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Hazard/Risk Assessment

Improving the Reliability and Ecological Validity of Pharmaceutical Risk Assessment: Turquoise Killifish (*Nothobranchius furzeri*) as a Model in Behavioral Ecotoxicology

Eli S.J. Thoré,[a,*] Laure Steenaerts,[a] Charlotte Philippe,[a,b] Arnout F. Grégoir,[a] Luc Brendonck,[a,c] and Tom Pinceel[a,d]

[a]Animal Ecology, Global Change and Sustainable Development, KU Leuven, Leuven, Belgium
[b]Systemic Physiological and Ecotoxicological Research, University of Antwerp, Antwerp, Belgium
[c]Water Research Group, Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa
[d]Centre for Environmental Management, University of the Free State, Bloemfontein, South Africa

Abstract: Pharmaceuticals are essential for human well-being, but their increasing and continuous use pollutes the environment. Although behavioral ecotoxicology is increasingly advocated to assess the effects of pharmaceutical pollution on wildlife and ecosystems, a consensus on the actual environmental risks is lacking for most compounds. The main limitation is the lack of standardized reproducible tests that are based on sensitive behavioral endpoints and that accommodate a high ecological relevance. In the present study, we assessed the impact of a 3-wk exposure to the antidepressant fluoxetine on multiple behavioral traits in the promising new model organism *Nothobranchius furzeri* (turquoise killifish). Overall, our study shows that fluoxetine can impact feeding behavior, habitat choice in a novel environment, and antipredator response of *N. furzeri* individuals; effects on spontaneous activity and exploration tendency were less pronounced. However, effects became only apparent when individuals were exposed to fluoxetine concentrations that were 10 times higher than typical concentrations in natural aquatic environments. Ecotoxicologists are challenged to maximize both the reliability and ecological validity of risk assessments of pollutants. Our study contributes to the development of a time- and cost-efficient, standardized ecotoxicological test based on sensitive, ecologically relevant behavioral endpoints in *N. furzeri*. *Environ Toxicol Chem* 2018;9999:1–9. © 2018 SETAC

Keywords: *Nothobranchius*; Ecotoxicology; Behavioral toxicology; Emerging pollutants; Fluoxetine

INTRODUCTION

Pharmaceuticals are of high socioeconomic importance, and their use (especially that of antidepressants) has increased enormously over the past decades (Gusmão et al. 2013). They constitute a novel class of environmental contaminants ("emerging"; Dzieweczynski and Hebert 2012; Loos et al. 2013; Brodin et al. 2014). Many pharmaceutical compounds are continuously discharged through domestic wastewater and are (pseudo-) persistent in the environment (Fent et al. 2006; Arnold et al. 2014). Although environmental concentrations are often low compared with traditional contaminants, pharmaceuticals are typically highly potent and designed to trigger specific pharmacological responses at low doses (Arnold et al. 2014). Pharmaceutical pollution is likely affecting aquatic wildlife because pharmaceutical products often target evolutionarily conserved pathways (Gunnarsson et al. 2008). Current ecotoxicity tests are generally designed to detect lethal, harmful, or stressful effects of exposure to traditional contaminants; they are less suitable for detecting specific pharmacological effects.

An improved assessment of the subtle effects of pollutants such as behavioral alteration is considered an essential step to gain an accurate estimation of the actual impact of pharmaceutical pollution on natural water bodies and their fauna (Fent et al. 2006; Brodin et al. 2014; Pyle and Ford 2017). Changes in behavioral expression have been shown to have direct (e.g. feeding rate, predator avoidance) and indirect (e.g. population dynamics, community structure) ecological consequences (Wolf and Weissing 2012; Brodin et al. 2014). In addition, because behavior is the integrative response to internal and external factors (Dell'Omo 2002; Levitis et al. 2009), it could be an

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* Address correspondence to eli.thore@kuleuven.be
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especially sensitive tool for ecotoxicologists (Melvin and Wilson 2013; Brodin et al. 2014).

