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Antidepressant exposure reduces body size, increases fecundity and alters social behavior in the short-lived killifish *Nothobranchius furzeri*☆

Eli S.J. Thoré [a, *], Charlotte Philippe [a], Luc Brendonck [a, b], Tom Pinceel [a, c]

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

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ABSTRACT

Social and mating behavior are fundamental fitness determinants in fish. Although fish are increasingly exposed to pharmaceutical compounds that may alter expression of such behavior, potential effects are understudied. Here, we examine the impact of lifelong exposure to two concentrations (0.7 and 5.3 μg/L) of the antidepressant fluoxetine on fecundity and social behavior (i.e. sociability and male-male aggression) in the turquoise killifish, *Nothobranchius furzeri*. When exposed to the highest concentration of fluoxetine (5.3 μg/L), fish were smaller at maturation but they more frequently engaged in mating. In addition, in both fluoxetine treatments females roughly doubled their overall fecundity while egg fertilization rates were the same for exposed and unexposed fish. Although aggression of male fish was not impacted by fluoxetine exposure, exposed male fish (5.3 μg/L) spent more time in the proximity of a group of conspecifics, which implies an increased sociability in these individuals. Overall, the results of this study indicate that exposure to fluoxetine may result in disrupted male sociability, increased mating frequency and an increased reproductive output in fish populations.

1. Introduction

Animal behavior is an integrative response to a range of internal and external stimuli and is of high ecological importance since it may directly determine performance and fitness at the population level (Brodin et al., 2014; Levitis et al., 2009). Changes in behavioral expression can be triggered by environmental changes of natural or anthropogenic origin (Brodin et al., 2014). One of the main causes of anthropogenic environmental change is pollution (Schwarzenbach et al., 2006) linked to activities such as agriculture, mining and transportation (Greaver et al., 2016; Lawrence and Vandecar, 2015; Pacifici, 2015). Typical pollutants in aquatic environments include pesticides (e.g. pyrethroids, organophosphates) and heavy metals (e.g. zinc, copper). While the potential harmful effects of these compounds are generally recognized (Santos et al., 2010; Sauvé and Desrosiers, 2014), there is a whole range of emerging contaminants, including pharmaceutical products, of which the potential threats are far less studied (Li, 2014).

Neuroactive pharmaceuticals, such as the selective serotonin reuptake inhibitor (SSRI) fluoxetine, are continuously used and discharged into surface waters where they may exert specific pharmacological effects on non-target organisms. Among neuroactive pharmaceuticals, fluoxetine is reported to have the highest acute toxicity (Puckowski et al., 2016), with a 48-h LC_{50} value for rainbow trout (*Oncorhynchus mykiss*) and fathead minnow (*Pimephales promelas*) of 2 mg/L and 705 μg/L, respectively (Brooks et al., 2003a). Fluoxetine is typically reported in surface waters at concentrations <600 ng/L (Saaristo et al., 2017) and in wastewater effluents up to 0.540 μg/L (Puckowski et al., 2016). While so far no studies reported fluoxetine to occur in the environment at concentrations that induce lethal effects, the environmental levels of fluoxetine are expected to increase because of its increasing use (Dzieweczynski et al., 2016; Winder et al., 2012). Moreover, antidepressants such as fluoxetine are relatively stable (Hazelton et al., 2014) and can accumulate in the environment and in organisms (Puckowski et al., 2016). For instance, Muir et al. (2017) reported on the bioaccumulation potential of pharmaceuticals and personal

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* Corresponding author. Charles Deberiotstraat 32, 3000, Leuven, Belgium.
E-mail address: eli.thore@kuleuven.be (E.S.J. Thoré).

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care products in blood plasma of wild goldfish in an urban wetland. Results of this study showed that out of a total of 64 detected compounds fluoxetine had the highest plasma bioaccumulation factor.

Although environmental concentrations of neuroactive pharmaceuticals are generally lower (ng-μg/L range) than those of traditional contaminants (see Mole and Brooks, 2019 for a recent report on the environmental occurrence of neuroactive pharmaceuticals), the compounds are highly potent and low doses can induce specific pharmacological effects that are often accompanied by behavioral modifications (Arnold et al., 2014; Mole and Brooks, 2019). Pharmaceuticals are designed to resist inactivation and are therefore persistent in their functionality. Combined with the fact that they are continuously discharged with domestic wastewater, these compounds persist and accumulate in natural environments where they remain potent over extended time periods (Arnold et al., 2014; Fent et al., 2006; Santos et al., 2010).

Because pharmacological target receptors are often evolutionarily conserved, pharmaceuticals may trigger effects in non-target organisms in their natural environment (Gunnarsson et al., 2008). In ecotoxicology, results of a battery of standard short-term exposure tests are typically combined to assess harmful, stressful or lethal effects of exposure to a specific compound (cf. OECD guidelines, OECD 203, 210, 212, 215, 229, 230, 234, 236, 240, 305 I, 305 II and US EPA guidelines OCSPP 850.1075, 890.1330 and 890.2200) (Klaminder et al., 2014; Thoré et al., 2018b). However, these tests do not address potential changes in behavior and generally fail to pick up on subtle and specific biological effects of pharmaceuticals (Arnold et al., 2014; Klaminder et al., 2014). Moreover, despite the fact that organisms are chronically exposed to pharmaceutical contaminants in their natural environment, effects of long-term exposure are often not assessed (Thoré et al., 2018b).

