maximino et al., 2010). Regardless of the underlying motivation, the chosen substrate has important consequences for egg survival by determining exposure to predation, physical disturbance, and other environmental pressures (Čech et al., 2012; Miano et al., 2019). Turquoise killifish (*Nothobranchius furzeri*), for example, are annual substrate-spawning fish that deposit their eggs in dark, clay-rich vertisol soils ('black cotton soil') in ephemeral African pools (Reichard et al., 2009; Watters, 2009). These soils retain moisture during the dry season and are crucial for preserving developing embryos (Cellerino et al., 2016; Thoré & Merckx, 2023). Understanding how pollutants influence spawning-site selection in such species is therefore essential for evaluating how laboratory-observed changes in reproduction may translate into ecologically meaningful outcomes.

Here, we test whether exposure to an environmentally relevant concentration (317 ng/L) of fluoxetine affects spawning-site selection in the turquoise killifish. We provided fish with a choice among substrates of contrasting colors and quantified spawning behavior in both single pairs and small groups, allowing us to explore whether fluoxetine's effects on reproduction are consistent across contexts. Based on the anxiolytic properties of fluoxetine (McDonald, 2017), and on evidence that avoidance of bright or open backgrounds is anxiety-related (Maximino et al., 2010), we hypothesized that exposed fish might show reduced discrimination among spawning substrates. We also expected fluoxetine to increase eggs production, as previously shown in this species (Thoré et al., 2020). Overall, our study aims to identify potentially overlooked behavioral mechanisms through which antidepressants may influence reproduction, thereby helping to connect simplified laboratory observations with behaviorally mediated processes that shape reproductive success in natural systems.

2. Materials and methods

2.1. Fish recruitment and maintenance

The experiment ran from 01 May 2022 to 28 July 2022 using *Nothobranchius furzeri* eggs obtained from a genetically diverse laboratory culture that originates from a natural population in Mozambique (MZCS-222). This culture had been maintained under optimal laboratory conditions for over five generations at the breeding facility of KU Leuven (Belgium).

Ninety fish were hatched by immersing ready-to-hatch eggs (following the method described by Philippe et al. (2018) in reconstituted water at a pH of 7.8. The water was prepared by adding Instant Ocean Salt mix (Blacksburg, VA, USA) into type III RO water until a conductivity of 600 μS/cm was achieved. Additionally, 1 g/L humic acid (53,680; Merck, Darmstadt, Germany) was added to the water.

Eight days after hatching, hatchlings were transferred to 10-L glass tanks (50 cm long × 20 cm wide × 17.5 cm high) with aerated reconstituted water. From 8 dph onward, all husbandry procedures described below were conducted in parallel for two experimental lines: a control line and a fluoxetine-exposed line (see *2.2 Experimental treatment and preparation of solutions*). The fish were kept in groups of 6–7 individuals per tank. At 43 days post-hatching (dph), all fish were presumed to have reached sexual maturity and were transferred to 2-L transparent glass jars, with one fish per jar, until 67 dph. This allowed for individual monitoring while still enabling visual interaction among the fish. Starting from 67 dph and until the end of the experiment, fish were transferred back to the 10-L glass tanks, but this time in social groups of two males and two females per tank.

Throughout the experiment, tanks and jars were cleaned once and twice per week, respectively. During each cleaning and water renewal, fish were gently transferred with a hand net into a clean tank or jar containing freshly prepared treatment water. No anesthetic was used during these routine transfers. Each time, the water was entirely renewed to maintain good water quality and ensure a stable concentration of fluoxetine (see *2.2 Experimental treatment and preparation of solutions*). Cleaning coincided with spawning tests and/or egg collection (see *2.3 Spawning tests with single male-female pairs* and *2.4 Spawning in social groups*) to minimize fish handling. Water quality was closely monitored at least twice per week (ammonium <0.2 mg/L, nitrate <25 mg/L, and nitrite <0.2 mg/L).

The fish tanks and jars were placed in heated water tubs (approx. 60 cm long × 200 cm wide × 20 cm high) at a constant temperature of 25 °C and subjected to a 14-h light: 10-h dark photo-regime. Each tub could house up to 9 tanks or 25–35 jars (depending on the housing setup). To minimize positional confounds, tanks and jars were randomly assigned to positions within each tub and re-randomized after each cleaning cycle. A schematic overview of the water-tub layout is provided in Supplementary Fig. S1. The tubs were made from grey polyvinyl chloride, ensuring a grey background for fish in the jars/tanks. Full-spectrum, white LED lights with a constant intensity of 2000 lux (at the jar level) were used, which were switched on at 8.00 a.m. and off at 10.00 p.m. daily.

Fish larvae were fed twice daily until satiation (at 8.30 a.m. and 6.30 p.m.) with live *Artemia franciscana* nauplii (Ocean Nutrition, Essen, Belgium) from 1 to 21 dph. From 14 dph, frozen *Chironomus* larvae (Ocean Nutrition, Essen, Belgium) were added to the *Artemia* diet once a day. Starting from 21 dph, fish were fed twice daily until satiation with frozen *Chironomus* larvae, and from 43 dph until the end of the experiment, they were fed once daily (at 8.30 a.m.). After each feeding session, any excess food was carefully removed from the tanks/jars using a glass pipette to maintain good water quality.

