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Long-term exposure to a pharmaceutical pollutant affects geotaxic behaviour in the adult but not juvenile life stage of killifish

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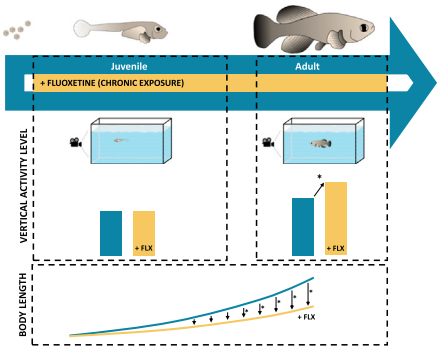
HIGHLIGHTS

- Impacts of chronic drug exposure across life stages of fish are hardly studied.
- We chronically exposed killifish to a field-realistic level of fluoxetine.
- Fish were smaller when exposed, which became more apparent as fish aged.
- Exposed adults but not juveniles showed effects in one of four geotaxic measures.
- Responses to drugs may only emerge later in time and during specific life stages.

GRAPHICAL ABSTRACT



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ABSTRACT

Ecosystems around the world are increasingly polluted with pharmaceutical compounds that may perturb wildlife behaviour. Because many pharmaceuticals are continuously present in the aquatic environment, animals are often exposed to them across several life stages or even their entire life. Despite a large body of literature showing various impacts of exposure to pharmaceuticals on fish, hardly any long-term studies across different life stages have been conducted which makes it hard to accurately estimate the ecological outcomes of pharmaceutical pollution. Here, we performed a laboratory experiment in which we exposed hatchlings of the fish model *Nothobranchius furzeri* to an environmentally relevant concentration (0.5 μg/L) of the antidepressant fluoxetine until well into adulthood. We monitored total body length and geotaxic behaviour (i.e. gravity-mediated activity) of each fish as two traits that are ecologically relevant and naturally differ between juvenile and adult killifish. Fish exposed to fluoxetine were smaller compared to control fish, an effect that became more apparent as fish aged. Even though fluoxetine did not affect average swimming depth of either juveniles or adults, nor the time spent at the surface or bottom of the water column, exposed fish changed their position in the water column (depth) more frequently in the adult but not juvenile phase. These results suggest that important morphological and behavioural responses to pharmaceutical exposure–and their potential ecological consequences–may only emerge later in time and/or during specific life stages. Therefore, our results highlight the importance of considering ecologically relevant timescales across developmental stages when studying the ecotoxicology of pharmaceuticals.

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1. Introduction

Aquatic ecosystems around the world become polluted at alarming rates with a growing amount and diversity of chemicals, including pharmaceuticals (Bernhardt et al., 2017; Orive et al., 2022). To date, environmental concentrations of most pharmaceuticals are still sufficiently low to not cause any direct lethal (or acutely toxic) effects in wildlife but they may still exert specific biological effects, such as behavioural changes that could have important ecological consequences (Brodin et al., 2013; Wong and Candolin, 2015). This is particularly the case for neuroactive drugs such as antidepressants and anxiolytics, which often end up in the environment because they are not effectively removed from domestic wastewater in treatment plants (Yang et al., 2017). Typically, such drugs act on target molecules that control various physiological and behavioural systems and that may be conserved across animal taxa (Gunnarsson et al., 2008), so that we can expect target-mediated effects in animals that are comparable to those in humans (Margiotta-Casaluci et al., 2014). For example, selective serotonin-reuptake inhibitors (SSRIs), such as fluoxetine, are typically used to treat depression and anxiety by binding to serotonin transporters, which leads to higher levels of serotonin in the synaptic cleft (McDonald, 2017). In fish, exposure to SSRIs may lead to changes in, among others, swimming activity (Ansai et al., 2016; Thoré et al., 2021a), propensity to take risks (Dzieweczynski et al., 2016; Martin et al., 2019), sociability (Ansai et al., 2016; Thoré et al., 2020) and daily rhythms in behaviour (Melvin, 2017; Thoré et al., 2021a). Furthermore, SSRIs may impact behaviours that are immediately linked to survival and reproduction, including antipredator behaviour (Martin et al., 2017; Saaristo et al., 2017), feeding behaviour (Weinberger and Klaper, 2014; Thoré et al., 2018) and mating behaviour (Bertram et al., 2018; Fursdon et al., 2019).

Even though the possible effects of pharmaceuticals on animal behaviour are increasingly well documented, it remains difficult to estimate the exact ecological outcomes. This is in part because conventional study approaches insufficiently address the complexity of real-world exposure scenarios, including ecologically relevant timescales (Pyle and Ford, 2017; Bertram et al., 2022). For example, the effects of long-term pharmaceutical exposure are not often studied in fish (but see e.g., Thoré et al., 2020, 2021a; Mason et al., 2021; Polverino et al., 2021) and, in particular, how long-term exposure may affect fish throughout their life or across life stages remains largely unexplored. Nevertheless, because many pharmaceutical compounds persist in the environment (Kwon and Armbrust, 2006; Puckowski et al., 2016), fish are often exposed to pharmaceuticals across several life stages and over periods that exceed typical study durations. Given that an organism's developmental stage can be a major source of intraspecific trait variation (Cope et al., 2022), generalising the effects of pharmaceutical exposure from one life stage to the other may not be straightforward. In particular, sensitivity to chemicals and how animals respond to exposure could differ substantially across the exposure period and between life stages, and could compromise our accuracy to predict the impact of pharmaceutical pollution.