Fish are particularly suitable model organisms for assessing the environmental impact of pharmaceutical pollution. Because their neuromuscular physiology is similar to that of humans and because pharmacological target molecules are often highly conserved, there is a high probability that fish are affected by human pharmaceuticals (Gunnarsson et al. 2008; Sakowski et al. 2012). The turquoise killifish (*Nothobranchius furzeri*) is a promising new model in many different biological disciplines (Cellerino et al. 2015), mainly because of its extremely fast maturation (<16 d) and short generation time (<3 mo). These traits enable *N. furzeri* to persist in temporary ponds with extremely short inundations in southeastern Africa (Cellerino et al. 2015; Polačik et al. 2016). Fast maturation likely trades off with lifespan: *N. furzeri* only lives for 5 to 6 mo under optimal laboratory conditions (Terzibasi et al. 2008; Wang et al. 2015; Polačik et al. 2016). This makes it an ideal model organism for whole-life studies and for studying aging-related processes. On reaching maturity, fish spawn daily and produce drought-resistant eggs that remain dormant in the sediment until the next inundation (Pinceel et al. 2015; Grégoir et al. 2017a). The fish produce large amounts of eggs that can easily be stored for up to several years and hatched synchronously for experimental purposes (Polačik et al. 2016; Grégoir et al. 2017b). Because of this unique trait set, *N. furzeri* has been introduced as a model species in traditional ecotoxicology (Philippe et al. 2017, 2018a–c). The available tools for *N. furzeri*, such as a whole-brain atlas (D'Angelo 2013), age-related histopathological analyses, an annotated genome and transcriptome (Di Cicco et al. 2011; Reichwald et al. 2015; Valenzano et al. 2015), and the generation of transgenic lines have added to the value of *N. furzeri* as a model species in ecotoxicology. Furthermore, as the need to unravel the underlying physiological and biochemical mechanisms of behavioral expression is increasingly emphasized (Sloman and McNeil 2012; Parker 2016, Thoré et al. 2018), these tools could aid in the further advancement of behavioral ecotoxicology. The overall aim of the present study was to investigate the potential of *N. furzeri* as a model in behavioral ecotoxicology.

Fluoxetine is the active compound of Prozac and is used as a selective serotonin reuptake inhibitor with antidepressant and anxiolytic effects (Winder et al. 2012). At the moment, the compound is often present in surface waters at concentrations that average about 0.5 μg/L (Winder et al. 2012). These levels are expected to increase further because fluoxetine use is continuously increasing (Winder et al. 2012; Dzieweczynski et al. 2016b). Fluoxetine is a well-studied compound in pharmacology and ecotoxicology. Although reference background data on its targeted molecular mode of action are widely available (Brodin et al. 2014; Parker 2016), standard tests to assess its impact on the fauna of aquatic ecosystems are lacking. Tests based on sensitive behavioral endpoints could accommodate high ecological relevance and provide the means for time- and cost-efficient risk assessment of fluoxetine and other emerging pharmaceutical compounds.

In the present study we investigated the potential of *N. furzeri* for behavioral ecotoxicology studies. We performed a 3-wk experiment and assessed the impact of exposure to environmentally relevant fluoxetine concentrations on activity, boldness, and exploration; we also chose the endpoints of feeding behavior, habitat choice, and antipredator response as behavioral traits with more direct ecological relevance. We selected this set of traits because they all have known fitness implications for fish (Brodin et al. 2014). Fluoxetine treatment reduced spontaneous locomotor activity and induced anxiolytic responses in medaka (*Oryzias latipes*; Ansai et al. 2016) and reduced anxiety-related behavior in zebrafish (*Danio rerio*; Wong et al. 2013). Congruent with these findings and given the anxiolytic properties of fluoxetine, we expected fluoxetine-exposed fish to exhibit less risk-averse behavior expressed as higher activity, boldness, and exploration levels, a high feeding rate, and a relaxed antipredator response.

MATERIALS AND METHODS

General setup and fish maintenance

The tested *N. furzeri* fish originated from a natural population in central Mozambique (MZCS-414). The laboratory population had been maintained for 3 generations under optimal common garden conditions prior to the onset of the experiment. Sixty-five experimental fish were hatched by inundating "ready-to-hatch eggs" (stage 43 sensu Wourms 1972) at 14 °C, according to the protocol of Polačik et al. (2016). Fish tanks were kept in a bain-marie system to ensure a constant water temperature (24.3 °C ± 1.09 standard deviation [SD]) at a 14:10-h light:dark regime.

The present study was approved by the ethical committee of KU Leuven (file no. P160/2016). All procedures performed conform to the legal requirements for animal research in Belgium. Individual condition and health of the fish were checked multiple times a day by 2 researchers separately (E.S.J. Thoré. and L. Steenaerts). Water parameters were measured daily in each tank to keep track of water quality. Animals were housed under optimal conditions, and the hand-made air-driven filter provided shelter in all tanks. Disturbance and handling was kept to a minimum.

Starting at 2 d post hatch, fish larvae were housed in 4-L tanks in groups of 20 individuals; 2 wk after hatching, fish were transferred to 10-L tanks in groups of 10 individuals. After 3 wk, fish were housed individually for individual monitoring in 9-L tanks (49 cm long × 19 cm wide × 16 cm high) with an air-driven filter. Tanks were visually separated from each other with opaque plastic to exclude social contact among individuals. One housing compartment/tank was delimited (~12 cm long × 19 cm wide) to resemble the tank setup for behavioral testing (see below).