2.2. Experimental treatment and preparation of solutions

At 8 dph, the fish were randomly assigned to two different conditions: the control group (n = 46: 22 males, 24 females) and the experimental group that was chronically exposed to fluoxetine (n = 44: 19 males, 25 females) at a nominal concentration of 0.5 μg/L until 88 dph. During the pre-sexual maturation phase (8–43 dph), fish were housed in 10-L tanks in groups of 6–7 individuals (see *2.1 Fish recruitment and maintenance*), resulting in 7 tanks for the control treatment and 7 tanks for the fluoxetine treatment. During the post-sexual maturation phase (43–67 dph), fish were housed individually in 2-L jars, resulting in 46 control jars and 44 fluoxetine jars (see *2.1 Fish recruitment and maintenance*). The nominal fluoxetine concentration falls within the upper

range of concentrations measured in surface waters worldwide (Mole & Brooks, 2019; Puckowski et al., 2016). Fluoxetine hydrochloride (CAS 56296-78-7; F-132; Merck, Darmstadt, Germany) was used to prepare a 5 mg/L stock solution (dissolved in reconstituted water). During water exchanges (once or twice per week, as described in *2.1 Fish Maintenance*), the stock solution was added to the tanks/jars to achieve the nominal concentration of 0.5 μg/L.

To verify the actual fluoxetine concentration during exposure, water samples were collected at six random time points, just prior to each water renewal. Each time, samples were taken from three randomly selected jars and pooled together to create a single sample replicate for both the fluoxetine-exposed condition (n = 6) and the control condition (n = 6). The concentration of fluoxetine in these pooled samples was determined using liquid chromatography coupled to mass spectrometry (LC/MS/MS). The measured concentration was 0.317 ± 0.029 μg/L (mean ± SD). No detectable levels of fluoxetine were found in the samples from the control condition.

2.3. Spawning tests with single male-female pairs

Between 43 and 67 dph, the fish were individually housed (as described in *2.1 Fish Maintenance*) and paired twice a week (1 male + 1 female) for spawning, for a total of five spawning sessions per individual (Fig. 1). This design allows for individual monitoring of fish without the need for marking, ensures standardized environmental and social conditions, and maximizes egg yield (Reichard et al., 2022).

For each spawning session, a female was transferred to a separate 10-L glass spawning tank (50 cm long × 20 cm wide × 17.5 cm high) containing 3 transparent plastic trays (16 cm long × 11 cm wide × 6.5 cm high) randomly positioned within the tank. Each tray was filled with spawning substrate of a different color: white, orange, or black (130 g per tray, ±1 cm depth). The spawning substrates used were Sansibar White, Sansibar Orange, or Sansibar Dark (JBL, Neuhofen, Germany), consisting of fine granite sand that was thoroughly rinsed and sieved to a grain size of 0.2–0.5 mm before use. Spawning tests were conducted in clean reconstituted water without added fluoxetine; thus, fish from the exposure treatment were temporarily removed from active fluoxetine exposure during the spawning sessions.

Upon introduction to the spawning tank, each female acclimated for 5 min before a randomly chosen male was added to the tank. The paired fish could then spawn for 2 h. After the spawning session, each fish was returned to its individual housing jar, and the sand in the trays was sieved (mesh size of 0.5 mm) to count the number of eggs deposited per substrate type. Additionally, the water in the spawning tank was sieved to account for any eggs that might have been deposited outside the spawning trays.

Spawning tests were consistently conducted between 10.00 a.m. and 3.00 p.m. to minimize potential confounds related to daily behavioral variation (Thoré, Brendonck, et al., 2021a). To facilitate this experimental schedule, fish from both the control and fluoxetine-exposed conditions were divided into two cohorts, ensuring that not all spawning tests had to be conducted on the same day. Fish from cohort 1 (23 control fish and 26 fluoxetine-exposed fish) spawned at 46, 51, 54, 58, and 61 dph, and fish from cohort 2 (23 control fish and 18 fluoxetine-exposed fish) spawned at 50, 53, 57, 60, and 64 dph. In cases where there were slightly more females than males, a small number of females were paired with a male from the other cohort, with these males randomly chosen each time. Despite male *N. furzeri* being constantly ready to coerce females into spawning (Cellerino et al., 2016), a minimum of 24 h of rest was always provided between spawning tests for the males.

One female from the control condition was lost due to early mortality at 60 dph and could only complete three out of the five spawning sessions, resulting in a total of 243 spawning sessions of single male-female pairs.

2.4. Spawning in social groups

From 67 to 88 dph, a total of 72 fish were randomly selected and housed in social groups (as described in *2.1 Fish Maintenance*), with each group consisting of two males and two females per tank. The composition of individuals within each group was randomly determined and remained unchanged throughout the experiment (n = 18 tanks: 9 control tanks + 9 fluoxetine tanks, each tank containing 4 fish).