To study how long-term exposure to SSRIs, specifically fluoxetine, may (differentially) affect fish across the juvenile and adult life stage, we performed a laboratory experiment in which we exposed new-born hatchlings of the fish model *Nothobranchius furzeri* to an environmentally relevant concentration (0.5 μg/L) of fluoxetine and maintained exposure until well into adulthood. Fluoxetine has been detected in natural surface waters at concentrations up to 0.33 μg/L and in sewage treatment plants effluents up to 0.54 μg/L, so that our chosen concentration is in the higher end of what has been detected in waterways around the world (Puckowski et al., 2016; Mole and Brooks, 2019; Gould et al., 2021). We monitored total body length and geotaxic behaviour of each individual throughout the experiment as two traits that are ecologically relevant and naturally differ between juvenile and adult fish. Fluoxetine has previously been shown to suppress appetite (Shimada et al., 2012; McDonald, 2017) and impair feeding behaviour (Weinberger and Klaper, 2014; Gaworecki and Klaine, 2008) in a variety of fish species, which may suppress somatic growth of fish. An earlier study with *N. furzeri* showed that exposure to 0.7–5.3 μg/L fluoxetine resulted in adult fish being smaller after 14 weeks of exposure (Thoré et al., 2020). This suggests an impaired growth rate but has until now not yet been formally tested. Based on these previous reports, we expect to confirm that fluoxetine exposure impairs *N. furzeri* growth. In particular, we expect fluoxetine-induced differences in body length to be most visible in adult compared to juvenile fish.

Besides body length, we monitored geotaxis of juvenile and adult fish, which can be defined as gravity-mediated activity, or movement away or towards the source of gravity ('diving tendency'). Many aquatic organisms, including several fishes, have a natural tendency to dive to the bottom to evade threats (such as bird predation) or when stressed (Parker, 2016; Doran et al., 2022), so that benthic behaviour can be considered more risk-averse than activity closer to the surface (Thoré et al., 2021b). SSRIs like fluoxetine are designed to mediate risk-perception and anxiety-related behaviour, and in fish can result in either anxiolytic (e.g., Wong et al., 2013; Ansai et al., 2016) or anxiogenic (e.g., Dzieweczynski et al., 2016; Saaristo et al., 2017) effects which are often concentration and/or sex-dependent (Martin et al., 2019). Earlier studies in *N. furzeri* showed that environmentally relevant concentrations of fluoxetine increased both freezing behaviour upon a simulated predator attack (Thoré et al., 2018) as well as thigmotaxis (i.e. activity in the periphery of an open area) (Thoré et al., 2021a), which suggests an increase in risk-averse behaviour. Based on these findings, we expect that fluoxetine exposure will lead to more activity near the bottom compared to the surface. Because adult fish are more conspicuous than juveniles and therefore more vulnerable to bird predation, we expect any anxiety-modulating effect of fluoxetine to be more pronounced in adult compared to juvenile fish.

2. Materials and methods

2.1. Study species and fish maintenance

The experiment was performed from 11 October 2016 till 04 January 2017. We used *Nothobranchius furzeri* as study species, which is an annual killifish that originates from temporary freshwater ponds in south-east Africa (Cellerino et al., 2015). These ponds only hold water during the rainy season and desiccate entirely during the dry season. *Nothobranchius* fish are adapted to this periodic drying by producing drought-resistant eggs that remain dormant in the sediment during the dry period (Pinceel et al., 2015). At the onset of the next rainy season, eggs hatch and the fish complete their life cycle in just a few months before the pond dries again (Pinceel et al., 2021). Because of the fast life cycle of *N. furzeri*, the species is particularly useful for research agendas that require time-efficient assessment across life stages and generations, including for aging research (Platzer and Englert, 2016) and ecotoxicology (Thoré et al., 2021c).

We hatched 54 fish by submerging ready-to-hatch eggs (stage 43, sensu Wourms, 1972) in reconstituted water (Instant Ocean Salt mix added to type III RO water until a conductivity of 600 μS/cm, pH 7.8) with 1 g/L humic acid (53,680; Sigma-Aldrich), after the protocol of Philippe et al. (2018). For this, we used eggs from a heterozygous *N. furzeri* laboratory strain, which originates from a natural population in Mozambique (MZCS-222) and which has been kept in optimal and standardised laboratory conditions for at least three generations. Two days post hatching (dph), each hatchling was transferred to a 1-L transparent, glass jar (with reconstituted water) and kept individually for the remainder of the experiment. Six weeks after hatching, fish were moved to 2-L glass jars to ensure sufficient water volume for each fish. This setup allowed to monitor each fish individually, while still allowing visual contact between fish and preventing agonistic encounters. Jars were cleaned three times per week (every Monday, Wednesday and Friday) during which the water of each jar was renewed completely to maintain good water quality and a stable fluoxetine concentration (see Section 2.3 Preparation of solutions). Cleaning coincided with the behavioural tests (see Section 2.2 Experimental setup and behavioural testing) to avoid unnecessary handling of the fish. Water quality was monitored at least three times per week (ammonium <0.2 mg/L, nitrate <25 mg/L, nitrite <0.2 mg/L). Fish were fed twice

daily until satiation with live *Artemia franciscana* nauplii (Ocean Nutrition, Essen, Belgium) until 32 dph. After that, and for the remainder of the experiment, fish were fed twice daily until satiation with frozen *Chironomus* larvae (Ocean Nutrition, Essen, Belgium). Any excess food was removed using a glass pipette to maintain good water quality and to prevent sorption of fluoxetine to the organic matter.

Throughout the experiment, fish jars were kept in a temperature-controlled room at a constant temperature of 28 °C and a 14 h light: 10 h dark photo-regime. Full-spectrum, white LED light was provided at a constant intensity of 2000 lx (jar level), and was switched on and off at 8.00 am and 10.00 pm, respectively.

2.2. Experimental setup and behavioural testing

At 2 dph, fish were randomly assigned to a control condition (n = 27: 16 males, 11 females) or a condition in which fish were continuously exposed to a nominal concentration of 0.5 μg/L fluoxetine (n = 27: 15 males, 12 females) until 85 dph (i.e. adult/late-adult stage). Note that fish were not yet exposed during the embryonal phase (i.e. starting immediately post-fertilisation) because, in order to simulate their natural life history, *N. furzeri* eggs are typically stored under dry conditions before being inundated to initiate hatching.