Although behavioral changes in response to pharmaceuticals have been reported, a consensus on associated ecological risks is lacking (Sumpter et al., 2014; Tanoue et al., 2019; Thoré et al., 2018b). Exposure to the antidepressant fluoxetine was shown to reduce anxiety-related behavior in zebrafish (*Danio rerio*) upon a one-week exposure to 100 μg/L and a two-week exposure to 33 μg/L of the compound (Wong et al., 2013). Also, anxiolytic responses upon a 10-day exposure to 100 μg/L of fluoxetine were demonstrated in medaka (*Oryzias latipes*)(Ansai et al., 2016). While these results suggest more risk-prone behavior upon exposure to high levels of fluoxetine, other studies have identified an inverse response upon exposure to lower levels of fluoxetine. Dzieweczynski et al. (2016), for instance, demonstrated a decreased boldness in Siamese fighting fish (*Betta splendens*) in response to a three-week exposure to 0.5 μg/L and 5 μg/L of fluoxetine. Likewise, guppies (*Poecilia reticulata*) were more shy and more anxious towards a predation threat after a 28-day fluoxetine exposure at 16 ng/L (Saaristo et al., 2017). Besides differences in the reported direction of the response to fluoxetine exposure, some studies report behavioral effects in response to fluoxetine at concentrations in the mg/L - g/L range (Kohlert et al., 2012; Lynn et al., 2016) whereas other studies report similar effects already in the ng/L to μg/L range (Barry, 2013; Thoré et al., 2018b).

Overall, discrepancies in fluoxetine's effects are likely to be at least partly due to methodological limitations. These include a lack of reproducible standard tests accompanied by ecologically relevant, sensitive endpoints to pick up on specific biological effects (Sumpter et al., 2014; Thoré et al., 2018b). Traditional model organisms, such as zebrafish, are impractical for whole-life and multigenerational testing of contaminant effects due to their long life-span. Therefore, the majority of standard ecotoxicological tests only focuses on the organismal effects of acute exposure to pollutants while exposure over more ecologically relevant timescales is rarely assessed (Philippe et al., 2018b; Philippe et al., 2017). The annual killifish *Nothobranchius furzeri* has been introduced as an alternative fish model to conduct much-needed chronic studies in a time- and cost efficient way (Cellerino et al., 2015; Harel et al., 2015; Thoré et al., 2018a). The fish inhabits temporary freshwaters in south-eastern Africa and has evolved to mature extremely fast (<16 days) and to reproduce before its habitat dries, with an average life expectancy of around 6 months (Polačik et al., 2016). Killifish populations survive the dry period by producing offspring under the form of drought resistant eggs that remain dormant in the sediment until the onset of the next rainy season (Grégoir et al., 2018, 2017; Pinceel et al., 2015). Because of its interesting life-history, *N. furzeri* has been introduced as a complementary fish model for ecotoxicological testing of chemical compounds (Philippe et al., 2018; Philippe et al., 2018a; Philippe et al., 2019).

Recently, the potential of *N. furzeri* as a model organism for behavioral ecotoxicology has been tested and the usefulness of *N. furzeri* as a behavioral model to assess the impact of antidepressant exposure was demonstrated (Thoré et al., 2019, 2018b; 2018a). When exposed to the antidepressant fluoxetine, fish displayed higher anxiety towards a simulated predation threat, be it only at fluoxetine concentrations that were at least ten times higher than typical environmental concentrations (Thoré et al., 2018b). Although this study could be considered a first fundamental step towards standardized testing of emerging contaminants at an ecologically relevant timescale, many potential effects of fluoxetine exposure remain unexplored. It is, for instance, important to assess potential effects on fecundity and social dynamics given the high relevance of such behavior for fitness (Thoré et al., 2018a). As a crucial link between standardized laboratory testing and the exact ecological implications of pharmaceutical pollution, it is imperative to assess how fecundity and social dynamics are impacted by exposure to the compound. To date, the impact of environmentally-relevant exposure to fluoxetine on reproductive behavior of fish has rarely been studied (Martin et al., 2019a) and the potential of fluoxetine to disrupt reproductive success remains poorly understood (Bertram et al., 2018). Moreover, although chronic exposure studies have been conducted, these tests often still only cover exposure in a limited timeframe of the organism's lifetime, whereas lifelong exposure, starting immediately after hatching, has largely been ignored.

In the current study, we investigate the effect of lifelong exposure to 0.7 μg/L and 5.3 μg/L fluoxetine on body size, fecundity, fertilization success and social behavior in the killifish *N. furzeri*. Our individual-level repeated measures design allows us to assess within- and among-individual behavioral variation to calculate repeatability of variation in social behavior (i.e. the fraction of behavioral variation that is due to differences between individuals) (Bell and Sih, 2007; Thoré et al., 2018a). This is especially relevant since insights in within-species individual behavioral variation and the repeatability of behavioral variation as fundamental baseline data are often lacking in behavioral ecotoxicology. Therefore, reaching robust conclusions on the effects of chemicals on animal behavior is complicated (Harris et al., 2014; Tanoue et al., 2019) and compiling the behavioral baseline of studied traits is essential (Tanoue et al., 2019; Thoré et al., 2018a). We hypothesize that exposed fish will exhibit lower aggressiveness, higher sociability and an increased reproductive output. In line with this hypothesis, a reduced territoriality in male Siamese fighting fish (*Betta splendens*) was shown after a 6-day exposure to fluoxetine at 540 ng/L (Forsatkar et al., 2014). Also, a 30-day exposure to environmentally realistic levels of fluoxetine increased male copulatory behavior and total sperm count in eastern mosquitofish (*Gambusia holbrooki*)(Bertram et al., 2018). Likewise, Martin et al. (2019a) showed that male mosquitofish spent more time pursuing females and

were more likely to engage in copulation upon a 35-day fluoxetine exposure at environmentally realistic concentrations (Martin et al., 2019a).

