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Article

Context is Key: Social Environment Mediates the Impacts of a Psychoactive Pollutant on Shoaling Behavior in Fish

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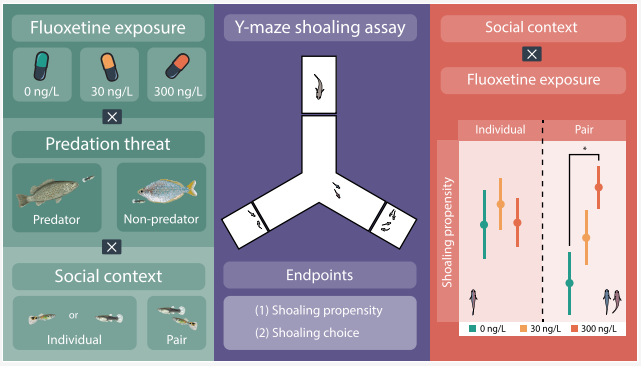
ABSTRACT: Behavior-modifying drugs, such as antidepressants, are increasingly being detected in waterways and aquatic wildlife around the globe. Typically, behavioral effects of these contaminants are assessed using animals tested in social isolation. However, for group-living species, effects seen in isolation may not reflect those occurring in realistic social settings. Furthermore, interactions between chemical pollution and other stressors, such as predation risk, are seldom considered. This is true even though animals in the wild are rarely, if ever, confronted by chemical pollution as a single stressor. Here, in a 2 year multigenerational experiment, we tested for effects of the antidepressant fluoxetine (measured concentrations [±SD]: 42.27 ± 36.14 and 359.06 ± 262.65 ng/L) on shoaling behavior in guppies (*Poecilia reticulata*) across different social contexts and under varying levels of perceived predation risk. Shoaling propensity and shoal choice (choice of groups with different densities) were assessed in a Y-maze under the presence of a predatory or nonpredatory heterospecific, with guppies tested individually and in male−female pairs. When tested individually, no effect of fluoxetine was seen on shoaling behavior. However, in paired trials, high-fluoxetine-exposed fish exhibited a significantly greater shoaling propensity. Hence, effects of fluoxetine were mediated by social context, highlighting the importance of this fundamental but rarely considered factor when evaluating impacts of environmental pollution.



KEYWORDS: *fluoxetine, pharmaceutical pollution, antidepressant, schooling, selective serotonin reuptake inhibitor*

1. INTRODUCTION

Pharmaceutical pollution is of mounting ecological concern, with pharmaceutical compounds having now been detected in ecosystems globally.[1−3] Many of these drugs are only partially metabolized after ingestion[4] and, after excretion, are often resistant to wastewater treatment processes.[5] As a consequence, pharmaceutical contaminants are frequently released into the environment via wastewater effluent discharge. Moreover, the threat posed by pharmaceutical pollution is rapidly escalating, with the production and diversification of pharmaceutical products currently growing at an unprecedented rate.[6]

One such group of pharmaceutical pollutants is antidepressants, which have been detected in aquatic environments worldwide.[7] One of the most common antidepressant contaminants is fluoxetine, with detected concentrations typically ranging between 0.2 and 373.8 ng/L in freshwater systems.[7] In addition to its extensive presence in aquatic ecosystems, fluoxetine is known to bioaccumulate in the tissues of wildlife (e.g., BFC in fish ranges from 2 to 500[8−14]). Through evolutionarily conserved molecular targets in aquatic organisms, such as fish, fluoxetine has the potential to alter a number of ecologically important traits,[15] with behavior being particularly sensitive.[16] Accumulating evidence suggests that fluoxetine exposure can disrupt a range of behaviors in nontarget species, including behaviors associated with reproduction,[17−19] antipredator responses,[20,21] activity,[22] aggression,[23] and anxiety,[24] and can produce sex-specific behavioral effects.[14,19,25] However, the majority of research to date investigating behavioral effects of exposure to fluoxetine—and environmental contaminants more generally—has measured the behavior of animals in social isolation, after acute exposure to a contaminant as a single stressor (discussed in ref 26−28). Additionally, studies have often overlooked potential differences in response between males and females and the complexity introduced by interactions between sexes, including through reproductively motivated

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behavior. Therefore, it remains unclear whether previously reported effects of fluoxetine on behavior are consistent when tested under more naturalistic settings.

Wild animals experience chemical pollution in complex and dynamic natural settings. For example, many aquatic species live in social groups and are therefore unlikely to experience stressors alone.[28] Indeed, it is well established that social interactions among members of a collective influence ecological traits, such as the behavior of individuals within a group.[29,30] Moreover, wildlife residing in pharmaceutical-contaminated ecosystems is rarely, if ever, confronted with pollution as the sole stressor. Instead, they are likely to be concurrently challenged by other natural and human-induced stressors (e.g., predation risk and temperature shifts[31,32]). The presence of multiple stressors could cause additive or antagonistic effects, making the realized ecological consequences of exposure difficult to predict from single-stressor studies alone.[33] For example, Thoré et al.[34] reported that in turquoise killifish (*Nothobranchius furzeri*), the impacts of fluoxetine exposure on activity levels and feeding were mediated by concurrent exposure to a pesticide. Given recent evidence that social context and additional stressors may mediate the effects of exposure to environmental pollutants, it is important that we measure how pollutants affect ecologically important traits such as animal behavior in more environmentally complex scenarios.