Fish geotaxis was monitored using a proof-of-principle diving test to which each individual fish was subjected five times per week (once daily, on Monday–Friday) and for 10 weeks in total (see Fig. 1). The diving test consisted of a barren glass arena (24.5 cm long x 6 cm wide x 17.5 cm high) that was filled with water to a height of 16 cm (approx. 2.4 L). The bottom, left- and right-sides of the arena were covered with a grey screen (opaque). The front side of the arena was transparent so that fish could be observed, while the backside was covered with a white screen to provide sufficient contrast between fish and background. Furthermore, the arena was virtually divided into 8 horizontal layers (height of 2 cm each) that were delineated by grey lines on the white background.

To start the diving test, each fish was individually transferred to the arena and allowed to settle for five minutes. Then, fish movements were recorded for 10 min using a Logitech C920 HD Pro webcam that was centred in front of the arena. All tests were conducted between 11.00 am and 2.00 pm to minimise confounds related to daily behavioural variation (Thoré et al., 2021a). Because juveniles could not be accurately detected by automated video-tracking software, all recordings were manually analysed (observer-blind). Due to logistical constraints, only 30 repeated tests per individual could be analysed (i.e. 24 repeated measures per individual as juveniles, and 6 repeated measures per individual as adults). Geotaxis of juveniles was scored at: 15, 16, 17, 21, 22, 23, 24, 27, 28, 29, 30, 31, 35, 36, 37, 38, 41, 42, 43, 44, 45, 48, 49 and 50 dph. Males reached sexual maturity at 53–60 dph as observed by the appearance of colouration in the fins (after Thoré et al., 2019) and all fish (i.e. also females) were assumed to have reached sexual maturity by 60 dph. Geotaxis of adults was scored at: 65, 66, 69, 70, 72 and 85 dph.

Four fish were lost due to early mortality (1 control fish at 69 dph, 2 fluoxetine fish at 65 dph, and 1 fluoxetine fish at 70 dph) and could not complete all trials. Additionally, 74 trials were not included in the dataset due to insufficient quality of the recording. This amounts to a total of 1528 processed trials, or approximately 255 h of observations.

To monitor total body length (including growth) through time, each individual was measured for a total of 19 times (at the ages of: 15, 16, 17, 21, 22, 23, 24, 27, 29, 31, 34, 36, 38, 41, 43, 45, 48, 50 and 83 dph). For this, each fish was briefly placed in a Petri dish with a small amount of water to avoid vertical movement. Then, a top-view, size-calibrated photograph was taken and analysed using the open source image processing software ImageJ v. 1.50i (Schneider et al., 2012) to assess the total length of each fish (i.e. tip of snout to tip of tail). These measurements were taken at the end of the diving tests and afterwards each fish was transferred back to its housing jar.

2.3. Preparation of solutions

Fluoxetine hydrochloride (F-132; Sigma-Aldrich; CAS 56296–78-7) was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 500 mg/L that was stored as aliquots at −18 °C until use. Aliquots were thawed and diluted (in reconstituted water of 600 mS/cm) till 5 mg/L to prepare working standard solutions. Working standard solution was added to the jars of fish belonging to the fluoxetine condition each time the water was exchanged (i.e. 3 times per week, see Section 2.1 Study

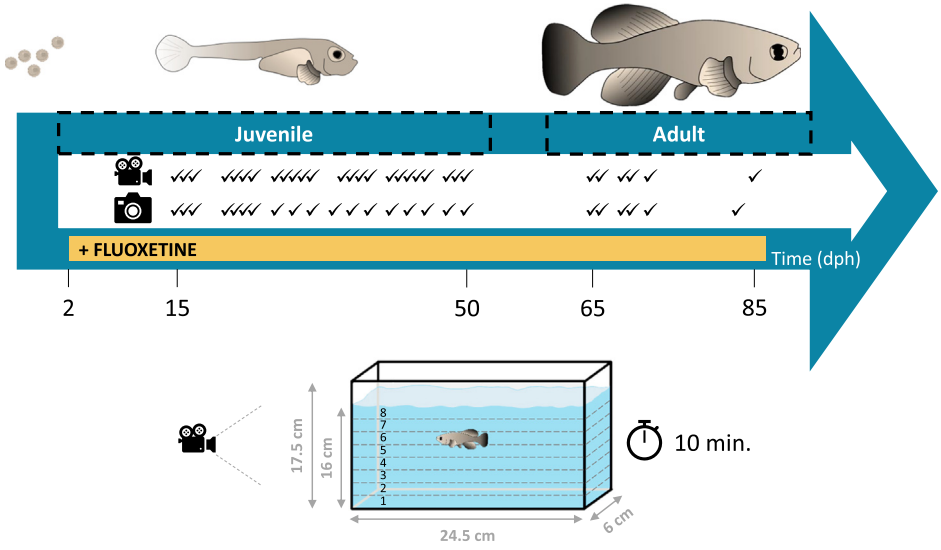

Fig. 1. Schematic figure of the experimental setup. Starting 2 days post hatching (dph), *Nothobranchius furzeri* were housed in individual (transparent) jars throughout the experiment. Fish were randomly assigned to either a control condition (n = 27) or a condition in which they were chronically exposed to 0.5 μg/L fluoxetine (n = 27). Fish were not yet exposed during the embryonal phase because, in order to simulate their natural life history, *N. furzeri* eggs are typically stored under dry conditions before being inundated to initiate hatching. First signs of maturation appeared at 53 dph, and all fish were assumed to have reached sexual maturity by 60 dph. Total body length of juveniles and adults was measured by taking size-calibrated, top-view pictures on select days (indicated by ticks on the lower timeline). Geotaxic behaviour was assessed by subjecting each fish to a diving test and monitoring their swimming activity for 10 min. This test was conducted five times per week (once daily, on Monday–Friday) and for 10 weeks in total. Thirty of these repeated trials (i.e. 30 trials per fish) were recorded and manually analysed (indicated by ticks on the upper timeline).