During this period, all tanks continuously contained three transparent trays, each filled with a different type of spawning substrate

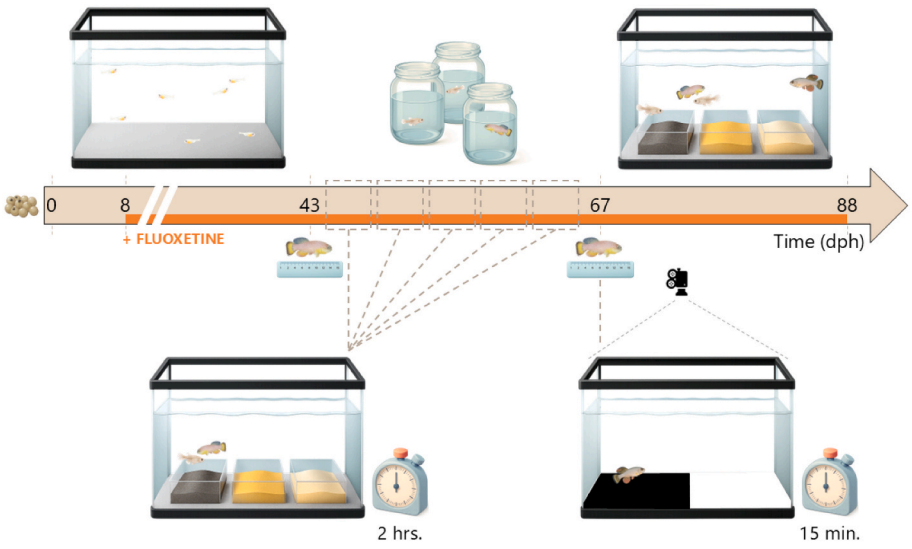

Fig. 1. Overview of the experimental design. From 43 to 67 days post-hatching (dph), adult fish were kept individually. Twice per week, each fish was randomly paired with a partner of the opposite sex (1 male + 1 female) and allowed to spawn for 2 h, resulting in 5 spawning sessions per individual. Three types of spawning substrate were available during these trials: white, orange, and black sand. At 67 dph, each fish underwent a 15-min habitat-choice test to evaluate preference for a white *vs.* black background outside of a mating context. Following this, fish were transferred to social groups of two males and two females per tank, where they could spawn freely for three weeks (eggs were collected weekly). Total body length was measured at 43 and 67 dph. A total of 44 individuals were chronically exposed to 317 ng/L fluoxetine from 8 to 88 dph, while 46 control fish remained unexposed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(white, orange, and black sand)—replicating the setup used during the spawning tests with single male-female pairs (see *2.3 Spawning tests with single male-female pairs*). Fish remained in their assigned treatment conditions, meaning that fluoxetine exposure was continuously maintained in the exposed tanks. Once weekly, at 74, 81, and 88 dph, coinciding with the regular tank cleaning schedule, all sand (and water) in the tanks was sieved to count the number of eggs deposited on each type of substrate.

2.5. Habitat-choice test

At 67 dph, prior to transferring the fish to tanks in social groups (as described in *2.1 Fish Maintenance* and *2.3 Spawning in Social Groups*), each individual fish was subjected to a habitat-choice test to assess its preference for a white *versus* black background in a non-mating context (Fig. 1). The habitat-choice test arena comprised a 10-L glass tank (50 cm long × 20 cm wide × 17.5 cm high) with the background divided into a white half and a black half, randomly oriented. The sides of the arena were covered with an opaque white screen to prevent disturbances during the test.

At the beginning of the habitat-choice test, each fish acclimated for 5 min. Subsequently, the fish's movement was recorded for 15 min (900 s) using a Logitech C920 HD Pro webcam positioned centrally above the arena (top-view). To minimize potential confounds related to daily behavioral variation (Thoré, Brendonck, et al., 2021a), all tests were consistently conducted between 10.30 a.m. and 3.00 p.m. After the test sessions, all recorded videos were manually analyzed in an observer-blind manner to determine the amount of time each fish spent in the white *versus* black zone of the arena. Habitat-choice tests were conducted in clean reconstituted water without added fluoxetine; thus, fish from the exposure treatment were temporarily removed from active fluoxetine exposure during the 15-min assay.

2.6. Total body length measurement

The total body length of each fish (i.e., from the tip of the snout to the tip of the tail) was measured at two time points: 43 dph, just before individual housing, and 67 dph, immediately following the habitat-choice test and before social housing (Fig. 1).

For this, each fish was gently placed in a Petri dish containing a small amount of water to minimize vertical movement and handling stress. No anesthetic was used during this procedure. Subsequently, the fish was carefully centered in the frame (dorsal view) of a Samsung Galaxy S8+ dual-pixel 12.0 MP AF F/1.7 camera. A size-calibrated photograph of each fish was taken and analyzed using ImageJ v. 1.50i software (Schneider et al., 2012).

2.7. Animal welfare note

All experimental procedures strictly adhered to the legal requirements for animal research in Belgium and were approved by the ethical committee of KU Leuven (file number: P113-2022). The condition and health of each individual fish were closely monitored at least twice per day to identify any signs of distress or health issues. The fish were maintained in tanks with optimal water quality, and standard *N. furzeri* husbandry procedures were followed to provide a suitable environment. To minimize potential stress to the fish, any disturbance and handling that were not strictly necessary for the experiment were kept at a minimum.

2.8. Statistical analyses

All statistical analyses were conducted using R version 4.0.5 (R Development Core Team, 2021) with a significance level set at $\alpha = 0.05$. Model assumptions, including distributional fit and homogeneity of variances, were visually verified. Gaussian error distributions were further confirmed using a Shapiro-Wilk test.