Following Organisation for Economic Co-operation and Development test guidelines 203 (1992) and 229 (2012), reconstituted water was used throughout the experiment by adding Instant Ocean salt mix to deionized water until a conductivity of 600 μS/cm was reached. Water was renewed every 2 d when larvae were housed in groups and once a week when fish were housed individually. This ensured good water

quality while limiting handling (pH mean ± SD 8.09 ± 0.33, ammonium <0.2 mg/L, nitrite <25 mg/L). When housed in groups, larvae were fed twice a day an ad libitum quantity of *Artemia franciscana* nauplii (Ocean Nutrition). Individually housed fish were fed ad libitum with *Chironomus* larvae (Ocean Nutrition) and twice a day with *Artemia* nauplii.

Starting at 4 wk post hatching, individual fish were subjected weekly to 4 behavioral tests, which were repeated every week for a total of 5 consecutive wk. The tests, which are explained in further detail below, included: 1) an emergence test, 2) a habitat choice test, 3) an open field test, and 4) a life skills test. For each test, each fish was transferred to an experimental arena, allowed to acclimate for 5 min, and video recorded from above using a digital camera (Logitech C920 HD Pro Webcam). Recordings were manually analyzed afterwards (observer-blind), except for open field data, which were analyzed using EthoVision XT Ver 9.0 video-tracking software (Noldus Information Technologies). After each behavioral test, fish were transferred back to their respective housing tanks. To minimize behavioral variation due to diel activity changes and to add to the logistic feasibility of the experiment, each sampling burst was restricted to a maximum of 3.5 h, and fish were randomly divided over 2 cohorts. Each cohort was subjected to 1 assay/d. Every Tuesday morning, the cohort 1 fish were subjected to the habitat choice test, and in the afternoon the cohort 2 fish were subjected to the emergence test. Every Wednesday, the cohorts 1 and 2 fish were subjected to the emergence test (afternoon) and the habitat choice test (morning), respectively. The same setup was repeated on Thursday and Friday, this time subjecting the fish to either the open field test or the life skills test. Fish were not fed for 24 h before the emergence and life skills test to stimulate exploration of the arena and to prevent disinterest in food. No behavioral tests were carried out on Saturday, Sunday, or Monday. Every Monday, water of the housing tanks was renewed.

At 6 wk post hatch (i.e., starting at the 3rd wk of the 5-wk test period), fish were randomly assigned to 1 of 3 treatments: F1, F2, and control. In the F1 condition (14 females, 9 males) fish were exposed to 0.5 μg/L fluoxetine hydrochloride (F-132; Sigma-Aldrich); in the F2 condition (11 females, 11 males) fish were exposed to 5 μg/L of the same compound. Because dimethyl sulfoxide (DMSO) was used as a solvent for preparation of the fluoxetine solution (see *Preparation of solutions* section), control fish (9 females, 11 males) were exposed to the same amount of DMSO as in the F2 condition (0.00001%). Treatments were applied during each water exchange (i.e., on Monday).

During the 5-wk test period, body size (tip of snout to tip of tail, dorsal view) and body width (at the pectoral fins, dorsal view) to approximate fish condition were monitored every Monday by briefly transferring every individual to a Petri dish with a small amount of water. Top-view, size-calibrated photographs were taken and analyzed using the open source image processing software ImageJ Ver 1.50i (Schneider et al. 2012).

Preparation of solutions

Stock aliquots (mL) were prepared by dissolving fluoxetine hydrochloride in DMSO to 500 mg/L and were preserved at –18 °C until use (maximum age 3 mo). Working standard solutions were prepared by thawing and diluting stock aliquots to 5 mg/L in fish rearing medium (reconstituted water at 600 μS/cm) and preserved at 4 °C. The DMSO working standard solutions were prepared by diluting DMSO with reconstituted water (600 μS/cm) to a 1% solution, and preserved at 4 °C.

Water samples were taken and analyzed for fluoxetine concentration in the last week of the test period on the 3rd and 5th d after administration. Actual concentrations of the F1 and F2 treatments on the 3rd and 5th d after administration, measured by liquid chromatrography–tandem mass spectrometry, were 0.37 (SD 0.17) and 4.41 (SD 0.90), and 0.32 (SD 0.21) and 4.47 (SD 0.93) μg/L, respectively.