2. Material and methods

2.1. Fish maintenance

For the purpose of the experiment, 126 fish were recruited from a *N. furzeri* laboratory strain that has been cultured in optimal common garden breeding conditions for three generations. This laboratory strain originates from a natural population in Mozambique (MZCS-222). At the onset of the experiment, eggs were hatched after the protocol of Polačik et al. (2016). Throughout the experiment, fish were kept under static conditions in aerated reconstituted water (Instant Ocean salt mix added to type III RO water, 7.8 pH, 600 μS/cm conductivity). Fish tanks were exposed to a 14 h light: 10 h dark photo-regime and at a constant water temperature of 24 °C. Two days post hatching, fish were transferred to glass 5L tanks (50 cm long x 20 cm wide x 17.5 cm high) in groups of seven fish. The water volume of the tanks was increased to 10 L at five weeks post hatching. At an age of eight weeks and for the remainder of the experiment, fish were transferred individually to 2L transparent glass jars to allow for individual monitoring. This setup still allowed for visual social contact between fish while limiting agonistic behavior to ensure animal welfare. During the first four weeks of the experiment, tanks were cleaned three times a week (every Monday, Wednesday and Friday) during which water in all tanks was renewed using reconstituted water (complete water renewal). After the first four weeks and for the remainder of the experiment, tanks were cleaned and water was renewed (complete water renewal) twice a week (every Monday and Friday). Water quality was monitored daily or every other day by measuring water parameters (7.8 pH, ammonium <0.2 mg/L, nitrate <25 mg/L, nitrite <0.2 mg/L). Juvenile fish were fed *ad libitum* with *Artemia franciscana* nauplii and *Chironomus* larvae twice a day. Starting four weeks post hatching, fish were fed *ad libitum* with *Chironomus* larvae once a day.

2.2. Experimental setup

Starting two days post hatching, fish were randomly assigned to one of three experimental conditions: a control condition and two conditions in which fish were chronically exposed to 0.7 μg/L or 5.3 μg/L fluoxetine hydrochloride (F-132; Sigma Aldrich), respectively, throughout the experiment and for a total of 14 weeks (Fig. 1). The low concentration of fluoxetine (0.7 μg/L) approximates the higher end of the range found in wastewater effluent (Dzieweczynski et al., 2016; Mole and Brooks, 2019; Puckowski et al., 2016) whereas the high concentration (5.3 μg/L) represents a pharmacologically relevant dose (Dzieweczynski et al., 2016). Control fish were exposed to an equal amount of dimethyl sulfoxide (DMSO, 0.00001%), the solvent used to prepare the fluoxetine stock solution (see Preparation of solutions section), as in the 5.3 μg/L fluoxetine condition. All treatments were applied during each water exchange.

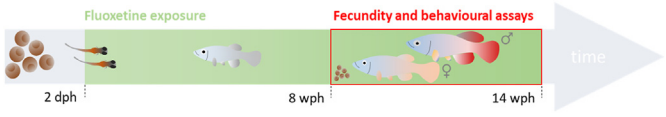

Fig. 1. Timeline of the experiment. dph = days post hatching; wph = weeks post hatching.

2.3. Behavioral setup

From eight weeks post hatching, fish were subjected to a fecundity test, a sociability test and an aggressiveness test (only males) once per week (Fig. 1). Fish were subjected to the fecundity test (each Wednesday) for a total of six repeated measures and to the sociability and aggressiveness test (each Thursday and Friday respectively) for a total of five repeated measures per individual. Per condition, 16 individuals of each sex were randomly selected to be used as experimental fish in the behavioral trials (i.e. total sample size of 96 fish). The remaining fish were assigned as non-experimental fish to be used in the sociability test as social conspecific group (see below).

All behavioral measures were top-view recorded using Logitech C920 HD Pro webcams. Fish behavioral data were collected for a maximum of five consecutive hours. Recordings were analyzed manually afterwards.

At the end of the experiment and upon completion of all behavioral tests (i.e. 14 weeks post hatching), body size (from the tip of the snout to the tip of the tail, dorsal view) of each fish was measured. To this end, each fish was transferred to a Petri dish with a small amount of water to prevent vertical movement. Top-view, size-calibrated photographs were taken and analyzed using the open source image processing software ImageJ v. 1.50i (Schneider et al., 2012).

2.4. Fecundity test

Per condition, fish were paired (16 pairs per condition) and allowed to spawn. Pairs were chosen so that a female was paired with the same male only once. At the onset of the fecundity test, each pair was transferred to a 1 L mating arena (18 cm long x 12 cm wide x 11.5 cm high) with a bottom layer of fine sand as spawning substrate. During a 5 min acclimation period, males and females were separated by means of an opaque plastic divider. At the onset of the trial, the divider was removed and fish were allowed to mate for 30 min. Afterwards, fish were transferred back to their respective housing jar and sand was sieved (mesh size of 1 mm) to count the number of deposited eggs. In addition, the fraction of fertilized eggs was assessed as a measure of fertilization effectiveness. Video recordings were analyzed to assess the time till first mating and the number of mating events.

2.5. Sociability test

To assess fish sociability, individual fish were transferred to a 7.5 L arena (50 cm long x 20 cm wide x 17.5 cm high) consisting of a large compartment separated by means of a transparent divider from a compartment with three non-experimental conspecifics of the same age (mixed sex). The focal individual was introduced in the large compartment and was allowed to visually interact with the conspecific group. After an acclimation period of 5 min, fish behavior was recorded for 20 min. The total time spent in proximity of the conspecific group (within 5 cm from the transparent divider, see Fig. 2A) was assessed as a proxy for sociability (cf. Cattelan et al., 2017).