species and fish maintenance) to reach a nominal concentration of 0.5 μg/L. Control fish were exposed to an equal amount of DMSO (0.00001 %). At random time-points during exposure, 10-mL water samples from three randomly selected jars were taken (just prior to water renewal) and pooled to create a single sample replicate ($n = 6$ for the fluoxetine condition; $n = 5$ for the control condition). The samples were analysed using liquid chromatography coupled to mass spectrometry (LC/MS/MS) to measure the concentration of fluoxetine hydrochloride. The mean detected level of fluoxetine in the samples from the treatment condition was 0.498 μg/L (STDEV: 0.069 μg/L). Noteworthy, fluoxetine is relatively recalcitrant to degradation with a > 100-days half-life in water (Kwon and Armbrust, 2006). No fluoxetine was detected in the samples from the control condition.

2.4. Animal welfare note

All procedures were conform to the legal requirements for animal research in Belgium and approved by the ethical committee of KU Leuven (file number: P070/2016). The condition and health of each individual fish was monitored at least twice per day. Optimal water conditions were provided, and water quality was measured at least three times per week. Any disturbance and handling that was not strictly necessary for the experiment was kept to a minimum to prevent and limit stress.

2.5. Statistical analyses

All statistical analyses were performed in R version 4.0.5 (R Core Team, 2016) at a significance level of $\alpha = 0.05$. Model assumptions, including distributional fit and homogeneity of variances, were verified graphically. Gaussian error distributions were additionally verified using a Shapiro-Wilk test.

To assess geotaxic behaviour, four response variables were calculated per fish per trial. (1) As a measure of 'vertical activity', we calculated the total number of times a fish changed depth in the 10-min timeframe (i.e. the frequency by which a fish switched from one horizontal layer to another, see Fig. 1). (2) The average depth of the fish in the water column was calculated as the cumulative time spent in each layer multiplied by a fixed score for that layer (score 1 for the bottom layer, score 8 for the surface layer, see Fig. 1), divided by the total observation time (600 s), after Thoré et al. (2021b). Hence, a higher score indicates that the fish spent more time closer to the surface, and *vice versa*. Furthermore, we calculated (3) the cumulative time spent in the upper 25 % (layer 7 + 8, see Fig. 1), and (4) the cumulative time spent in the lower 25 % (layer 1 + 2, see Fig. 1) of the water column.

Each of the four behavioural response variables was analysed by means of a linear mixed-effects model (lme4 package, Bates et al., 2017). A Gaussian error distribution was assumed for all models, except for vertical activity (i.e. number of layer changes) for which a Poisson error distribution was assumed. Condition (control vs. fluoxetine), sex (male vs. female) and life stage (juvenile vs. adult) were added as fixed factors to each model, including the interaction between condition and sex, and between condition and life stage. The three-way interaction term and the interaction between sex and life stage were initially also included but these had no significant effect and were dropped from the final model to improve the fit (based on AIC comparisons). In addition, total body length at 43 dph was added as a covariate (mean-centred and scaled). Fish identity and trial number of the diving test were added as random effects. We also added an observation-level random effect in the model for vertical activity to account for overdispersion.

Similarly, total body length was analysed by means of a linear mixed-effects model with Gaussian error distribution. Condition, sex and trial (referring to the repeated measures) were added as fixed factors, including their full interaction. Fish identity was added as a random effect. For further scrutiny, we additionally calculated the Von Bertalanffy growth rate (nlstools package, Baty et al., 2015) for each individual, and compared the overall difference in growth between the control and fluoxetine-treated fish using a *t*-test.

For all mixed-effects models, type III Wald chisquare tests were used to test the significance of the fixed effects and the interaction terms. Post-hoc differences were assessed by means of Tukey-corrected pairwise comparisons (lsmeans package, Lenth and Love, 2017). For the sake of completeness, we also determined if individual variation in the behavioural measures was repeatable. For this, we calculated repeatability values as the between-individual variance over the sum of between-individual and residual variance (based on above mixed-effects models) (Nakagawa and Schielzeth, 2010). The statistical significance of the repeatability values was tested by means of likelihood-ratio tests that compare the model with and without the fish identity random effect (rptR package, Stoffel et al., 2018).

3. Results

Except for time spent at the bottom of the water column, all variables related to geotaxic behaviour were differently expressed in juvenile vs. adult fish (Table 1). When only considering control fish, there was a trend for adults (mean ± SE 118 ± 30 times) to change their position in the water column (depth) 59 % more frequently (Tukey $P = 0.070$; Fig. 2A) than juveniles (mean ± SE 74 ± 12 times). Furthermore, adults (mean ± SE 3.96 ± 0.31 depth score) swam on average 21.5 % deeper (Tukey $P = 0.008$; Fig. 2B) in the water column compared to juveniles (mean ± SE 4.81 ± 0.20 depth score), and adults (mean ± SE 152.73 ± 31.26 s) spent 40 % less time at the surface (Tukey $P = 0.002$; Fig. 2C) than juveniles (mean ± SE 254.73 ± 20.37 s). All behavioural measures were significantly repeatable (Table S1).

For fish exposed to fluoxetine, we observed similar patterns. Adults (mean ± SE 181 ± 46 times) that were exposed to fluoxetine changed their position in the water column (depth) 141 % more frequently (Tukey $P < 0.001$; Fig. 2A) than juveniles (mean ± SE 75 ± 12 times). Average swimming depth of adults (mean ± SE 4.00 ± 0.32 depth score) was similar (Tukey $P = 0.168$; Fig. 2B) to that of juveniles (mean ± SE 4.43 ± 0.20 depth score), even though adults (mean ± SE 135.99 ± 31.59 s) spent 37.4 % less time at the surface (Tukey $P = 0.012$; Fig. 2C) than juveniles (mean ± SE 217.25 ± 20.42 s).