Behavioral tests

Emergence test. The emergence test arena resembled the housing tank setup (Figure 1A). Fish were introduced to the smaller "start" compartment, after which a doorway was opened, allowing the individual to enter and explore the "novel" larger compartment during the next 45 min. Latency time to enter the novel compartment as a measure of exploration tendency was recorded, and a maximum score of 45 min was assigned to fish that failed to enter the novel environment (33% of all data points). In addition, the novel compartment was equally divided into a barren zone (risk-prone zone) and a zone holding artificial plants as shelter (risk-averse zone). Fish preference for the zones as a measure for risk aversion in this novel compartment (calculated as the amount of time spent in the barren zone compared with the total amount of time spent in the novel compartment) was recorded for 30 min.

Open field test. In the open field arena (Figure 1B), spontaneous activity was recorded for 20 min. Total distance moved was monitored as a measure of locomotor activity. Moreover, the open field arena was virtually divided into a centrum (50% of arena length and width) and a peripheral zone. Activity in the centrum zone is considered more risk-prone behavior (boldness) compared with activity in the peripheral zone (Ansai et al. 2016). Thus the number of times the fish entered the centrum, the latency time to enter the centrum for the first time, and the cumulative duration spent in the centrum were assessed as a measure of boldness.

Habitat choice test. The habitat choice test arena was equally divided into a barren zone and a zone holding artificial plants for shelter (Figure 1C). Fish preference for the zones (calculated as the relative amount of time spent in the barren zone) was recorded for 30 min.

Life skills test. Using a life skills test arena (Figure 1D), feeding behavior and antipredator response were assessed. The arena was virtually divided into 4 equally sized zones. When fish entered zone 1 or 4, *Chironomus* larvae were added in zone 3, and latency time to initiate feeding was assessed. Fish that did not feed within 15 min were given the maximum score of 15 min. As soon as fish started feeding, a suspended 15-mL Falcon tube

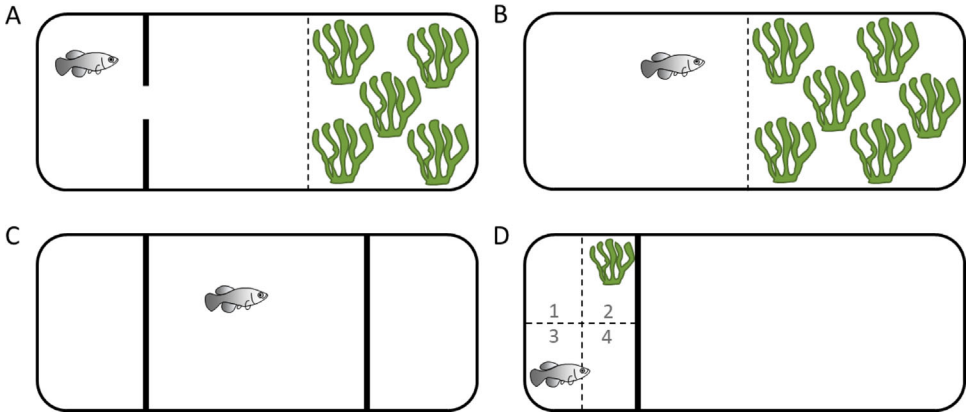

FIGURE 1: Schematic representation of the different test arenas used (dorsal view). All tanks are L × W × H 49 cm long × 19 cm wide × 16 cm high and hold 9 L of water, except for the open field arena, which only holds water to a height of 2 cm. (**A**) Experimental setup for the emergence test. The start compartment resembles the housing conditions. A doorway (diameter 20 mm) allows individuals to explore the novel, larger part of the tank that is equally divided into an open, barren part and a part provided with artificial plants as shelter. (**B**) Experimental setup for the habitat choice test. The tank is equally divided into an open, barren part and a part provided with artificial plants as shelter. The dotted line represents a virtual barrier. (**C**) Open field experimental setup. (**D**) Experimental setup for the life skills test, used to characterize feeding and antipredator behavior. The experimental compartment was virtually divided into 4 equally sized zones (delineated by the dotted lines). Zone 2 holds an artificial plant as shelter, whereas both feeding stimulus and simulated avian attack were applied in zone 3.

(weighted, opaque) was dropped and allowed to touch the water surface in zone 3 as simulation of an avian predator attack. The time until movement and the time needed to resume feeding for fish that froze or swam away were assessed. The test was terminated 45 min after the simulated predator attack. Fish that did not resume feeding were given the maximum score of 45 min.

