Improving the reliability and ecological validity of pharmaceutical risk assessment: turquoise killifish (Nothobranchius furzeri) as a model in behavioral ecotoxicology

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Eli S.J. Thoré, Laure Steenaerts, Charlotte Philippe, Arnout F. Grégoir, Luc Brendonck, Tom Pinceel

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Improving the reliability and ecological validity of pharmaceutical risk assessment – Turquoise killifish (*Nothobranchius furzeri*) as a model in behavioural ecotoxicology

**Running head:** Killifish in behavioural ecotoxicology

Eli S.J. Thoré, Laure Steenaerts, Charlotte Philippe, Arnout F. Grégoir, Luc Brendonck, Tom Pinceel

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*a* Animal Ecology, Global Change and Sustainable Development, KU Leuven, Ch. Deberiotstraat 32, 3000 Leuven, Belgium

*b* Systemic Physiological and Ecotoxicological Research, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium

*c* Water Research Group, Unit for Environmental Sciences and Management, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa

*d* Centre for Environmental Management, University of the Free State, P.O. Box 339, Bloemfontein, 9300 South Africa

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* Corresponding author: Eli S.J. Thoré, Charles Deberiotstraat 32, 3000 Leuven, Belgium, e-mail: eli.thore@kuleuven.be, Phone nr: +32 16 32 68 60

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Abstract (words: 192)

Pharmaceuticals are essential for human wellbeing but their increasing and continuous use pollutes the environment. Although behavioural 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 standardised reproducible tests that are based on sensitive behavioural endpoints and that accommodate a high ecological relevance. In the current study, we assessed the impact of a three-week exposure to the antidepressant fluoxetine on multiple behavioural traits in the upcoming model organism *Nothobranchius furzeri* (Turquoise killifish). Overall, this study shows that fluoxetine can impact feeding behaviour, habitat choice in a novel environment and antipredator response of *N. furzeri* individuals while effects on spontaneous activity and exploration tendency were less pronounced. However, effects became only apparent when individuals were exposed to fluoxetine concentrations that were ten 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 a time- and cost-efficient, standardised ecotoxicological test based on sensitive, ecologically relevant behavioural endpoints in *N. furzeri*. This article is protected by copyright.

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Keywords: *Nothobranchius*, ecotoxicology, behavioural toxicology, emerging pollutants, fluoxetine
1. Introduction

Pharmaceuticals are of high socioeconomic importance. Their use, especially that of antidepressants, has increased enormously over the past decades (Gusmão et al. 2013). This has turned them into a novel class of contaminants (‘emerging contaminants’) in the environment (Dzieweczynski & 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). While environmental concentrations are often low compared to 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 since pharmaceutical products often target evolutionary conserved pathways (Gunnarsson et al. 2008). While current ecotoxicity tests are generally designed to detect lethal, harmful or stressful effects of exposure to traditional contaminants, they are less suitable to detect specific pharmacological effects.

An improved assessment of subtle effects, such as behavioural alteration, of pollutants 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 & Ford 2017). After all, changed behavioural expression has been shown to have direct (e.g. feeding rate, predator avoidance) and indirect (e.g. population dynamics, community structure) ecological consequences (Wolf & Weissing 2012; Brodin et al. 2014). In addition, since behaviour is the integrative response to internal and external factors (Dell’Omo 2002; Levitis et al. 2009), it could be an especially sensitive tool for ecotoxicologists (Melvin & Wilson 2013; Brodin et al. 2014).
Fish are particularly suitable model organisms to assess the environmental impact of pharmaceutical pollution. Since their neuromuscular physiology is similar to that of humans and pharmacological target molecules are often highly conserved, there is a high probability that they are affected by human pharmaceuticals (Gunnarsson et al. 2008; Sakowksi et al. 2012). The turquoise killifish (*Nothobranchius furzeri*) is an upcoming model in many different biological disciplines (Cellerino et al. 2015). Its popularity mainly derives from its extremely fast maturation (<16 days) and short generation time (<3 months). These traits enable *N. furzeri* to persist in temporary ponds with extremely short inundations in south-eastern Africa (Cellerino et al. 2015; Polačik et al. 2016). Fast maturation likely trades-off with lifespan since *N. furzeri* only lives for 5-6 months 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 ageing related processes. Upon 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). *N. furzeri* produces 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, annotated genome and transcriptome (Di Cocco et al. 2011; Reichwald et al. 2015; Valenzano et al. 2015) and the generation of transgenic lines add 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 behavioural expression is increasingly emphasized (Sloman & McNeil 2012; Parker 2016, Thoré et al. 2018), these tools could aid in the further advancement
of behavioural ecotoxicology. The overall aim of this study is to investigate the potential of \textit{N. furzeri} as a model in behavioural ecotoxicology.