For the analysis of the number of eggs deposited per substrate type during the spawning tests with single male-female pairs (43–67 dph), a linear mixed-effects model *lme4* package (Bates, 2016) with Poisson error distribution was used. The fixed factors included in the model were experimental condition (control, fluoxetine), substrate type (white, orange, or black sand), and total body length at 43 dph (mean-centered and scaled). The model also included the two-way interaction terms between each of these factors. Initially, the three-way interaction term was included, but it turned out to be non-significant and was therefore excluded from the final model based on AIC comparisons. Random effects included the identity of females and session number (referring to the repeated measures: 1–5). Additionally, an observation-level random effect was included to account for overdispersion. The cohort factor (cohort 1, cohort 2) was initially considered as an additional random effect but was dropped from the final model (after AIC comparison and a likelihood-ratio test that compares the model with and without the cohort random affect structure). Only actual clutches were considered, meaning that females had to deposit at least one egg, irrespective of whether it was placed in the white, orange, or black sand. In cases where females did not produce a minimum of one egg, the spawning was deemed unsuccessful and treated as a missing value (19 out of 243 spawning sessions).

Similarly, the number of eggs deposited per substrate type during the spawning in social groups (67–88 dph) was analyzed using a linear mixed-effect model with Poisson error distribution. The fixed factors in this model were experimental condition and substrate type, along with their interaction. Random effects included group identity (tank number) and session number (repeated measures: 1–3). An observation-level random effect was included to account for overdispersion.

Type 3 Wald chi-square tests were used to test the significance of the fixed effects and interaction terms. Post-hoc differences were evaluated using Tukey-corrected pairwise comparisons *lsmeans* package (Lenth, 2016).

To analyze female fecundity, the total number of deposited eggs per session was assessed for both single male-female pairs and social groups, regardless of the color of the substrate in which they were deposited. The analysis included zero values, which accounted for instances where no eggs were deposited during a session. Similar models were used as described above, with the factor substrate type removed from the models.

To evaluate habitat choice during the habitat-choice test (in a non-mating context), a linear model with Gaussian error distribution was constructed. The response variable was measured as the time spent in the white zone *minus* the time spent in the black zone (in seconds). A positive score indicates a preference for the white zone, while a negative score indicates a preference for the black zone. A score of 0 indicates an equal amount of time spent in both zones. The fixed factors included in the model were condition (control, fluoxetine), sex (male, female), and total body length at 67 dph (mean-centered and scaled). The model also included the two-way interaction terms between each of these factors. Additionally, a one-sample *t*-test with $\mu = 0$ was conducted separately for control fish and fish treated with fluoxetine to assess whether the fish on average preferred one habitat over the other (as opposed to having no preference).

3. Results

Females deposited a total of 16,372 eggs, with 5895 eggs during the single-pair spawning trials and 10,477 eggs during the 3-week spawning in social groups. Most of these eggs (16,193 eggs, 99%) were deposited within the spawning trays, while only a minimal fraction (179 eggs, 1%) was found outside of the trays. When considering only the eggs deposited within the trays, fish exhibited a clear preference for depositing eggs on black sand in both the single-pair and social settings. This preference was independent of female size and unaffected by fluoxetine exposure

Table 1
Output of the linear mixed-effects models to analyze the number of deposited eggs (i.e., clutch size) per substrate type during spawning tests with single male-female pairs (43–67 dph) and during spawning in social groups (67–88 dph) in *Nothobranchius furzeri*.

	Clutch size of single male-female pairs		Weekly clutch size of social groups	
Effect	χ^2	*P* value	χ^2	*P* value
Condition	3.526	0.060	1.387	0.239
Substrate	208.921	**< 0.001**	137.506	**< 0.001**
Size	0.068	0.794	-	-
Condition × Substrate	0.655	0.721	1.006	0.605
Condition × Size	0.669	0.413	-	-
Substrate × Size	2.030	0.362	-	-

(Table 1; Fig. 2).

For single pairs of the control condition, the average clutch size on black sand (mean ± SD; 14 ± 13 eggs) was 3.5 times higher (Tukey $P < 0.001$; Fig. 2A) than that on orange (4 ± 6 eggs) and white sand (4 ± 8 eggs). Similarly, for single pairs exposed to fluoxetine, the average clutch size on black sand (20 ± 16 eggs) was 4 times higher (Tukey $P < 0.001$; Fig. 2B) compared to that on orange (5 ± 7 eggs) and white sand (5 ± 11 eggs). For social groups under control conditions, the average weekly clutch size on black sand (123 ± 58 eggs) was 3.5 times higher (Tukey $P < 0.001$; Fig. 2C) than that on orange sand (34 ± 32 eggs), which in turn was approximately twice as high as that on white sand (16 ± 18 eggs). Similarly, fluoxetine-exposed social groups had a higher average weekly clutch size on black sand (140 ± 91 eggs), which was 3.5 times higher (Tukey $P < 0.001$; Fig. 2D) compared to that on orange sand (51 ± 58 eggs) which, in turn, was over twice as high compared to that on white sand (24 ± 29 eggs).