Statistical analyses

All statistical analyses were performed in R 3.3.1 (R Development Core Team 2016) at a significance level of 0.05. Model assumptions including homogeneity of variance and distributional fit were verified graphically for all analyses. For all behavioral response variables, linear mixed models with Gaussian error distribution were fitted (lme4 package) with treatment (control, F1, F2) and sex (male, female) as fixed factors. The interaction term between treatment and sex was nonsignificant for all models and was therefore excluded from the final models. Fish identity, trial number (referring to the repeated measures), and cohort were added to the model as random factors. Only behavioral responses after treatment were considered in the analyses (i.e., 3 repeated measures/individual), because the first 2 trials were used to habituate fish to the experimental setup. Condition of the fish was approximated by body width measurements corrected for body size and was analyzed using a linear mixed model with Gaussian error distribution with treatment, trial, and their interaction as fixed factors. Sex was added to the model as a predictor variable and fish identity as a random factor. Differences between groups were assessed using Wald chi-square tests (car package) and Tukey-corrected pairwise comparisons (lsmeans package). Behavioral response variables/behavioral test are given in Table 1, including the applied transformation to meet model assumptions.

RESULTS

Emergence test

Overall, latency time to enter the novel environment did not differ among treatments ($\chi^2 = 0.2536$, $p = 0.8809$), and also males and females did not differ in their exploration tendency ($\chi^2 = 0.0045$, $p = 0.9467$).

Habitat preference in the novel environment differed among treatments ($\chi^2 = 6.5129$, $p = 0.0385$), with fish from the F1 condition having a higher preference for the sheltered area compared with control fish (Figure 2A). Sexes differed significantly in habitat preference ($\chi^2 = 10.0152$, $p = 0.0015$), with females spending more time in the sheltered area compared with males.

Open field test

Overall, total distance moved did not differ among treatments ($\chi^2 = 0.8052$, $p = 0.6686$), nor did sexes differ in activity ($\chi^2 = 0.0881$, $p = 0.7667$).

TABLE 1: Behavioral response variables/behavioral test[a]

Behavioral test	Behavioral response
Emergence	Latency time to enter novel environment (log)
	Habitat choice
Open field	Total distance moved
	Number of times the fish entered centrum (log+1)
	Latency time to enter centrum for the first time (log)
	Cumulative duration in centrum (log+1)
Habitat choice	Habitat choice
Life skills	Latency time to feed before attack (double log)
	Latency time to resume feeding (log)
	Time until movement after attack (log)

[a]To meet model assumptions, variables were transformed (indicated in parentheses) except for habitat choice and total distance moved.