Fluoxetine is the active compound of Prozac and is used as a selective-serotonin reuptake-inhibitor (SSRI) 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 0.5µg/L (Winder et al. 2012). These levels are expected to increase further since fluoxetine use is continuously increasing (Winder et al. 2012; Dzieweczynski et al. 2016b). Fluoxetine is a well-studied compound in pharmacology and ecotoxicology. While reference background data on its targeted molecular mode of action is widely available (Brodin et al. 2014; Parker 2016), standard tests to assess its impact on the fauna of aquatic ecosystems are lacking. Tests that are based on sensitive behavioural 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 current study, we investigate the potential of \textit{N. furzeri} for behavioural ecotoxicology. For this, we perform a three-week experiment and assess the impact of exposure to environmentally relevant fluoxetine concentrations on activity, boldness and exploration as well as on feeding behaviour, habitat choice and antipredator response as behavioural traits with more direct ecological relevance. We selected this set of traits since they all have known fitness-implications for fish (Brodin et al. 2014). Fluoxetine treatment reduced spontaneous locomotor activity and induced anxiolytic responses in medaka (\textit{Oryzias latipes}) (Ansai et al. 2016) and reduced anxiety-related behaviour in zebrafish (\textit{Danio rerio}) (Wong et al. 2013). Congruent with these findings and given the anxiolytic properties of fluoxetine, we expect fluoxetine-exposed fish to
exhibit less risk-averse behaviour expressed as higher activity, boldness and exploration levels, a high feeding rate and a relaxed anti-predator response.

2. Materials and methods

2.1 General setup and fish maintenance

The tested *N. furzeri* fish originate from a natural population in central Mozambique (MZCS-414). The laboratory population had been maintained for three generations under optimal common garden conditions prior to the onset of the experiment. A total of 65 experimental fish was hatched by inundating ‘ready-to-hatch eggs’ (stage 43 *sensu* Wourms, 1972) at 14°C, protocol after Polačík et al (2016). Fish tanks were kept in a bain-marie system to ensure a constant water temperature (24.3 °C ± sd 1.09) at a 14h: 10h light: dark regime.

Starting two days post-hatching, fish larvae were housed in 4L tanks in groups of 20 individuals, and two weeks after hatching fish were transferred to 10L tanks in groups of 10 individuals. After three weeks, fish were housed individually for individual monitoring in 9L tanks (LxWxH 49x19x16cm) with an air-driven filter. Tanks were visually separated from each other with opaque plastic to exclude social contact among individuals. Per tank, one housing compartment was delimited (approx. LxW 12 x 19cm) to resemble the tank setup for behavioural testing (see below).

Following OECD guidelines for testing of chemicals (e.g. OECD TG 203, 229), reconstituted water was used throughout the experiment by adding Instant Ocean salt mix to deionized water until a conductivity of 600µS/cm was reached. Water was renewed every two days 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 < 0.2mg/L,
nitrite < 25mg/L). When housed in groups, larvae were fed twice a day an *ad libitum* quantity of *Artemia franciscana* nauplii (Ocean Nutrition, Essen, Belgium). Individually housed fish were fed *ad libitum* with *Chironomus* larvae (Ocean Nutrition, Essen, Belgium) and *Artemia* nauplii, twice a day.