There was no difference in geotaxic behaviour between males and females (Table 1). Even though the model output suggests that there was a sex-specific effect of fluoxetine exposure on time spent at the bottom 25 % of the water column, post-hoc analysis did not reveal any differences in behaviour between control and fluoxetine-exposed females (Tukey $P = 0.175$) or between control and fluoxetine-exposed males (Tukey $P = 0.193$) (Table 1; Fig. S1). Fluoxetine-exposed males spent more time at the bottom than fluoxetine-exposed females ($P = 0.040$, uncorrected *P*-value) but no such difference emerged after correcting for multiple comparisons (Tukey $P = 0.165$).

Except for time spent at the surface (top 25 % of the water column), there was a signal that fluoxetine exposure differentially impacts geotaxic behaviour in the juvenile vs. adult phase. Specifically, the model output suggests life stage-specific effects of fluoxetine exposure on time spent at the bottom ($\chi^2 = 6.443$; $P = 0.011$; Table 1), frequency by which fish changed their position in the water column ($\chi^2 = 12.120$; $P < 0.001$; Table 1), and a trend for average swimming depth ($\chi^2 = 3.514$; $P = 0.057$; Table 1). However, post-hoc analysis that corrected for multiple comparisons only revealed significant differences for frequency by which fish changed their position in the water column ('vertical activity'). Specifically, juveniles that were exposed to fluoxetine (mean ± SE 75 ± 12 times) changed their position in the water column (depth) as much (Tukey $P = 0.981$; Fig. 2A) as juveniles that were not exposed to fluoxetine (mean ± SE 74 ± 12 times). In contrast, adults that were exposed to fluoxetine (mean ± SE 183 ± 46 times) changed their position in the water column 54 % more frequently (Tukey $P = 0.025$; Fig. 2A) than adults that were not exposed to fluoxetine (mean ± SE 119 ± 30 times).

Because *N. furzeri* is sexually dimorphic in terms of body size, with males being larger than females, total body length differed between both

Table 1
Output of the linear mixed-effects models to analyse geotaxic behaviour of *Nothobranchius furzeri*.

	Frequency of layer changes		Average depth		Time at the surface		Time at the bottom	
Effect	χ^2	*P*-value	χ^2	*P*-value	χ^2	*P*-value	χ^2	*P*-value
Condition	1.466	0.226	0.153	0.696	0.691	0.406	0.012	0.918
Sex	0.151	0.697	0.601	0.438	0.364	0.546	0.747	0.387
Life stage	7.265	**0.007**	4.953	**0.026**	10.204	**0.001**	0.847	0.357
Body length	2.288	0.130	0.626	0.429	1.118	0.290	0.249	0.618
Condition*Sex	0.098	0.754	3.753	0.053	2.596	0.107	4.312	**0.038**
Condition*Life stage	11.806	**<0.001**	3.690	0.055	1.016	0.313	6.438	**0.011**

P-values <0.05 are bold and underlined. *P*-values that are only slightly higher than 0.05 are underlined.

sexes (Table 2). For example, at 83 dph, males (mean ± SE 41.7 ± 0.23 mm) were 23.7 % larger than females (mean ± SE 33.7 ± 0.29 mm). Irrespective of sex, fish exposed to fluoxetine were smaller compared to control fish (Table 2; Fig. 3). This difference emerged for 12 out of 19 times that body length was assessed throughout the experiment, specifically at 21 and 22 dph, and consistently as of 31 dph until the end of the experiment (Fig. 3). Furthermore, there was a trend (t_{52} = 1.981; *P* = 0.053) for 19 % lower growth of fluoxetine-treated fish (mean ± SE 0.013 ± 0.006 Von Bertalanffy K) compared to control fish (mean ± SE 0.016 ± 0.005 Von Bertalanffy K).

4. Discussion

Pharmaceutical pollution is a threat to aquatic wildlife but we still hardly understand how long-term exposure affects fish across life stages. Because an animal's developmental stage can be a major source of