Regarding female fecundity, the total number of deposited eggs was higher in fluoxetine-exposed fish during the single-pair spawning sessions ($\chi^2 = 8.816$, $P < 0.001$; Fig. 3A). Fluoxetine-exposed fish (30 ± 20 eggs) produced, on average, 1.5 times more eggs compared to control fish (20 ± 16 eggs). The size of the females did not affect the number of deposited eggs ($\chi^2 = 0.761$, $P = 0.383$), nor was the effect of fluoxetine size-dependent ($\chi^2 = 3.569$, $P = 0.056$). In social groups, fluoxetine did not appear to affect female fecundity ($\chi^2 = 2.446$, $P = 0.118$; Fig. 3B).

Both control fish ($t_{44} = -2.170$; $P = 0.035$; Fig. 4) and fluoxetine-treated fish ($t_{44} = -2.407$; $P = 0.021$; Fig. 4) preferred the black zone over the white zone in a non-mating context. Specifically, control fish spent on average 517 s (±208 s, SD) in the black zone (i.e., ~57% of the time), and fluoxetine-exposed fish spent on average 530 s (±218 s, SD) in the black zone (i.e., ~59% of the time). This preference did not differ between control fish and fluoxetine-exposed fish (Table 2; Fig. 4).

4. Discussion

This study examined whether fluoxetine, at an environmentally realistic concentration, influences where substrate-spawning fish such as the turquoise killifish (*Nothobranchius furzeri*) choose to spawn and how many eggs they deposit. Reproductive decision-making integrates various motivational states and environmental cues, and understanding how these processes respond—or fail to respond—to pharmaceutical pollution is essential for evaluating ecological risk. We predicted that fluoxetine would reduce preference for darker spawning substrates—given its anxiolytic effects—and increase reproductive output. Instead, fish consistently preferred darker substrate regardless of treatment, whereas fluoxetine increased fecundity only in single-pair spawning sessions. These findings suggest that components of reproductive decision-making may differ in their sensitivity to fluoxetine, and they highlight the need to identify which behavioral pathways are most vulnerable to pharmaceutical pollution.

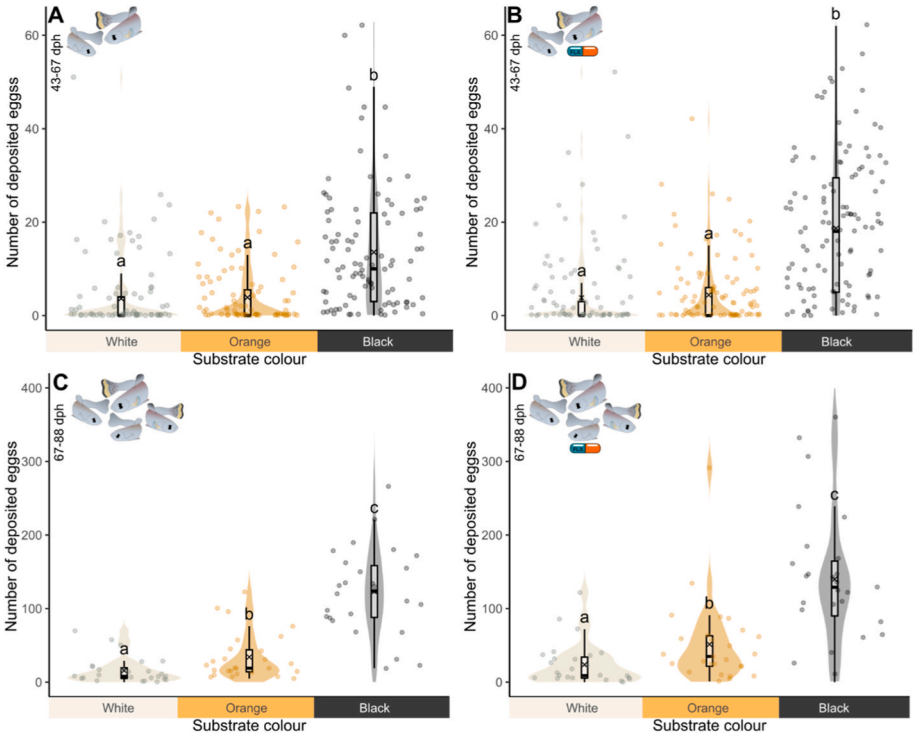

Fig. 2. Number of deposited eggs per substrate type. (A–B) Number of eggs deposited in white, orange, or black sand during 2-h spawning trials with single male-female pairs (46–64 dph) under (A) control conditions or (B) chronic exposure to 317 ng/L fluoxetine. **(C–D)** Weekly number of eggs deposited in white, orange, or black sand during social housing of two males and two females (67–88 dph) under (C) control conditions or (D) chronic exposure to 317 ng/L fluoxetine. Boxplots display the 25th, 50th (median), and 75th quartiles, with the mean indicated by ' × '. Different letters indicate significant differences. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