2.6. Aggressiveness test

To assess fish aggressiveness, male individuals were transferred to a 7.5 L tank (50 cm long x 20 cm wide x 17.5 cm high), divided in two equally-sized, visually separated compartments by means of an opaque plastic divider. In each compartment, a mirror was attached to the side (see Fig. 2B). Using this setup, fish behavior could be recorded for two males simultaneously. In each compartment, a

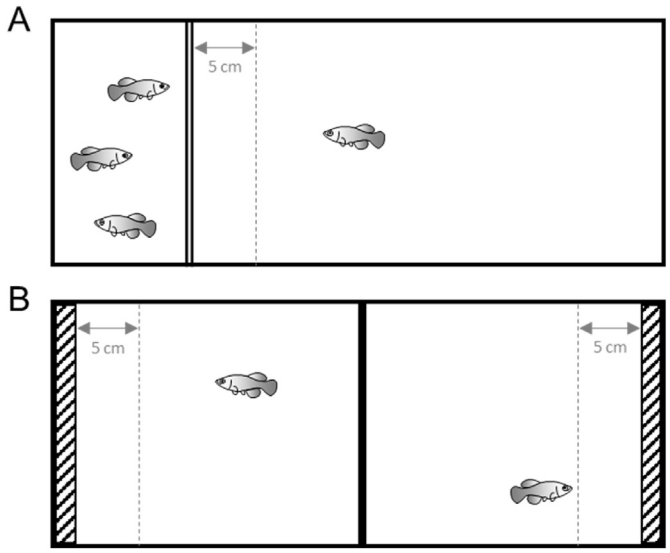

Fig. 2. Schematic representation of the test arenas. Test arena (top view) used for (A) the sociability test and (B) the aggressiveness test. All tanks are LxWxH 49 × 19 × 16 cm and hold 7.5L of water.

focal male was introduced and was allowed to visually interact with his mirror image. After an acclimation period of 5 min, fish behavior was recorded for 20 min. The time spent in proximity of the mirror (within 5 cm from the mirror, see Fig. 2B) was assessed as a proxy for fish aggressiveness (Ansai et al., 2016).

2.7. Preparation of solutions

Fluoxetine hydrochloride (Sigma F-132) 500 mg/L stock solution was prepared by dissolving fluoxetine hydrochloride in dimethyl sulfoxide (DMSO) and was stored as aliquots at −20 °C. Working solutions were prepared by diluting the stock solution to a concentration of 5 mg/L using reconstituted water at 600 μS/cm. This solution was added to the fish tanks each time the water was renewed (see above) to establish the target concentrations of the respective experimental conditions. Fluoxetine was previously shown to be relatively resistant to hydrolysis, photolysis and microbial degradation in aqueous solutions, with half-lives exceeding 100 days (Kwon and Armbrust, 2006). Water samples from three random jars per condition were collected once a week before renewal of the medium, starting from nine weeks post hatching for a total of four weeks. Fluoxetine concentrations were measured at the University of Ghent (Department of Crop Protection) by means of liquid chromatography (LC/MS/MS) with ESI (Waters ACQUITY UPLC, Xevo TQD mass spectrometer). Actual concentrations of fluoxetine in the low and high fluoxetine condition were 0.708 ± sd 0.0982 μg/L (n = 12) and 5.342 ± sd 1.490 μg/L (n = 12) respectively.

2.8. Statistical analyses

All statistical analyses were conducted in R 3.3.1 (R Development Core Team, 2016) at a significance level of alpha = 0.05. Model assumptions, including distributional fit and homogeneity of variances, were verified graphically for all analyses. A linear model with Gaussian error distribution was used to analyze body size. Condition (control, 0.7 μg/L, 5.3 μg/L) and sex (male, female) were added to the model as fixed factors, including their interaction. Effects of fluoxetine exposure on time spent in proximity of a social group as a measure for sociability, time spent in proximity of a mirror as a measure for aggressiveness and latency time to initiate mating were analyzed by means of linear mixed models with Gaussian error distribution using the lme4 package (Bates et al., 2017). Condition and body size were added as fixed factors, while fish identity and trial number (referring to the repeated measures) were added as random effects to these models. In addition, for sociability, sex and the interaction term between sex and condition were added as fixed factors. Latency time to initiate mating was log-transformed to improve the distributional fit. The number of deposited eggs and the number of mating events were analyzed using linear mixed models with a Poisson error distribution. A linear mixed model with binomial error distribution was used to analyze the fraction of fertilized eggs over the total egg count as measure for fertilization effectiveness in response to fluoxetine. Condition and body size were added as fixed factors, while fish identity and trial number were added as random effects. In addition, an observation-level random effect was modelled to accommodate overdispersion.

Significance of the fixed effects was tested using parametric bootstrapping with 1000 simulations using the afex package (Singmann et al., 2017) and post-hoc differences were assessed by means of Tukey-corrected pairwise comparisons using the lsmeans package (Lenth and Love, 2017).

To determine if individual variation in aggressiveness and sociability is repeatable, repeatability measures were calculated as the between-individual variance over the sum of between-individual and residual variance (Nakagawa and Schielzeth, 2010) using the rptR package (Stoffel et al., 2018). To test the statistical significance of the repeatability values, likelihood-ratio tests that compare the model with and without the fish identity random effect were performed in the rptR package.

3. Results

Adult body size differed significantly between conditions ($F = 27.617$, $\mathbf{p < 0.001}$) and sexes ($F = 512.300$, $\mathbf{p < 0.001}$) and body size differences between conditions did not depend on sex ($F = 658.000$, $p = 0.936$). Fish exposed to 5.3 μg/L were smaller as adults compared to control fish or fish exposed to 0.7 μg/L of fluoxetine (Fig. 3A). Males of the control, 0.7 μg/L and 5.3 μg/L condition had an average adult body size of 37.597 mm (±0.259 SE), 37.127 mm (±0.259 SE) and 35.887 mm (±0.259 SE), respectively. Females had an average adult body size of 33.109 mm (±0.237 SE), 32.511 mm (±0.237 SE) and 31.225 mm (±0.237 SE), respectively (Fig. 3A).