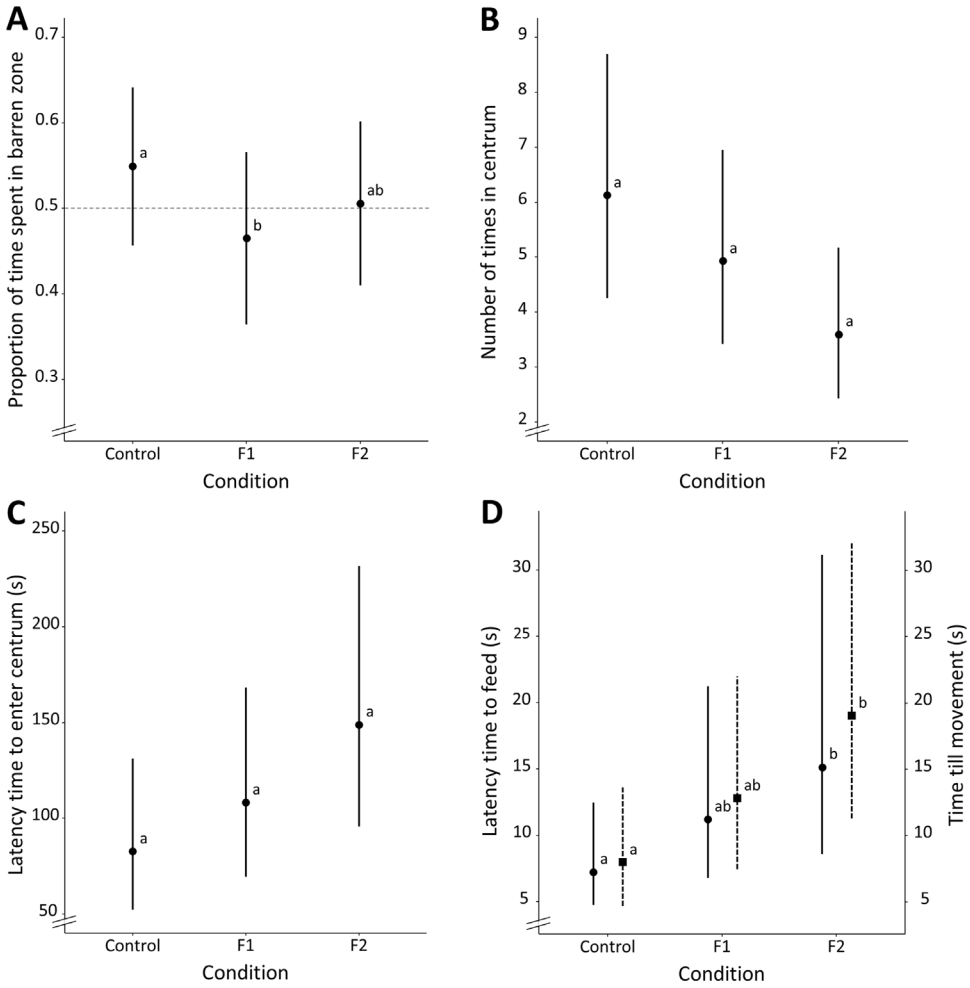

FIGURE 2: Average behavioral response for control fish, fish exposed to 0.5 μg/L fluoxetine (F1) and 5 μg/L fluoxetine (F2). (**A**) Habitat preference in the emergence test setup, with smaller values indicating a higher preference for the sheltered zone as opposed to the open, barren zone of the arena. (**B**) Number of times the fish entered the centrum of the open field test setup. (**C**) Latency time (in seconds) to enter the centrum zone of the open field arena for the first time. (**D**) Latency time to feed (in seconds) before simulated predator attack (circles and solid lines) and time until movement (in seconds) after simulated predator attack (squares and dashed lines) in the life skills test setup. All behavioral response variables are presented in original scale. Whiskers delineate the upper and lower 95% confidence limit. Letters indicate significant differences based on Tukey-corrected post hoc tests.

Although overall the number of times that fish entered the centrum was generally lower under increasing fluoxetine concentrations, this was not significant ($\chi^2 = 5.8944$, $p = 0.0525$; Figure 2B). Also, there was a trend for a higher latency time to enter the centrum with an increasing concentration of fluoxetine (Figure 2C; $\chi^2 = 4.6428$, $p = 0.0981$). Sexes did not differ in the number of times fish entered the centrum ($\chi^2 = 0.0329$, $p = 0.8561$) nor in latency time to enter the centrum (for the first time; $\chi^2 = 0.0217$, $p = 0.8830$).

Cumulative duration spent in the centrum did not differ among treatments ($\chi^2 = 3.5750$, $p = 0.1674$), or between sexes ($\chi^2 = 0.0346$, $p = 0.8525$).

Habitat choice test

Habitat preference did not differ among treatments ($\chi^2 = 2.4846$, $p = 0.2887$). Sexes differed in habitat preference ($\chi^2 = 4.5772$, $p = 0.0324$), with females having a higher preference for the sheltered area compared with males.

Life skills test

Overall, fluoxetine exposure did not impact the latency time to feed before ($\chi^2 = 5.8910$, $p = 0.0526$) or after ($\chi^2 = 4.3180$, $p = 0.1154$) the simulated predator attack. Although the overall model result is only marginally significant, post hoc analysis revealed that fish from the F2 condition have a significantly higher latency time to feed before a simulated attack compared with fish from the control condition (Figure 2D). After a simulated predator attack, a trend was observed for a higher latency time to resume feeding with increasing fluoxetine concentration (Supplemental Data, Figure S1). Sexes did not differ in latency time to feed before ($\chi^2 = 0.1376$, $p = 0.7107$) or after ($\chi^2 = 0.0088$, $p = 0.9254$) the simulated predator attack.

Overall, the time until movement after a simulated predator attack differed among treatments ($\chi^2 = 7.9736$, $p = 0.0186$), with F2 fish waiting longer before resuming activity compared with control fish (Figure 2D). The time until movement after a

simulated predator attack did not differ between sexes ($\chi^2 = 0.2005$, $p = 0.6543$).

Fish condition

Fish condition, measured as body width to size ratio, did not differ among trials ($\chi^2 = 1.1259$, $p = 0.5695$) or treatments ($\chi^2 = 0.9123$, $p = 0.6337$), nor was the effect of trial dependent on treatment ($\chi^2 = 6.5529$, $p = 0.1615$). The width to size ratio differed between sexes ($\chi^2 = 3.8959$, $p = 0.0484$), with males having a smaller width to size ratio than females.

DISCUSSION

Overall, the present study shows the presence of fluoxetine-induced alterations in feeding behavior, habitat choice, and antipredator response of *N. furzeri* individuals. However, with the exception of habitat choice in a novel environment, these effects only emerged at a 10-fold higher concentration of fluoxetine than that typically reported in the environment. In contrast to our hypotheses, spontaneous activity, boldness, and exploration behavior were not impacted by fluoxetine exposure.