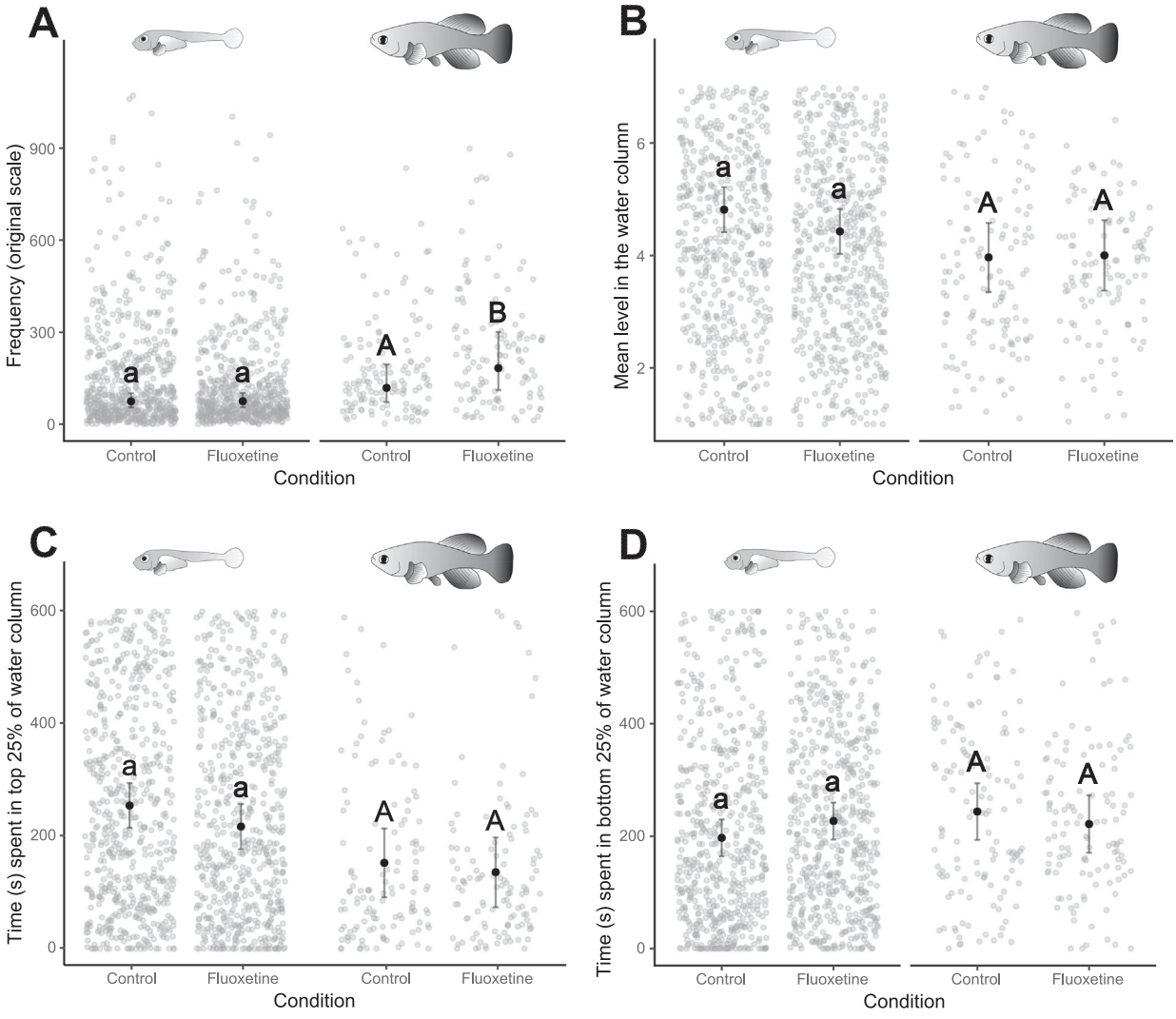

Fig. 2. Geotaxic behaviour (mean ± standard error) during a 10-min diving test of *Nothobranchius furzeri* juveniles and adults in relation to fluoxetine exposure. Different letters indicate significant differences. (A) Frequency by which fish changed their position in the water column was assessed as a measure of 'vertical activity', and was affected by fluoxetine exposure in the adult but not juvenile phase. (B) The average depth of the fish in the water column was not affected by fluoxetine exposure, regardless of life stage. (C) Juveniles spent more time at the surface (upper 25 % of the water column) than adults but time spent at the surface was not affected by fluoxetine exposure. (D) Fluoxetine exposure did not affect time spent at the bottom (lower 25 % of the water column), regardless of life stage.

Table 2
Output of the linear mixed-effects model to analyse total body length of *Nothobranchius furzeri*.

	Frequency of layer changes	
Effect	χ^2	*P*-value
Condition	12.355	**<0.001**
Sex	21.376	**<0.001**
Trial	51,884.139	**<0.001**
Condition*Sex	0.874	0.350
Condition*Trial	30.177	**0.036**
Sex*Trial	679.569	**<0.001**
Condition*Sex*Trial	5.814	0.997

P-values <0.05 are bold and underlined.

intraspecific trait variation, simply extrapolating responses from one life stage to another may be flawed. This, in turn, makes it hard to accurately estimate the ecological outcomes of pharmaceutical pollution. Here, we assessed how long-term exposure to fluoxetine affects body length and geotaxic behaviour in juvenile vs. adult fish. Our results show that chronic exposure to fluoxetine impairs somatic growth and impacts at least one measure of geotaxic behaviour in the adult but not juvenile phase. Overall, our results suggest that important morphological and behavioural responses to pharmaceutical exposure may only emerge later in time and/or during specific life stages. This highlights the importance of considering ecologically relevant timescales across developmental stages when studying the ecotoxicology of pharmaceuticals.

4.1. Effects on body length

As expected, fish exposed to 0.5 μg/L fluoxetine were smaller than unexposed fish. This confirms the results of a previous study with *N. furzeri*, which showed that exposure to 0.7–5.3 μg/L fluoxetine resulted in adult fish being smaller after 14 weeks of exposure (Thoré et al., 2020). In humans, fluoxetine has anorexigenic effects and is known to impact body weight (Halford et al., 2005). Likewise, in fish, fluoxetine often suppresses feeding behaviour (McDonald, 2017), which could translate into reduced somatic growth. For example, feeding behaviour of hybrid striped bass (*Morone saxatilis* × *M. chrysops*) was impaired after six days of exposure