Females exposed to fluoxetine roughly doubled their reproductive output compared to control fish ($\chi^2 = 9.791$, $\mathbf{p = 0.014}$), with females from the control, 0.7 μg/L and 5.3 μg/L condition producing an average of 1.228 (±0.351 SE), 3.871 (±0.332 SE) and 4.810 (±0.349 SE) eggs per spawning session, respectively (Fig. 3B). The number of mating events per spawning session was doubled for fish exposed to 5.3 μg/L of fluoxetine compared to control fish ($\chi^2 = 6.503$, $\mathbf{p = 0.049}$), with an average of 1.186 (±0.370 SE), 2.859 (±0.353 SE) and 3.715 (±0.368 SE) mating events per spawning session for control, 0.7 μg/L and 5.3 μg/L fish, respectively (Fig. 4A). The number of produced eggs and the number of mating events were positively correlated with each other (Spearman rank correlation = 0.94, $\mathbf{p < 0.001}$). Although latency time to initiate mating decreased with increasing fluoxetine concentrations (Fig. 4B), this effect was not significant ($\chi^2 = 4.285$, $p = 0.133$). In addition, the percentage of fertilized eggs did not differ between conditions ($\chi^2 = 0.561$, $p = 0.804$), with a fertilization success of 49.7% (±0.248 SE), 45.6% (±0.211 SE) and 49.5% (±0.219 SE) for control, 0.7 μg/L and 5.3 μg/L fish, respectively. Female adult body size did not impact fecundity ($\chi^2 = 0.178$, $p = 0.694$), the number of mating events per spawning session ($\chi^2 = 0.120$, $p = 0.744$), the

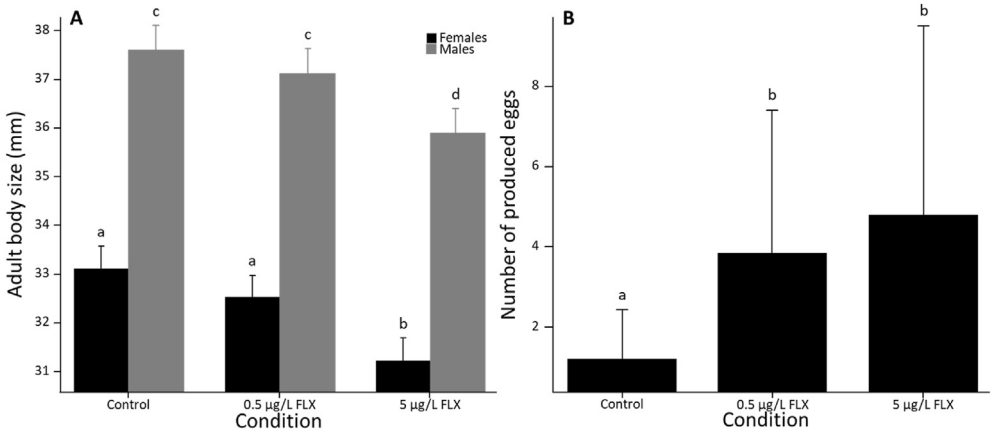

Fig. 3. Impact of fluoxetine-exposure on adult body size and fecundity. (A) Adult body size in relation to fluoxetine treatment for females (black) and males (grey). (B) Female fecundity (number of produced eggs) in relation to fluoxetine treatment. Whiskers delineate the upper and lower 95% confidence limit. Letters indicate significant differences based on Tukey-corrected post-hoc tests. FLX = fluoxetine.

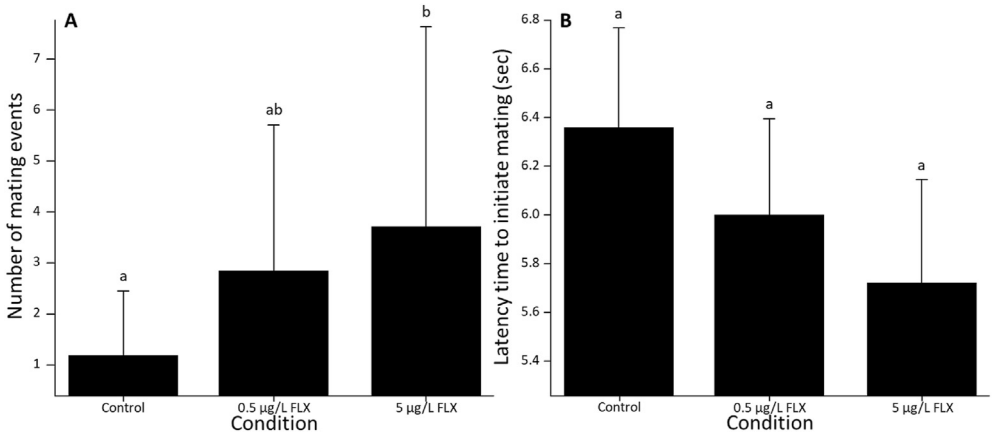

Fig. 4. Impact of fluoxetine-exposure on mating behavior. (A) Number of mating events in relation to fluoxetine treatment. (B) Latency time to initiate mating in relation to fluoxetine treatment (in seconds). Whiskers delineate the upper and lower 95% confidence limit. Letters indicate significant differences based on Tukey-corrected post-hoc tests. FLX = fluoxetine.

latency time to mate ($\chi^2 = 0.042$, $p = 0.824$) or the fertilization success ($\chi^2 = 0.060$, $p = 0.818$).

Across conditions, males interacted with the mirror for 58% of the time and spent 42% of the time in the rest of the tank. Male aggressiveness, measured as the time spent in proximity of a mirror (mirror test), did not differ between conditions ($\chi^2 = 1.324$, $p = 0.536$) (Fig. S1) and was independent of adult body size ($\chi^2 = 0.789$, $p = 0.376$). Individual aggressiveness was significantly repeatable with R = 0.360 (***p* < 0.001**).