Starting at an age of four weeks post-hatching, individual fish were weekly subjected to four behavioural tests, repeated every week for a total of five consecutive weeks. These behavioural tests included 1) an emergence test, 2) a habitat choice test, 3) an open field test and 4) a life skills test and are explained in further detail below. For each test, each fish was transferred to an experimental arena, allowed to acclimate for 5 minutes and video recorded from above using a digital camera (Logitech C920 HD Pro webcams, www.logitech.com). Recordings were manually analysed afterwards (observer-blind), except for open field data which were analysed using EthoVision XT Version 9.0 video-tracking software (Noldus Information Technologies Inc; www.noldus.com). After each behavioural test, fish were transferred back to their respective housing tanks. To minimize behavioural 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 hours and fish were randomly divided over two cohorts. Each cohort was subjected to one assay per day. Every Tuesday morning, fish of cohort 1 were subjected to the habitat choice test, while in the afternoon fish of cohort 2 were subjected to the emergence test. Every Wednesday, fish of cohort 1 and 2 were subjected to the emergence test (afternoon) and the habitat choice test (morning) respectively. The same set-up 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 hours before the emergence and life skills test to stimulate exploration of the arena and to
prevent disinterest in food. No behavioural tests were carried out on Saturday, Sunday and Monday. Every Monday, water of the housing tanks was renewed.

At an age of 6 weeks post hatching (i.e. starting at the third week of the 5-week test period), fish were randomly assigned to one of three treatments: F1, F2 and control. In the F1 condition (14 females, 9 males) fish were exposed to 0.5µg/L fluoxetine hydrochloride (Sigma F-132), while in the F2 condition (11 females, 11 males) fish were exposed to 5µg/L of the same compound. As DMSO was used as a solvent for fluoxetine solution preparation (see below), control fish (9 females, 11 males) were exposed to an equal amount of DMSO as applied in the F2 condition (0.00001 %). Treatments were applied during each water exchange (i.e. on Monday).

During the 5-week 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 was 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 analysed using open source image processing software ImageJ 1.50i (Schneider et al. 2012).

2.2 Preparation of solutions

Fluoxetine hydrochloride (FLX) was purchased from Sigma-Aldrich (Sigma F-132). Stock aliquots (mL) were prepared by dissolving FLX in DMSO to 500mg/L and were preserved at -18°C until use (maximum age of three months). Working standard solutions were prepared by thawing and diluting stock aliquots to 5mg/L in fish rearing medium (reconstituted water at 600µS/cm) and preserved at 4°C.

DMSO working standard solutions were prepared by diluting DMSO with reconstituted water (600µS/cm) to a 1 % solution, and preserved at 4°C.

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Water samples were taken and analysed for fluoxetine concentration in the last week of the test-period on the third and fifth day after administration. Actual concentrations of the F1 and F2 treatment on the third and fifth day after administration, measured by means of LC-MS/MS, equalled 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.

2.3 Behavioural tests

2.3.1 Emergence test

The emergence test arena resembled the housing tank setup (Fig 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 minutes. Latency time to enter the novel compartment as a measure of exploration tendency was recorded and a maximum score of 45 minutes was assigned to fish that failed to enter the novel environment (33% of all data points). In addition, the novel compartment was equally divided in 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 to the total amount of time spent in the novel compartment) was recorded for 30 minutes.

2.3.2 Open field test

In the open field arena (Fig 1B), spontaneous activity was recorded for 20 minutes. Total distance moved was monitored as a measure of locomotor activity. Moreover, the open field arena was virtually divided in a centrum (50% of arena length and width) and peripheral zone. Activity in the centrum zone is considered more risk-prone behaviour (boldness) compared to
activity in the peripheral zone (Ansai et al. 2016). As such, the number of times the fish entered the centrum, 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.