Although fluoxetine has been shown to impact basic behavioral traits including activity and boldness, even at concentrations as low as 0.3 μg/L, in several fish species (Barry 2013; Brodin et al. 2014; Dzieweczynski et al. 2016b), such effects could not be entirely confirmed for *N. furzeri* in our study. Our results do show, however, that feeding behavior, habitat choice, and antipredator response of *N. furzeri* are directly impacted by chronic fluoxetine exposure. For instance, fish that were exposed to 5 μg/L fluoxetine exhibited a higher latency time to initiate feeding and to resume feeding after a simulated predator attack (trend) compared with control fish. Because increased latency time to feed was not associated with a reduction in body width to length ratio in the present study, a higher latency time could reflect a decrease in the propensity to take risks. This could be true given that energy intake is known to often trade off against predation risk (Lima et al. 1985). In favor of this hypothesis, fluoxetine-exposed fish waited longer before resuming activity after a simulated predator attack, possibly indicating decreased boldness due to fluoxetine exposure. Alternatively, and nonmutually exclusively, these results could also be a reflection of a decrease in appetite: fluoxetine is known to have anorexigenic properties (Halford et al. 2005). Conners et al. (2009), for instance, showed a reduced growth of African clawed frog (*Xenopus laevis*) tadpoles after fluoxetine exposure and argued that this effect could be driven by reduced food intake. Whether the observed effect on feeding behavior is due to a decrease in appetite or a reduced propensity to take risks should be the subject of further investigation. Finally, although fish that were exposed to fluoxetine exhibited a higher preference for the sheltered area in a novel environment (emergence test), this effect was only present in fish exposed to 0.5 μg/L fluoxetine and could not be replicated in a familiar environment (habitat choice test). Whether this result is indeed biologically meaningful, reflects a false positive, or is due to a differential feeding status between the 2 tests (fish were not fed for 24 h before the emergence test) remains to be confirmed. Surprisingly, responses to fluoxetine exposure were the opposite of what was hypothesized. Despite the anxiolytic properties of fluoxetine, *N. furzeri* displayed more risk-averse behavior in response to fluoxetine exposure in the present study. Similar findings on the behavior-modulating impact of fluoxetine exposure in fish have been reported in the literature. For instance, Siamese fighting fish (*Betta splendens*) were less bold (Dzieweczynski et al. 2016a) and less exploratory (Dzieweczynski et al. 2016b) after fluoxetine exposure. Gaworecki and Klaine (2008) showed that hybrid striped bass (*Morone saxatilis* × *M. chrysops*) exhibited a decrease in ability to capture prey in response to fluoxetine treatment. A similar decrease in ability to capture prey after fluoxetine exposure was demonstrated in fathead minnow (*Pimephales promelas*; Weinberger and Klaper, 2014). In another study, wild guppies (*Poecilia reticulata*) were found to wait longer before resuming activity after a simulated predator attack and spent more time under plant cover (Saaristo et al. 2017). Although the effects of fluoxetine exposure on behavioral expression in nontarget organisms has received ample attention in the literature, the results are diverse and the underlying behavioral mechanism of action through which fluoxetine exerts its effect on fish remains poorly understood. Underlying mechanisms could include general motor sedation or a decreased arousal to external stimuli (Eisenreich and Szalda-Petree, 2015). Future research is needed to improve our understanding of the fluoxetine-induced behavioral effects reported in literature.

Behavioral ecotoxicology is gaining in popularity, especially with regard to detecting the effects of pharmaceutical pollution, not only because a multitude of pharmaceutical compounds are specifically designed to induce behavioral alterations but also because behavioral endpoints are generally more sensitive compared with traditional endpoints in ecotoxicology (Robinson 2009; Melvin and Wilson 2013; Sumpter et al. 2014). Ecologists and ecotoxicologists have increasingly emphasized the importance of ecotoxicological tests that take natural conditions into better consideration and stress the need for realistic exposure tests to further increase the ecological validity and reliability of ecological risk assessments (Arnold et al. 2014; Backhaus 2014). For instance, the impact and implications of pharmaceutical exposure for wildlife and ecosystems over ecologically relevant time periods remain poorly studied (Fent et al. 2006; Arnold et al. 2014). Moreover, multigenerational setups represent an even higher level of realism compared with chronic toxicity tests that are restricted to one generation. Such tests are particularly relevant because effects of pollutants may only emerge after several generations of exposure or organisms could adapt to the situation and become less sensitive (Goussen et al. 2013; Parker 2016). Fluoxetine, for instance, has recently been shown to induce chromatin changes in "brain reward regions" leading to epigenetic inhibition of behaviorally relevant gene expression (Robison et al. 2014). Accordingly, parental exposure is likely to have consequences for future generations through (transgenerational) epigenetic inheritance, which makes multigenerational testing highly relevant (Parker 2016). The short generation time of *N. furzeri* allows for relatively time-efficient whole-life and

multigenerational setups to study the impact of pharmaceutical exposure in vertebrate nontarget organisms.