Males that were exposed to 5.3 µg/L of fluoxetine spent more time in the proximity of conspecifics as a measure for fish sociability compared to unexposed fish, whereas such an effect did not emerge in females ($\chi^2 = 8.110$, ***p* = 0.022**)(Fig. 5). When exposed to 5.3 µg/L fluoxetine, males showed a 34% increase in time spent in the proximity of conspecifics (802.639 s ± 41.338 SE) compared to control males (597.962 s ± 45.016 SE). Males that were exposed to 0.7 µg/L fluoxetine spent an intermediate amount of time in proximity of conspecifics (676.287 s ± 43.752 SE). An overall effect of fluoxetine treatment on fish sociability was not found ($\chi^2 = 6.497$, $p = 0.063$). However, an overall difference in sociability between sexes emerged with males spending more time in proximity than females ($\chi^2 = 6.929$, ***p* = 0.015**). Individual sociability was significantly repeatable with R = 0.195 (***p* < 0.001**).

4. Discussion

The use of pharmaceuticals has vastly increased over the past decades. While continuous discharge of active compounds into natural ecosystems may trigger undesired behavioral changes in the fauna of these systems, such effects and associated risks remain poorly understood. With this study, we demonstrate that lifelong exposure to 5.3 µg/L of the antidepressant fluoxetine disturbs social behavior and that reproductive behavior in the killifish *N. furzeri* is already affected at concentrations as low as 0.7 µg/L. Furthermore, adult body size of fish was significantly reduced due to fluoxetine-exposure at 5.3 µg/L. These findings are of particular relevance given the increased prevalence and persistence of fluoxetine in surface waters and the strong fitness implications of the observed phenotypic changes.

4.1. Fluoxetine exposure induces reproductive behavior and fecundity

Exposure to fluoxetine enhanced the fecundity of the tested killifish. The number of eggs produced more than doubled for exposed compared to unexposed females, both at 0.7 µg/L and at 5.3 µg/L fluoxetine. Given that the fertilization success of eggs was

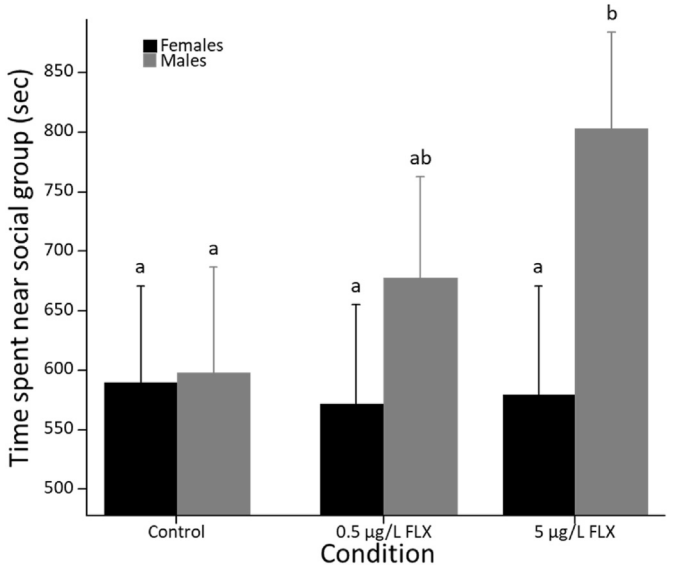

Fig. 5. Impact of fluoxetine-exposure on fish sociability. Time spent in proximity of a social group of conspecifics for females (black) and males (grey) in relation to fluoxetine treatment (in seconds). Whiskers delineate the upper and lower 95% confidence limit. Letters indicate significant differences based on Tukey-corrected post-hoc tests. FLX = fluoxetine.

equally high in exposed fish as in unexposed fish, this finding may have major fitness consequences since it implies a potential doubling of the next generation. To date, reports on potential effects of fluoxetine exposure on reproduction are highly incongruous. Likely, the sensitivity to the compound is species-specific due to, for instance, differences in the mode of reproduction among organisms (e.g. internal versus external fertilization) that have so far been tested (Bertram et al., 2018). However, differences in experimental results could also derive from differences in the adopted methodology. For instance, next to differences in water chemistry that may influence the bioavailability and uptake of compounds (e.g. pH (Martin et al., 2019b)), the exposure regime (e.g. dosage, duration) often varies and also the reproductive traits that were assessed (e.g. number of deposited eggs, parental care, mating intent) are rarely identical (Bertram et al., 2018; Sumpter et al., 2014). For instance, Lister et al. (2009) reported a 4.5 fold decrease in egg production of zebrafish when fish were exposed to 32 µg/L of fluoxetine over a 7-day period. In another short-term study, male Siamese fighting fish (*Betta splendens*) were exposed to a lower concentration of the compound (540 ng/L) for 6 days and were shown to exhibit a reduced territoriality with a suggested reduction in parental care and reproductive success (Forsatkar et al., 2014). A reduced fecundity was also shown in invertebrate species, such as in *Ceriodaphnia dubia* after exposure to 223 µg/L of fluoxetine for 7 days (Brooks et al., 2003b) and antidepressant exposure consistently impaired reproduction in mollusks (reviewed by Fong and Ford (2014). In contrast, Foran et al. (2004) exposed Japanese medaka (*Oryzias latipes*) to 0.1 µg/L, 0.5 µg/L, 1 µg/L and 5 µg/L of fluoxetine over a four week period and reported no significant impact on egg production, fertilization rate and spawning. However, consistent with our findings, other studies do report an increase in reproduction or reproductive behavior after continued exposure to fluoxetine. Bertram et al. (2018) exposed eastern mosquitofish (*Gambusia holbrooki*) to 40 ng/L and 400 ng/L of fluoxetine for 30 days and showed that the number of copulation attempts and total sperm count increased upon exposure. Likewise, male mosquitofish spent more time associating with females when fish were exposed for 35 days to 31 ng/L and 374 ng/L of fluoxetine (Martin et al., 2019a). A 28-day exposure to 350 ng/L of fluoxetine was also shown to increase male coercive 'sneak' copulations in guppies (Fursdon et al., 2019). Analogous to these findings, exposure for 10–14 days to SSRIs (selective serotonine reuptake inhibitors) including fluoxetine (10, 40, 80 µg/L) and fluvoxamine (3, 7, 30 µg/L), was shown to stimulate reproductive output of the invertebrate waterflea *Daphnia magna* (Campos et al., 2012; Rivetti et al., 2016).