2.3.3 Habitat choice test

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

2.3.4 Life skills test

Using a life skills test arena (Fig 1D), feeding behaviour and antipredator response were assessed. The arena was virtually divided in four 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 minutes were given the maximum score of 15 minutes. As soon as fish started feeding, a suspended 15 mL falcon tube (weighted, opaque) was dropped and allowed to touch the water surface in zone 3 as simulation of an avian predator attack. The time till movement and the time needed to resume feeding for fish that froze or swam away were assessed. The test was terminated 45 minutes after the simulated predator attack. Fish that did not resume feeding were given the maximum score of 45 minutes.
2.4 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 behavioural response variables, linear mixed models with Gaussian error distribution were fitted (lme4 package) with treatment (control, F1, F2) and sex (male, female) as fixed factor. The interaction term between treatment and sex was non-significant 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 behavioural responses after treatment are considered in the analyses (i.e. three repeated measures per individual), as the first two 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 analysed 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 predictor variable and fish identity as random factor. Differences between groups were assessed using Wald chi-square tests (car package) and Tukey-corrected pairwise comparisons (lsmeans package). Behavioural response variables per behavioural test are given in Table 1, including the applied transformation to meet model assumptions.
Table 1. Behavioural response variables per behavioural test. To meet model assumptions, variables were transformed (indicated between brackets) except for habitat choice and total distance moved.

<table>
<thead>
<tr>
<th>Behavioural test</th>
<th>Behavioural response</th>
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<td>Emergence test</td>
<td>Latency time to enter novel environment (log)</td>
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<td></td>
<td>Habitat choice</td>
</tr>
<tr>
<td>Open field test</td>
<td>Total distance moved</td>
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<tr>
<td></td>
<td>Number of times the fish entered centrum (log+1)</td>
</tr>
<tr>
<td></td>
<td>Latency time to enter centrum for the first time (log)</td>
</tr>
<tr>
<td></td>
<td>Cumulative duration in centrum (log+1)</td>
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<tr>
<td>Habitat choice test</td>
<td>Habitat choice</td>
</tr>
<tr>
<td>Life skills test</td>
<td>Latency time to feed before attack (double log)</td>
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<td></td>
<td>Latency time to resume feeding (log)</td>
</tr>
<tr>
<td></td>
<td>Time till movement after attack (log)</td>
</tr>
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</table>
3. Results

3.1 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 to control fish (Fig. 2A). Sexes differed significantly in habitat preference ($\chi^2 = 10.0152, p = 0.0015$), with females spending more time in the sheltered area compared to males.

3.2 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 ($\chi^2 = 0.0881, p = 0.7667$).

Although overall the number of times that fish entered the centrum was generally lower under increasing fluoxetine concentrations, this was not significant ($\chi^2 = 5.8944, p = 0.0525$) (Fig. 2B). Also, there was a trend for a higher latency time to enter the centrum with an increasing concentration of fluoxetine (Fig. 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 ($\chi^2 = 3.5750, p = 0.1674$), nor between sexes ($\chi^2 = 0.0346, p = 0.8525$).
3.3 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 to males.

3.4 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 to fish from the control condition (Fig. 2D). After a simulated predator attack, a trend was observed for a higher latency time to resume feeding with increasing fluoxetine concentration (Fig. A1, see supplementary material). 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 till 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 to control fish (Fig. 2D). The time till movement after a simulated predator attack did not differ between sexes ($\chi^2 = 0.2005, p = 0.6543$).

3.5 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.
4. Discussion

Overall, this study shows fluoxetine-induced alterations in feeding behaviour, 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 tenfold higher concentration of fluoxetine than that which is typically reported in the environment. In contrast to our hypotheses, spontaneous activity, boldness and exploration behaviour were not impacted by fluoxetine exposure.