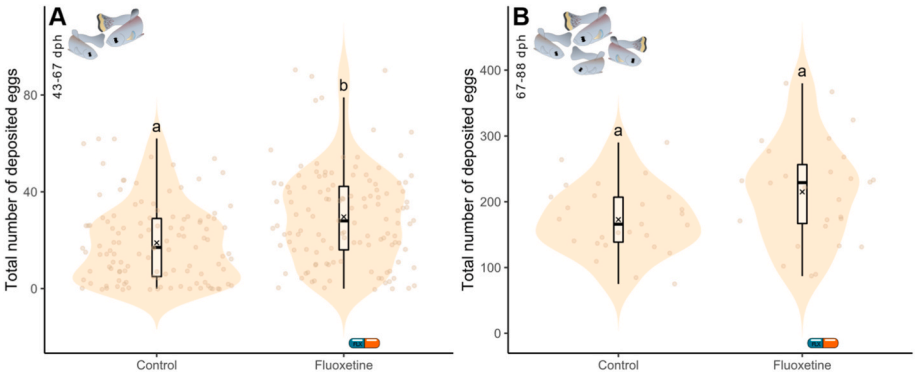

Fig. 3. Total number of deposited eggs per spawning session in relation to fluoxetine exposure. (A) Total number of deposited eggs during 2-h spawning trials with single male-female pairs (46–64 dph) kept under control conditions or chronically exposed to 0.5 μg/L fluoxetine. **(B)** Weekly total number of deposited eggs during social housing of two males and two females (67–88 dph) kept under control conditions or chronically exposed to 0.5 μg/L fluoxetine. Boxplots display the 25th, 50th (median), and 75th quartiles, with the mean indicated by ' × '. Different letters indicate significant differences.

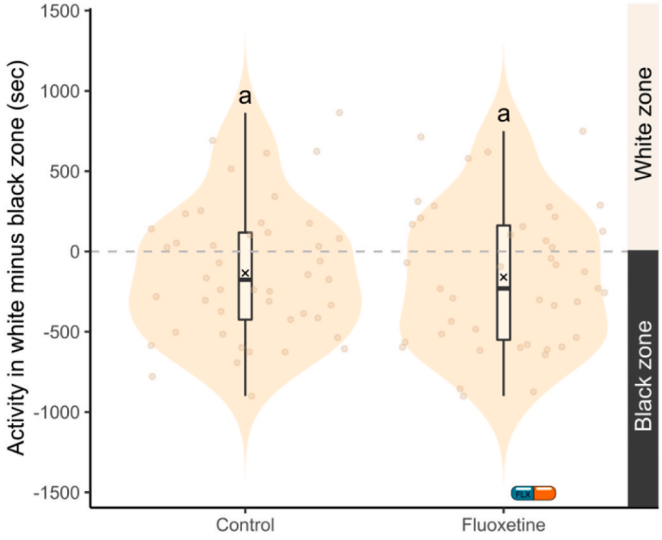

Fig. 4. Habitat preference in a non-mating context, measured as the time spent in the white zone *minus* time spent in the black zone, for both control and fluoxetine-treated fish. A negative score indicates that a fish spent more time in the black compared to the white zone, and *vice versa*. Boxplots display the 25th, 50th (median), and 75th quartiles, with the mean indicated by ' × '. Different letters indicate significant differences.

Table 2
Output of the linear model to analyze habitat preference of *Nothobranchius furzeri* in a non-mating context.

	Time spent in white minus black zone		
Effect	F	Df	*P* value
Sex	0.108	1	0.744
Condition	0.009	1	0.925
Body length	0.269	1	0.605
Condition × Sex	0.005	1	0.945
Sex × Body length	1.211	1	0.274
Condition × Body length	0.001	1	0.981
Residuals		81	

4.1. Spawning-site preference

Across all trials, killifish consistently preferred darker substrate, a pattern that aligns with their natural history. *Nothobranchius furzeri* occurs in ephemeral savannah pools formed on clay-rich vertisol soils, which are dark in color and provide the hydration capacity and structural stability needed for egg survival during desiccation (Cellerino et al., 2016; Matias & Adrias, 2010; Reichard et al., 2009; Wildekamp, 2004). By contrast, pools developed on exclusively laterite soils, which are typically rust-colored and retain less water, are unable to support killifish populations (Matias & Adrias, 2010). The strong preference for dark substrate in captivity therefore mirrors selection pressures experienced in the wild and suggests that color or brightness cues associated with vertisols are important oviposition cues (Thoré & Merckx, 2023).

Under group conditions, egg deposition on orange substrate was intermediate—higher than on white but lower than on black—whereas this distinction was absent in single-pair trials. This context dependence likely reflects social dynamics: although males do not defend strict territories, they form dominance hierarchies (Polačik & Reichard, 2009) in which dominant males gain privileged access to preferred spawning sites (Cellerino et al., 2016). In group tanks, dominant males likely monopolized the black tray, forcing subordinate males to use the next-best option. Orange substrate, being visually less bright and somewhat closer to natural soil appearance than white, plausibly served as this second-choice environment. Thus, the orange > white pattern seen only in groups probably reflects hierarchy-mediated spatial segregation rather than an intrinsic preference for orange *per se*.

Background preference may also serve an anti-predator function (Maximino et al., 2010). Many fishes, including *Nothobranchius*, display scototaxis—a preference for darker environments—which reduces visibility to visually hunting predators (de Abreu et al., 2020; Kysil et al., 2017). Fish in our study spent more time over darker backgrounds in both mating and non-mating contexts, consistent with this tendency. Surprisingly, fluoxetine exposure did not influence substrate choice or background use, despite evidence that fluoxetine can alter anti-predator and (other) anxiety-related behaviors in fishes (Ansai et al., 2016; Saaristo et al., 2017)—including in *N. furzeri* when different assays are used (Thoré et al., 2019; Thoré, Van Hooreweghe et al., 2021c). The absence of a treatment effect also suggests that fluoxetine did not appear to grossly disrupt the fish's ability to discriminate among visually distinct environmental backgrounds under the conditions tested. While our study was not designed to assess visual acuity directly, the persistence of clear color-/brightness-based preferences indicates that the observed behavioral patterns are unlikely to be explained by a general impairment of visual cue perception. One interpretation is that the motivational systems driving habitat choice and spawning-site selection are less responsive to chemical perturbation than other commonly assayed endpoints, at least in *N. furzeri*. This is ecologically relevant, because pollutant-induced shifts in habitat use or attraction–avoidance behavior can potentially expose animals to suboptimal or risky environments (Islam et al., 2025; Sandoval Herrera et al., 2025; Stremmel et al., 2023), with downstream consequences for foraging, predation