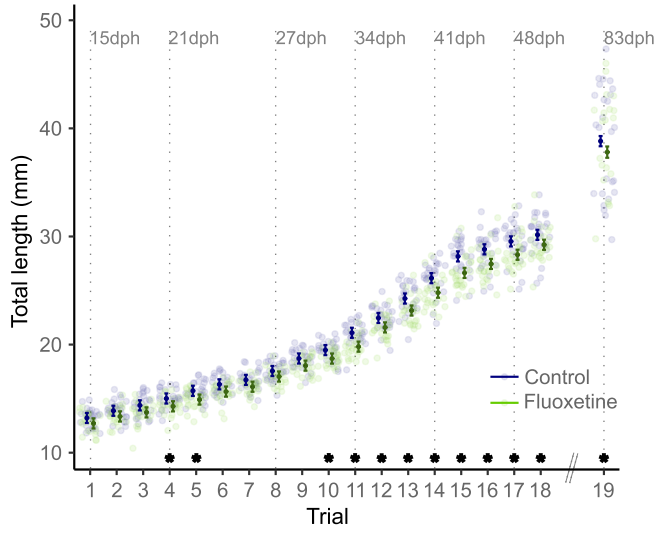

Fig. 3. Total body length (mean ± standard error) of *Nothobranchius furzeri* over the course of the experiment, in relation to fluoxetine exposure. Significant differences between control fish (blue) and fish exposed to fluoxetine (green) are shown with an asterisk (*). Fish exposed to fluoxetine were larger than control fish at 21 (trial 4) and 22 (trial 5) dph, and as of 31 (trial 10) dph onwards. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to 23 μg/L fluoxetine (Gaworecki and Klaine, 2008). Likewise, in fathead minnows (*Pimephales promelas*), a 7-day exposure to 51 μg/L fluoxetine reduced feeding rate and growth (Stanley et al., 2007). Another study showed that feeding rate of fathead minnows decreased after four weeks of exposure to 10 μg/L fluoxetine (Weinberger and Klaper, 2014). In contrast, exposure for 28 days to 0.06–0.35 μg/L of fluoxetine did not affect standard length, weight or body condition in guppies (*Poecilia reticulata*) (Fursdon et al., 2019; Martin et al., 2019). Although not tested in the current study, it is possible that reduced food intake of *N. furzeri* in response to fluoxetine exposure underlies the observed reduction in body size. This hypothesis is supported by the results of an earlier study which showed that three weeks of exposure to 5 μg/L fluoxetine inhibits feeding behaviour in *N. furzeri* (Thoré et al., 2018). The observation that fluoxetine-exposed fish are only consistently smaller than unexposed fish as of 31 dph onwards could be because fluoxetine (and its metabolite norfluoxetine, which is formed by cytochrome P-450 by demethylation; Kwon and Armbrust, 2006; Puckowski et al., 2016) may bioaccumulate over time and only reach high enough levels to exert its effect after some time of exposure (Nakamura et al., 2008; Martin et al., 2019). Even regardless of bioaccumulation, SSRIs such as fluoxetine may only exert their full pharmacological effect after a few weeks of exposure (Gaworecki and Klaine, 2008). This is because the compound may not (only) act–directly and immediately–by inhibiting serotonin transporters but also by delayed compensatory responses in the brain which take longer to develop (Andrews et al., 2015). Alternatively, and not mutually exclusively, if reduced food intake is responsible for lower somatic growth, then it may be reasonable to expect some delay before a change in feeding behaviour is translated to morphological changes.

Because a previous study already showed that chronic exposure to fluoxetine leads to smaller body sizes in *N. furzeri* (Thoré et al., 2020), this finding is, in itself, not entirely novel. Nevertheless, it is important (and novel) to find that this effect is repeatable. Already for about a decade, researchers have voiced concerns over inconsistent results in literature about the impact of fluoxetine on fish, and expressed that it is unlikely that all reported effects will be repeatable (Sumpter et al., 2014). Even though replicability is a core tenet of the scientific method (Mebane et al., 2019), logistical and ethical constraints result in limited attempts to replicate studies which means that it is currently still difficult to reach any evidence-based consensus on how wildlife may, or may not, be affected by fluoxetine (Melvin, 2017; Thoré et al., 2021a). Confirming that chronic fluoxetine exposure leads to smaller body sizes in *N. furzeri,* hence, adds confidence to the credibility of this finding.

4.2. Effects on geotaxic behaviours

Consistent with our expectations, adult fish spent less time at the water surface and, on average, resided deeper in the water column than juveniles. Because adult fish are more conspicuous to bird predators, avoiding the surface and swimming in deeper water may be an antipredator strategy. More generally, many aquatic organisms have a natural tendency to avoid the surface and dive to the bottom to avoid risk (Parker, 2016; Doran et al., 2022), so that our observation suggests that adults are more risk-averse than juveniles. Similar observations were made for other inhabitants of temporary ponds, including *Branchipodopsis wolfi* fairy shrimps. In this species, larger females–which are typically more susceptible to visual predation by birds (Brendonck et al., 1995)–were more benthic than smaller males (Thoré et al., 2021b). Such differences between males and females were, however, not observed for *N. furzeri* in the current- nor in previous studies (Evsiukova et al., 2021).

Based on earlier studies that showed more risk-averse behaviour of *N. furzeri* after exposure to fluoxetine (Thoré et al., 2018; Thoré et al., 2021a), we expected that fluoxetine would lead to more benthic and less active behaviour. However, regardless of life stage, fluoxetine did not affect the average swimming depth, nor the amount of time spent at the surface or the bottom of the water column. Similarly, fathead minnows (adults) exposed for 28 days to 0.1–16 μg/L fluoxetine did not show any change in

geotaxic behaviour, except at higher concentrations of 38–72 μg/L (Margiotta-Casaluci et al., 2014). Such concentration-dependency was also observed in other studies. For example, larval and juvenile brown trout (*Salmo trutta*) exposed to 0–1000 μg/L of another SSRI, venlafaxine, spent more time at the surface than unexposed fish but only at a concentration of 100 μg/L or higher (Ziegler et al., 2021).