Physiologically, altered reproductive output upon fluoxetine exposure in fish could be associated with a hormonal disruption. In teleost fish, reproduction is regulated by the hypothalamic-pituitary-gonadal (HPG) axis and exposure to SSRIs has been shown to disrupt the HPG axis (Mcdonald, 2017). Elevated concentrations of extracellular serotonin are known to interact with the HPG axis at all levels (reviewed by Mcdonald, 2017). This stimulates the release of gonadotropin releasing hormone from the hypothalamus (Mennigen et al., 2011) and the release of gonadotropins and luteinising hormone at the level of the pituitary (Bertram et al., 2018; Lorenzi et al., 2009; Mennigen et al., 2008). At the gonadal level, elevated levels of extracellular serotonin increase the gonadosomatic index in fish and facilitate oocyte maturation (Prasad et al., 2015) which could lead to increased egg production in female fish as observed in our study.

In addition to disrupting physiology (e.g. leading to alterations in gamete production), changes in social and sexual dynamics due to fluoxetine exposure could underpin changes in reproductive output (Martin et al., 2019a; Mcdonald, 2017). Our findings suggest that the observed fluoxetine-enhanced reproductive output could be partly mediated by changes in social and sexual behavior. Consistent with the findings of recent studies in which mosquitofish and guppies were chronically exposed to environmentally relevant levels of fluoxetine (Bertram et al., 2018; Fursdon et al., 2019; Martin et al., 2019a), the number of mating attempts in *N. furzeri* exposed to 5.3 µg/L of fluoxetine more than doubled and a trend for a decreased latency time to initiate mating behavior with increasing fluoxetine concentrations was observed. It should be noted that *Nothobranchius* killifish have a different mating strategy than mosquitofish or guppies. *Nothobranchius* killifish have evolved a polygynandrous mating system in which males and females mate with multiple partners to fertilize a single batch of fully developed eggs (Cellerino et al., 2015). Males compete for access to mates and actively explore the habitat searching for females (Cellerino et al., 2015; Thoré et al., 2019). When a male encounters a female, he coerces her into spawning to deposit a single egg (Cellerino et al., 2015; Haas, 1976). In this way, females typically deposit 20–50 eggs per day (Cellerino et al., 2015). While a higher number of mating attempts could be associated with a reduced fertilization success, this was not the case in our study.

According to our results, there was a trend for an increased sociability with increasing fluoxetine concentrations in *N. furzeri* males, with males that were exposed to 5.3 µg/L fluoxetine spending significantly more time in association with a group of conspecifics compared to unexposed males. While this might indicate an increased male mating intent, it could also indicate an increased aggressiveness. However, this explanation seems less likely given that no fluoxetine-enhanced aggressiveness could be confirmed with a mirror test in the current study. Also, Sebire et al. (2015), who exposed male stickleback (*Gasterosteus aculeatus*) to environmentally relevant concentrations of fluoxetine for 21 days, could not confirm any fluoxetine-induced effects on aggressiveness. Moreover, exposure to fluoxetine even resulted in a decrease in aggressiveness in Siamese fighting fish (*Betta splendens*) (Dzieweczynski and Hebert, 2012; Kohlert et al., 2012) and medaka (*Oryzias latipes*)(Ansai et al., 2016). Whether or not the observed change in male sociability in the current study is driven by changes

in non-reproductive sociability or rather reflects an altered reproductive motivation should be subject to further investigation (e.g. by using single-sex social groups as opposed to mixed-sex groups).

Furthermore, while male sociability was affected by fluoxetine exposure at 5.3 μg/L, an effect of fluoxetine exposure on female sociability did not emerge, suggesting that fluoxetine might impact male and female fish differently. Sex-specific effects of fluoxetine exposure on fish behavior have also been demonstrated in other studies. For instance, male fathead minnows were shown to invest more in nest building and defending their nest upon a 4-week exposure to 1 μg/L fluoxetine whereas mating behavior of females was largely unaffected by fluoxetine (Weinberger II and Klaper, 2014). Nevertheless - and alternatively to the previous explanations - the observed increase in number of spawning events upon 5.3 μg/L fluoxetine exposure might also be mediated by a higher female mating intent and receptiveness. Future research is needed to further elucidate the potential underlying behavioral mechanism of an increased number of spawning events and associated increase in reproductive output in *N. furzeri* upon fluoxetine exposure.

4.2. Fluoxetine exposure reduces adult body size

Fish of both sexes had a smaller adult body size after life-long exposure to fluoxetine compared to control fish in our study. Fluoxetine is known to have anorexigenic properties (Halford et al., 2005) and exposure to fluoxetine suppresses feeding behavior in a range of fish species (Mcdonald, 2017). Although not directly tested in this study, a smaller body size in fluoxetine-exposed fish could derive from a decreased food intake and 3-weeks of exposure to 5 μg/L fluoxetine was previously shown to inhibit feeding in *N. furzeri* individuals (Thoré et al., 2018b). In addition, Conners et al. (2009), for instance, found a reduced growth rate in African clawed frog (*Xenopus laevis*) tadpoles that was likely due to a reduced food intake upon fluoxetine exposure.