In addition to the major challenge of ecological validity for behavioral ecotoxicology, maximizing test–retest reliability is also of pivotal importance to the field (Parker 2016). Although there is a vast body of literature on the effects of fluoxetine on fish species, the results from these studies are highly diverse, implying that the potency of fluoxetine is variable (Sumpter et al. 2014). Some of these studies report fluoxetine-induced behavioral effects at levels within the g/L to μg/L range (Kohlert et al. 2012; Lynn et al. 2016), whereas others report that even concentrations as low as ng/l or pg/L can induce differential behavioral expression (Dzieweczynski and Hebert 2012; Barry 2013; Sumpter et al. 2014). Therefore, despite the vast amount of studies that have examined the impact of fluoxetine exposure on aquatic organisms, it remains impossible to reach any consensus on the actual environmental risks of the compound. Sumpter et al. (2014) ascribed the divergence in the literature in large part to the lack of high-quality reproducible research using standard endpoints. Not only is reproducibility fundamental to good scientific practice, it is also essential to ensure reliable risk assessments. Repeatability measures per behavioral endpoint, measured as the between-individual variance in behavioral expression over the sum of between-individual and residual variance, can serve as a first indication for test–retest reliability (Wolak et al. 2012). All behavioral measures in the present study system were shown to be repeatable, as reported by Thoré et al. (2018).

Generally, reliability trades off against ecological validity (Carter et al. 2013; Parker 2016). Thus reaching an equilibrium to maximize both reliability and validity is believed to be one of the challenges in ecotoxicology (Parker 2016). To this end, standardized (reproducible) ecotoxicological tests that allow for testing over ecologically relevant time periods are pivotal, especially for (pseudo-)persistent contaminants such as pharmaceuticals. However, such tests should be time and cost efficient. Traditional model organisms such as zebrafish (*Danio rerio*) do not allow for this because of their slow life cycle and long lifespan of up to 5 yr (Harel et al. 2015). A standardized ecotoxicological test, based on sensitive, ecologically relevant behavioral endpoints in the model organism *N. furzeri*, has high potential; the use of *N. furzeri* combines the advantages of traditional fish model organisms with the benefit of a short-generation time. This allows for whole-life and even multigenerational studies at a reasonable monetary and time cost. In addition to high reliability and ecological validity, a sensitive and standardized test for *N. furzeri* could reduce laboratory animal suffering whereas increased experimental reproducibility would avoid redundant duplication (Parker 2016) and add to a reduction in numbers of laboratory animals.

CONCLUSIONS AND FUTURE PERSPECTIVES

Behavioral endpoints should be incorporated into ecotoxicological testing to increase ecological realism (Pyle and Ford 2017). However, standardized tests are lacking and current ecotoxicity tests are not suitable to detect specific pharmacological effects. The results of our study indicate that fluoxetine may alter ecologically relevant behavior of the promising model organism *N. furzeri*. Although behavioral alterations can have important ecological consequences, and although the behavioral endpoints examined are known to be of high ecological relevance in fish, such effects still need to be related quantitatively to environmental protection goals before they can be used as endpoints in environmental risk assessments. This is a major goal of future research. Standardized behavior-based tests with *N. furzeri* could substantially improve the reliability and ecological validity of ecotoxicology. Future efforts should develop this potential and fuel the launch of a reproducible standard test that meets the need for ecological validity, specifically with regard to whole-life or multigenerational setups. A crucial step will be to establish individual variability in behavioral endpoints and to examine how environmental conditions affect baseline behavioral expression (Sumpter et al. 2014). Individual-based studies over ecologically relevant time periods will allow us to unravel and account for individual behavioral variation (Parker 2016) and will be of primary importance to elucidate behavioral expression with relation to underlying physiological traits, life-history expression, and development (Clutton-Brock and Sheldon 2010). Furthermore, a standardized behavior-based test using *N. furzeri* could easily be combined with systematic environmental heterogenization in an attempt to improve reliability even further (Richter et al. 2010; Parker 2016). The unique life-history of *N. furzeri* along with the readily available biomedical and ecological background will drive further advances in behavioral ecotoxicology.

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