Surprisingly, *N. furzeri* adults that were exposed to fluoxetine changed their position in the water column (depth) more frequently and were more 'vertically active' than unexposed fish. This observation seems inconsistent with earlier findings on *N. furzeri* which showed that fluoxetine increased anxiety-related behaviour (Thoré et al., 2018) and led to lower activity levels (as measured in a shallow-water test) (Thoré et al., 2021a). Furthermore, other studies reported that chronic exposure to fluoxetine did not affect total distance travelled–a measure of fish activity–during a diving test in adult fathead minnows (28 days at 38–72 μg/L; Margiotta-Casaluci et al., 2014) and adult zebrafish (*Danio rerio*) (14 days at 50–100 μg/L; Egan et al., 2010). Even though one could reasonably assume concentration- and/or species-dependency to underlie this variation in reported effects, also the context may determine how animals respond to a pollutant. For example, when eastern mosquitofish (*Gambusia holbrooki*) were exposed for 30 days to 0.4 μg/L fluoxetine, males would attempt to mate more frequently in the absence but not in the presence of a male competitor (Bertram et al., 2018). Furthermore, it is important to note that geotaxis of aquatic animals reflects more than only anxiety-related behaviour and activity level. Instead, it is the result of various, interconnected motivational drivers that may influence how an animal responds to chemical exposure. For example, animals may also adjust their swimming depth–or the frequency by which they do so–to optimise the abiotic (e.g. temperature, light, dissolved oxygen level) and biotic environment (e.g. probability of encountering prey, mates, parasites) (Lester, 1971; Decaestecker et al., 2002; Hurst et al., 2009).

Potential life stage-associated behavioural change adds further to this complexity. In the present study, the increase in vertical activity after exposure to fluoxetine was only observed in the adult but not juvenile phase. This suggests that life stage can determine how animals respond to chemical exposure and should be considered when studying the ecotoxicology of pharmaceuticals. The same applies, in fact, to responses to other global-change stressors (see e.g. Komoroske et al., 2014; Ortiz-Santaliestra et al., 2006). It is worth noting that we cannot entirely exclude the possibility that the different response between juveniles and adults is due to the difference in exposure duration rather than an effect of life stage *per se*. After all, fluoxetine and its metabolites may bioaccumulate and it may take a few weeks before fluoxetine exerts its full pharmacological effect (Gaworecki and Klaine, 2008; Andrews et al., 2015). However, juveniles were exposed for longer than just a few weeks (> 7 weeks), so that it is not likely that the absence of an effect in juveniles vs. adults is merely related to the exposure duration. Indeed, previous studies on *N. furzeri* already found effects of fluoxetine exposure on feeding, anxiety-related behaviour and anti-predator response after as little as 3 weeks (Thoré et al., 2018). Future studies should aim to uncouple life stage from exposure duration to better assess how life stage, in itself, influences responses to chemicals. Furthermore, to gain more confidence in this result, future research should assess whether or not it is repeatable, under similar conditions as well as in varying contexts.

4.3. Life stage should not be overlooked to assess the ecological outcomes of pharmaceutical pollution

Given that the biology and ecology of animals can change substantially across their development (Choh et al., 2012; Ward et al., 2020), it comes as no surprise that responses can be life stage-specific. However, this factor has not received much attention in behavioural ecotoxicology. This is likely because, in practice, long-term exposure that covers several life stages of fish is often hard to implement. The relatively long life cycle of most test species brings logistical and financial constraints (Thoré et al., 2021d). In this regard, species with relatively short life cycles, such as *N. furzeri*, offer promising and cost-efficient alternatives.

Exposure across several life stages of fish approximates the complexity of real-world situations better than classic study designs. Still, it remains challenging to translate changes in body length and geotaxic behaviour under simplified laboratory conditions into field-realistic consequences. Body size is one of the most fundamental traits of an organism, and strongly impacts the structure and dynamics of ecological networks (Woodward et al., 2005). Likewise, changes in geotaxic behaviour may carry important fitness consequences and could have cascading effects across multiple scales of organisation. For example, killifish are gape-limited predators, which means that juvenile killifish are more likely to eat smaller (species of) prey than adult killifish (Pinceel et al., 2021). Invertebrate prey, including many zooplankton and larger branchiopod crustaceans, are known to daily migrate up and down the water column to limit fish predation (Gliwicz, 1986; Brendonck et al., 1995). Therefore, any pollutant-induced change in body size and/or vertical activity of fish could affect these predator-prey interactions in a life-stage dependent manner. Resulting changes in zooplankton population- and community composition could, in turn, translate into differences in grazing on phytoplankton and eventually affect clear-water states (Peretyatko et al., 2012; Gianuca et al., 2016). This is speculative, however, and what the observed shifts under laboratory conditions mean in the complexity of natural ecosystems still largely eludes us. Nevertheless, explicitly considering intraspecific trait variation, particularly biological and ecological differences related to the developmental stages of animals, is necessary to advance our understanding of how to protect natural ecosystems in an increasingly medicated world.

5. Conclusions

We assessed how long-term exposure to fluoxetine affects body length and geotaxic behaviour in juvenile vs. adult fish under simple laboratory conditions. Our results show that chronic exposure to fluoxetine impairs somatic growth and impacts at least one measure of geotaxic behaviour in the adult but not juvenile phase. Specifically, even though we expected an increase in risk-averse behaviour upon fluoxetine exposure, adults changed their position in the water column (depth) more frequently than unexposed fish. This could suggest a decrease in risk-averse behaviour but may well be confounded by various other motivational drivers that underlie this behavioural response. As expected, such effects were not visible in juvenile fish which suggests that life stage can determine how animals respond to chemical exposure. Ideally, however, future studies should aim to uncouple life stage from exposure duration to better assess how life stage, in itself, influences responses to chemicals. Even though it remains challenging to translate these changes into field-realistic consequences, our results suggest that important morphological and behavioural responses to pharmaceutical exposure may only emerge later in time and/or during specific life stages. This highlights the importance of considering ecologically relevant timescales across developmental stages when studying the ecotoxicology of pharmaceuticals.

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CRediT authorship contribution statement

Eli S. J. Thoré: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Visualisation. **Birgit Vanden Berghen**: Methodology, Writing – review & editing. **Luc Brendonck**: Writing review & editing. **Tom Pinceel**: Writing – review & editing.

Data availability

The data that support the findings of this study are openly available in figshare at https://doi.org/10.6084/m9.figshare.22253434.v1.

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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