Despite the reduction in body size, exposed females had a higher reproductive output. A limited energy budget implies life-history trade-offs, in which energy-allocation to reproduction is offset by investment in somatic growth (Stearns, 1989; Thoré et al., 2019). Therefore, our findings suggest that fish exposed to fluoxetine shift their energy allocation towards reproduction (i.e. number of deposited eggs, mating intent and effort) rather than somatic growth and the decrease in body size upon fluoxetine exposure might not solely be due to impaired feeding behavior. To further elucidate this apparent shift in energy allocation, future research should complement life-history with behavioral and physiological analyses (including integrated data on food intake and locomotor activity) to gain more understanding of the mechanisms that underlie these observations.

Indirectly, the fitness consequences of a reduced body-size can be very large. In killifish, males establish a hierarchy based on male size to access females, with dominant males having a tendency to be larger than subordinate males (Cellerino et al., 2015; Polačik and Reichard, 2009). Therefore, male body size could be an important mediator of sexual selection through, for instance, male-male competition for mates, coercive copulation with females or female mate choice. Although Polačik and Reichard (2009) could not confirm female mate preference with respect to male dominance and male body size in terms of number of deposited eggs in *Nothobranchius korthausae,* the relationship between male dominance and body size with reproductive success in the wild remains poorly understood for *Nothobranchius* fish. Additional research is needed to assess the importance of male body size as a mediator of sexual selection. Fluoxetine-induced changes in body size can entail indirect fitness consequences through altered dominance hierarchy or male-male competition, through changes in the effectiveness of coercive mating and through female mate choice for larger males. The current experimental setup did not allow for male-male competition or mate choice since only pairs of one female and one male were formed to assess fecundity. While an increased reproductive output in response to fluoxetine exposure was observed in the current study, allowing fish to spawn in a social context that allows for more complex social interactions might yield differential effects of fluoxetine on reproductive output. Bertram et al. (2018), for instance, exposed eastern mosquitofish (*Gambusia holbrooki*) to environmentally relevant doses of fluoxetine for 30 days and found an increase in male copulatory behavior in the absence of male-male competition whereas no effect was detected when male-male competition was allowed.

Tightly controlled experimental setups allow researchers to gain more insight in the exact underlying mechanisms of enhanced reproductive success by gradually building towards more ecologically relevant setups that more closely approximate natural conditions. For instance, while an increased mating effort and elevated number of deposited eggs might have short-term population fitness consequences, such fluoxetine-induced effects may be negative in the longer term. Increased short-term reproductive success might lead to deprivation of nutrient resources and is likely to trade-off with survival due to energetic constraints (Stearns, 1989; Thoré et al., 2019). Moreover, increased mating intent and sexual harassment might entail fitness costs for both males and females. Pilastro et al. (2003), for instance, showed that foraging efficiency of female mosquitofish was reduced with over 50% by sexual harassment. Sexual conflict can also entail fitness costs for the males. Fluoxetine-exposed males, with a higher mating intent, might experience a reduced reproductive success if increased sexual harassment leads to females minimizing the cost of sexual conflict by associating with males with lower mating intent (Bertram et al., 2018; Pilastro et al., 2003). In addition, fluoxetine-modulated changes in sexual conflicts can alter the strength and direction of sexual selection by affecting the quality of the produced offspring, with potential consequences for population demographics (Martin et al., 2019a; Wong and Candolin, 2015). Therefore, to improve our understanding of the fitness-consequences of fluoxetine exposure, it is important to also consider other determinants of fitness including egg quality, hatching success and the overall offspring quality. Given that reproduction is energetically costly, an increase in offspring production could trade off with offspring viability or quality. Campos et al. (2012), for instance, showed that an increased offspring production and shorter maturation time in SSRI-exposed *Daphnia magna* was offset by a smaller size of the neonates. Lastly, even though fluoxetine-exposure might enhance reproduction and potentially enhance fitness, such effects are likely to be detrimental at the community and ecosystem level.

N. furzeri is highly cost- and time-efficient as a model to perform pharmaceutical exposure trials with a high ecological relevance and realism. In the current study, we show that lifelong exposure to fluoxetine can impact sociability and mating behavior of fish at 5.3 μg/L as well as impact reproductive output already at concentrations as low as 0.7 μg/L. It is worth noting, however, that fish in natural ecosystems could have a spatio-temporal mosaic of exposure throughout their lifetime and adding such patterns of exposure would add an interesting next level of ecological relevance to studies. In the current study, we also show that individual-level variation in aggressive and social behavior in *N. furzeri* reflects stable individual differences. This observation provides fundamental information on the baseline behavior of *N. furzeri*, as it is important in behavioral ecotoxicology to fully characterize the 'behavioral norm' of test organisms (Tanoue et al., 2019). Even though our understanding of the environmental impact of

fluoxetine is still very basic, the current study not only adds to the further establishment of *N. furzeri* as a novel model organism for behavioral ecotoxicology but it also provides a first important link between standardized laboratory testing of *N. furzeri* and the exact ecological implications of pharmaceutical pollution.

Animal welfare note

All procedures and methods are in accordance with the legal requirements for animal research in Belgium and were approved by the ethical committee of KU Leuven (file number: P070/2016). The condition and health of individual fish was checked multiple times a day by two researchers separately (E. S. J. Thoré and B. De Rijck). Animals were housed under optimal conditions and water quality was monitored daily or every other day by measuring water parameters (7.8 pH, ammonium <0.2 mg/L, nitrate <25 mg/L, nitrite <0.2 mg/L). To prevent and limit stress, disturbance and handling was kept to a minimum.

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CRediT authorship contribution statement

Eli S.J. Thoré: Conceptualization, Methodology, Formal analysis, Writing - original draft. **Charlotte Philippe:** Writing - original draft. **Luc Brendonck:** Supervision. **Tom Pinceel:** Writing - original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115068.

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