risk, and reproduction. In the present case, however, the persistence of a strong preference for darker, more naturalistic spawning environments suggests that this aspect of reproductive decision-making may remain relatively robust under environmentally realistic fluoxetine exposure. This cautions against assuming uniform fluoxetine effects across behavioral contexts or species without understanding the specific motivational drivers of each behavior.

4.2. Fecundity and mating activity

In single-pair assays, fluoxetine-exposed fish deposited ~1.5 × more eggs than controls. Because *Nothobranchius* mating typically yields one egg per successful copulation, increased egg counts are a direct indicator of increased mating activity. This result is consistent with several reports of elevated mating behavior and egg deposition following fluoxetine exposure in fish, including turquoise killifish (Thoré et al., 2020), fathead minnows (Weinberger II and Klaper, 2014), mosquitofish (Bertram et al., 2018), and guppies (Fursdon et al., 2019). Yet, the literature is heterogenous: some studies report no effect (Foran et al., 2004; Schultz et al., 2011; Weinberger II and Klaper, 2014) or even decreases in reproductive effort (Lister et al., 2009) depending on species, dose, and exposure duration.

Although female body size did not significantly predict egg production, the near-significant interaction between size and fluoxetine exposure suggests that the reproductive effects of fluoxetine may not be entirely phenotype-independent. We observed little association between female size and egg production in control fish, whereas larger fluoxetine-exposed females tended to produce more eggs. In many fishes, larger females often have greater energetic reserves and reproductive capacity, and it is therefore possible that fluoxetine amplifies fecundity most strongly in individuals able to invest more heavily in reproduction. At the same time, this pattern should be interpreted cautiously, as the interaction did not reach statistical significance. Interestingly, in a previous study on the same species, lifelong fluoxetine exposure reduced body size at maturation while still increasing mating frequency and fecundity (Thoré et al., 2020), suggesting that elevated egg output is unlikely to be explained by body size alone. Instead, fluoxetine may influence reproduction through altered mating behavior and shifts in energetic allocation or neuroendocrine pathways. Because we did not measure feeding rate, body condition, or gonadal investment here, we cannot distinguish among these mechanisms.

In contrast to the single-pair results, no fluoxetine effect on recovered egg numbers was detectable when fish spawned in social groups. Several non-exclusive mechanisms could explain this discrepancy. First, social dynamics—dominance, competition, mate choice, and courtship interference—may mask treatment effects that are readily expressed in isolated pairs. In support of this possibility, previous research has shown increased courtship in fluoxetine-exposed mosquitofish in the absence but not in the presence of competing males (Bertram et al., 2018). More broadly, fluoxetine may alter multiple behavioral processes that become especially relevant in group settings, including sociability, activity, spatial use, encounter rates between the sexes, and the extent to which dominant individuals can monopolize access to mates or preferred spawning sites. In our species, fluoxetine has previously been shown to increase sociability and mating frequency (Thoré et al., 2020), suggesting that its behavioral effects are not limited to courtship alone. In a social environment, however, such changes could either enhance or disrupt realized reproductive output depending on how they interact with hierarchy formation, social interference, and opportunities for mating. These context-dependent behavioral pathways may therefore help explain why increased egg production was evident in isolated pairs but not detectable in groups. Second, egg cannibalism—a known behavior in *Nothobranchius* (Polačik et al., 2016)—is more likely during continuous group housing and could reduce apparent fecundity and increase noise. Importantly, the egg counts recovered from the group tanks represent a net outcome of egg production and egg loss, rather than egg production alone. This may be particularly relevant under fluoxetine exposure, because fluoxetine can alter feeding-related behavior in fish (Weinberger II and Klaper, 2014; Thoré et al., 2019). If exposed fish differed in their tendency to consume eggs, treatment effects on actual reproductive output may have been partially masked or distorted. Because egg cannibalism was not quantified directly in the present study, we cannot determine whether it contributed to the absence of a detectable fluoxetine effect in social groups, but it remains a plausible and important mechanism. Third, age differences may also matter. Killifish fecundity peaks early in adulthood and declines thereafter (Blažek et al., 2013; Kim et al., 2016); our single-pair trials occurred during this peak, whereas group trials occurred when fish were older. If fluoxetine amplifies peak reproductive output rather than sustained output, its effects would be most visible earlier in the reproductive period. Further, chronic exposure may induce physiological desensitization or behavioral habituation (Bertram et al., 2025; Blumstein, 2016), attenuating drug effects over time. Finally, the group experiment had lower statistical power, making small effects harder to detect.