Although fluoxetine has been shown to impact basic behavioural traits including activity and boldness, even at concentrations as low as 0.3µg/L, in several fish species (Barry 2013; Brodin et al. 2014; Dzieweczynski et al. 2016b), such effects could not entirely be confirmed for *N. furzeri* in our study. Our results do show, however, that feeding behaviour, habitat choice and antipredator response of *N. furzeri* is directly impacted by chronic fluoxetine exposure. For instance, fish that were exposed to 5µg/L fluoxetine exhibited a higher latency time to initiate feeding and to resume feeding after a simulated predator attack (trend) compared to control fish.

Since increased latency time to feed was not associated with a reduction in body width to length ratio in the current study, a higher latency time could be reflective of 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 favour of this hypothesis, fluoxetine exposed fish waited longer before resuming activity after a simulated predator attack, possibly indicating a decreased boldness due to fluoxetine exposure. Alternatively and non-mutually exclusive, these results could also be a reflection of a decrease in appetite as 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 a reduced food intake. Whether or not the observed effect on feeding behaviour is due to a decrease in appetite or a reduced propensity to take risks should be subject to further investigation. Finally, while fish that were exposed to fluoxetine exhibited a higher preference for sheltered area in a novel environment (emergence test), this effect was only present in fish exposed to 0.5µg/L fluoxetine and could not be replicated in a familiar environment (habitat choice test). Whether or not this result is indeed biologically meaningful, reflects a false positive or is due to a differential feeding status between the two tests (fish were not fed for 24 hours before the emergence test) remains to be confirmed. Surprisingly, responses to fluoxetine exposure are in opposite direction to what was hypothesised. Despite the anxiolytic properties of fluoxetine, *N. furzeri* displayed more risk-averse behaviour in response to fluoxetine exposure in the current study. Similar findings on the behaviour-modulating impact of fluoxetine exposure in fish have been reported in 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 & 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 & 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 behavioural expression in non-target organisms has received ample attention in literature, results are diverse and the underlying behavioural mechanism of action through which fluoxetine exerts its effect in fish remains poorly understood. Underlying mechanisms could include general motor sedation or a
decreased arousal to external stimuli (Eisenreich & Szalda-Petree, 2015). Future research is needed to improve our understanding of the fluoxetine-induced behavioural effects reported in literature.

Behavioural ecotoxicology is gaining in popularity, especially with regard to detecting effects of pharmaceutical pollution. This is not only because a multitude of pharmaceutical compounds are specifically designed to induce behavioural alterations but also because behavioural endpoints are generally more sensitive compared to traditional endpoints in ecotoxicology (Robinson 2009; Melvin & Wilson 2013; Sumpter et al. 2014). Ecologists and ecotoxicologists increasingly emphasize 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 to chronic toxicity tests that are restricted to one generation. Such tests are particularly relevant since 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 behaviourally relevant gene expression (Robinson et al. 2014). Accordingly, parental exposure is likely to exert consequences in future generations through (transgenerational) epigenetic inheritance which makes multi-generational 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 non-target organisms.

Besides the major challenge of ecological validity for behavioural ecotoxicology, also maximizing test-retest reliability is of pivotal importance to the field (Parker 2016). While there is a vast body of literature on the effects of fluoxetine on fish species, results from these studies are highly diverse. This implies that the potency of fluoxetine seems variable (Sumpter et al. 2014). Some of these studies report fluoxetine-induced behavioural effects at levels within the g/L to µg/L range (Kohlert et al. 2012; Lynn et al. 2016) while others report that even concentrations as low as within ng/l or pg/L exert differential behavioural expression (Dzieweczynski & 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 actual environmental risks of the compound. Sumpter et al. (2014) ascribed the divergence in literature in large part to the lack of high-quality reproducible research on standard endpoints. Not only is reproducibility fundamental to good scientific practice, it is also essential to ensure reliable risk assessments. Repeatability measures per behavioural endpoint, measured as the between-individual variance in behavioural 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 behavioural measures in the current 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). In light of this, reaching an equilibrium to maximize both reliability and validity is believed to be one of the challenges in ecotoxicology (Parker 2016). To this end, standardised (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 due to a slow life cycle and long lifespan of up to 5 years (Harel et al. 2015). A standardised ecotoxicological test, based on sensitive, ecologically relevant behavioural endpoints in the model organism *N. furzeri* offers high potential. *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 standardised test for *N. furzeri* could reduce laboratory animal suffering while increased experimental reproducibility avoids redundant duplication (Parker 2016) and adds to a reduction in numbers of laboratory animals.