4.3. Ecological implications

The increase in fecundity under fluoxetine exposure in pairs indicates that environmentally realistic concentrations of this neuroactive pollutant have the potential to alter reproductive investment. Whether such changes translate into ecological consequences will depend on how they interact with variation in social structure, habitat conditions, and life-history trade-offs in natural settings. Because *Nothobranchius* populations pass through a single, compressed breeding season (Cellerino et al., 2016), even moderate increases in mating frequency—if expressed under realistic ecological conditions—could influence cohort size and population trajectories. By contrast, the absence of fluoxetine effects on spawning-site selection suggests that certain reproductive decisions may be less plastic and less susceptible to chemical perturbation. If substrate choice is strongly canalized by evolutionary pressures related to egg survival, it may remain stable even under pharmaceutical exposure. These contrasting patterns underscore the value of distinguishing behavioral pathways that are sensitive to pollutants from those that are behaviorally or evolutionarily constrained, as this distinction determines the predictability of pharmaceutical impacts on wild populations.

Mechanistically, fluoxetine's effects on reproduction could arise via multiple, interacting pathways. Serotonergic signaling, the main target of fluoxetine, influences reproductive physiology at several stages of the hypothalamic–pituitary–gonadal axis, including modulation of GnRH release, gonadotropin and luteinizing hormone secretion, and processes regulating gametogenesis (McDonald, 2017). Changes in serotonergic signaling can also alter the production of androgens known to regulate sexual behaviors in fish (Borg, 1994; Munakata & Kobayashi, 2010). Such neuroendocrine effects provide plausible mechanisms through which fluoxetine could increase reproductive output without altering the motivational systems governing spawning-site selection.

4.4. Limitations and future directions

Several limitations should be considered when interpreting these findings. In addition, we tested only a single fluoxetine concentration. Although this concentration falls within the upper range reported from contaminated surface waters, behavioral and reproductive responses to pharmaceuticals are often non-linear and may occur at lower concentrations as well. Future work should therefore incorporate broader concentration gradients to determine whether the effects observed here are dose-dependent, non-monotonic, or restricted to the upper end of environmentally realistic exposure scenarios.

Most importantly, the two experimental phases differed in age, exposure duration, social context, and opportunities for egg

cannibalism. In addition, the single-pair spawning and habitat-choice assays were conducted in clean water rather than under active fluoxetine exposure, meaning that exposed fish experienced a brief interruption of exposure during those tests. However, given that fish had been chronically exposed throughout development and adulthood, and that these interruptions lasted only 2 h and 15 min, respectively, substantial recovery from fluoxetine-induced effects during the assays is unlikely. Nevertheless, future studies could explicitly compare behavioral responses under maintained *versus* interrupted exposure conditions to test this assumption more directly. This design precludes attributing context-dependent effects to any single cause. Future studies should therefore manipulate these components independently—for example by establishing controlled dominance hierarchies, preventing egg cannibalism (e.g., through mesh-protected substrates)—and/or monitor spawning behavior and cannibalism continuously through video tracking.

Our substrate-choice assays reflect preferences within a restricted set of options. Rearing on grey tank backgrounds may have influenced color-associated decisions, and laboratory substrates represent simplified analogues of natural soils (Thoré & Merckx, 2023). Incorporating natural substrates and broader environmental variation would improve inference. Ontogenetic shifts in background preference are another potential confound (Bai et al., 2016; Lau et al., 2011), as the two experimental phases occurred at different ages. Longitudinal designs following the same individuals across life stages would help clarify how background and substrate preferences develop over time (Thoré et al., 2023).

Finally, a clearer mechanistic understanding of fluoxetine's effects on reproduction will require integrated behavioral–physiological approaches. Quantifying hormone levels, gonadal development, gamete maturation, or neuroendocrine gene expression would help determine whether fluoxetine increases egg output by altering mating motivation, enhancing gametogenesis, or both. Equally important will be the direct quantification of behavioral processes likely to mediate realized reproductive success, such as courtship, aggression, activity, spatial interactions, and egg cannibalism. Combining these behavioral endpoints with reproductive measurements will be essential for understanding how pharmaceutical exposure translates into altered fitness-related outcomes. Given the heterogeneity of findings across species and contexts, and exposure regimes, mechanistic studies that consider the broader behavioral context will be key for understanding when fluoxetine amplifies reproductive effort, suppresses it, or has no effect.

5. Conclusions

Environmentally realistic fluoxetine exposure increased reproductive output in turquoise killifish during single-pair spawning, but did not affect spawning-site selection or habitat preference. Fish consistently preferred dark substrates across all contexts, likely reflecting strong evolutionary constraints on oviposition behavior. These results show that fluoxetine can alter some components of reproduction without disrupting others, highlighting the importance of distinguishing sensitive behavioral pathways from those that remain robust to pharmaceutical pollutants. Understanding which reproductive behaviors are chemically responsive will improve predictions of how neuroactive contaminants shape population dynamics in natural systems.

CRediT authorship contribution statement

Theresia J. Kimario: Writing – original draft. **Nicole J. Goede:** Writing – review & editing. **Noémie Buratto:** Writing – review & editing. **Yusuph A. Kafula:** Writing – review & editing. **Omayma Missawi:** Writing – review & editing, Supervision. **Eli S.J. Thoré:** Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2026.128153.

Data availability

All datasets and R scripts used in this study are openly available on FigShare at https://doi.org/10.6084/m9.figshare.30865793.

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