4.1 Conclusion and future perspectives

Behavioural endpoints should be incorporated to increase the ecological realism of ecotoxicological testing (Pyle & Ford 2017). Yet, standardised tests are lacking and current ecotoxicity tests are not suitable to detect specific pharmacological effects. The results from our study indicate that fluoxetine may alter ecologically relevant behaviour of the upcoming model organism *N. furzeri*. While behavioural alterations can have important ecological consequences, and although the examined behavioural endpoints are known to be of high ecological relevance in fish, such effects still need to be related quantitatively to environmental protection goals to be used as endpoints in environmental risk assessments, which represents a major goal for future research. Standardised behaviour-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 behavioural endpoints and to examine how environmental conditions affect baseline behavioural expression (Sumpter et al. 2014). Individual-based studies over ecologically relevant time periods will allow to unravel and account for individual behavioural variation (Parker 2016) and be of primary importance to elucidate behavioural expression with relation to underlying physiological traits, life-history expression and development (Clutton-Brock & Sheldon 2010). Furthermore, a standardised behaviour-based test using *N. furzeri* could easily be combined with systematic environmental heterogenisation 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 advancement of behavioural ecotoxicology.

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**Author contributions:** The study was designed by ESJT and TP and performed by ESJT and LS. Data was analysed by ESJT. The manuscript was written by ESJT and reviewed by TP, LS, CP and LB. All authors gave final approval for publication.

**Data accessibility:** (Data will be accessible at the FigShare repository upon acceptance of the manuscript for publication.) Data pertaining to this manuscript are deposited in FigShare at DOI:xxxx.

**Ethical statement:** This study was approved by the ethical committee of KU Leuven (file number: P160/2016). All performed procedures are conform the legal requirements for animal research in Belgium. Individual condition and health was checked multiple times a day by two researchers separately (ESJT and LS). In addition, water parameters were measured daily in each tank to keep track of water quality. Animals were housed under optimal conditions and the handmade air-driven filter provided shelter in all tanks. Disturbance and handling was kept to a minimum.

**Literature**


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in *Betta splendens*. Advances in Physiology Education. 31, 358–363. DOI: 10.1152/advan.00024.2007.


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FIGURE CAPTIONS

Figure 1. Schematic representation of the different test arenas used (dorsal view). All tanks are LxWxH 49x19x16cm and hold 9L water, except for the open field arena which only holds water to a height of 2cm. A. Experimental setup for the emergence test. The start compartment resembles the housing conditions. A doorway (diameter 20mm) allows individuals to explore the novel, larger part of the tank that is equally divided in 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 in 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 behaviour. The experimental compartment was virtually divided in four 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.

Figure 2. Average behavioural response for control fish, fish exposed to 0.5µg/L fluoxetine (F1) and 5µg/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 sec) to enter the centrum zone of the open field arena for the first time. D. Latency time to feed (in sec) before simulated predator attack (circles and solid lines) and time till movement (in sec) after simulated predator attack (squares and dashed lines) in the life skills test setup. All behavioural 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.

Figure A1. Average latency time to resume feeding (sec) after simulated predator attack for control fish, fish exposed to 0.5µg/L fluoxetine (F1) and 5µg/L fluoxetine (F2) in the life skills test setup. The behavioural response is presented in original scale. Whiskers delineate the upper and lower 95% confidence limit. Letters indicate significant differences based on Tukey-corrected post-hoc tests.
Figure 1
Figure 2