Here, we used a 2 year multigenerational experiment to investigate the effects of environmentally realistic fluoxetine exposure on the shoaling behavior of a freshwater fish, the guppy (*Poecilia reticulata*), using both males and females under varying levels of predation risk and in two different social contexts. Shoaling in fish is a widespread phenomenon and is critical to individual and collective fitness,[35,36] including in guppies.[37] Group formation can incur both costs and benefits to individual group members. For example, shoaling may not only increase disease transmission and competition for resources[38,39] but also confer a number of potential benefits to members of the collective, particularly in regard to predator avoidance.[37,40] Indeed, social behavior and antipredator behavior are closely linked in many social-living species but are, again, seldom considered together when addressing the impacts of human-mediated disturbance, such as chemical pollution.

We employed a 3 × 2 × 2 factorial design to investigate the effects of environmentally relevant fluoxetine exposure (nominal concentrations: 0, 30, and 300 ng/L) on the shoaling behavior of male and female guppies tested at two levels of perceived predation risk (predator presence or absence) and in two different social contexts (individually or in male−female pairs). More specifically, we had two primary aims: (1) to identify whether potential effects of fluoxetine on shoaling behavior were mediated by predator treatment and (2) to uncover whether potential effects of fluoxetine were influenced by the social context in which fish were tested. We predicted that fluoxetine exposure would decrease shoaling behavior and that this effect would be more pronounced when the perceived risk of predation was heightened (i.e., when the predator was present). This is because fluoxetine has been shown to reduce antipredator behavior and anxiety-related behavior in fish,[14,21,24,25,41] and thus, fluoxetine may reduce the tendency for fish to shoal. Regarding the effects of fluoxetine across the two social contexts, we had two alternative predictions. First, the paired context could result in less shoaling and less-pronounced effects of fluoxetine because, in pairs, the perceived risk of predation on either one of the individuals may be reduced (i.e., dilution effect[42]). Second, as fluoxetine exposure has consistently been shown to increase male reproductive behavior,[12,13,19,43] females may increase shoaling in an attempt to avoid male harassment.[44−46] Furthermore, if females increase shoaling, males are likely to follow them and thereby also show higher shoaling propensity.

2. MATERIALS AND METHODS

2.1. Mesocosm System and Fluoxetine Exposure. Male and female guppies were sourced from 12 mesocosm populations, which were subjected to one of three exposure treatments over 27 months, representing approximately 2−4 overlapping generations.[47] This system was designed to replicate ecologically realistic conditions for the guppy populations, comprising multiple overlapping and interacting generations, which is reflective of populations in nature.[48] Four mesocosm populations were allocated to each of the following treatments: unexposed (0 ng/L), low fluoxetine (nominal concentration: 30 ng/L), or high fluoxetine (nominal concentration: 300 ng/L). The low treatment represented fluoxetine concentrations detected in aquatic ecosystems worldwide (0.4−49.2 ng/L; 1−99th percentile in freshwater[7]), while the high treatment represented levels detected in direct effluents and the highest levels detected in effluent-dominated aquatic ecosystems (2.4−689.4 ng/L; 1−99th percentile in effluents[7]).

The long-term mesocosm system and fluoxetine exposure protocol are described in detail in ref 49. Briefly, each of the 12 mesocosm populations was founded with 300 fish of equal sex ratio, from Alligator Creek, Townsville, Australia. Each population was held in a stainless-steel tank (180 × 60 × 60 cm, length × width × height; water depth: 30 cm) with aquatic plants (*Taxiphyllum barbieri*). All tanks were measured weekly for pH (mean = 7.47, SD = 0.67, and n = 1324) and temperature (mean = 23.4, SD = 1.19, and n = 1315). Fish were fed to satiation with commercial food pellets every second day (Aquasonic Nutra Xtreme C1 pellets, 0.8 mm). Tanks were dosed with fluoxetine twice weekly, and partial water changes (20%) were conducted every week. Once per month, water samples were collected for analytical verification of fluoxetine levels. Water analysis was performed using gas chromatography−tandem mass spectrometry (7000C Triple Quadrupole GC−MS/MS, Agilent Technologies, Delaware, USA; minimum detection limit: 2 ng/L) following protocols described in ref 17 and was conducted by Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025). The mean exposure concentrations for the low and high fluoxetine treatments were 42.27 ng/L (SD = 36.14 and n = 96) and 359.06 ng/L (SD = 262.65 and n = 96), respectively. All control tank samples indicated no contamination with fluoxetine (n = 49; limit of quantification: 2 ng/L).

On the morning of behavioral experiments, focal guppies were transferred from the mesocosm system to individual housing tanks (25 × 15 × 15 cm; water depth: 10 cm). Individual housing tanks were filled with water from their respective mesocosm tanks to ensure that the exposure treatments were maintained throughout the experimental period (3 days per fish).

2.2. Stimulus Predator and Nonpredator. Spangled perch (*Leiopotherapon unicolor*) and eastern rainbowfish

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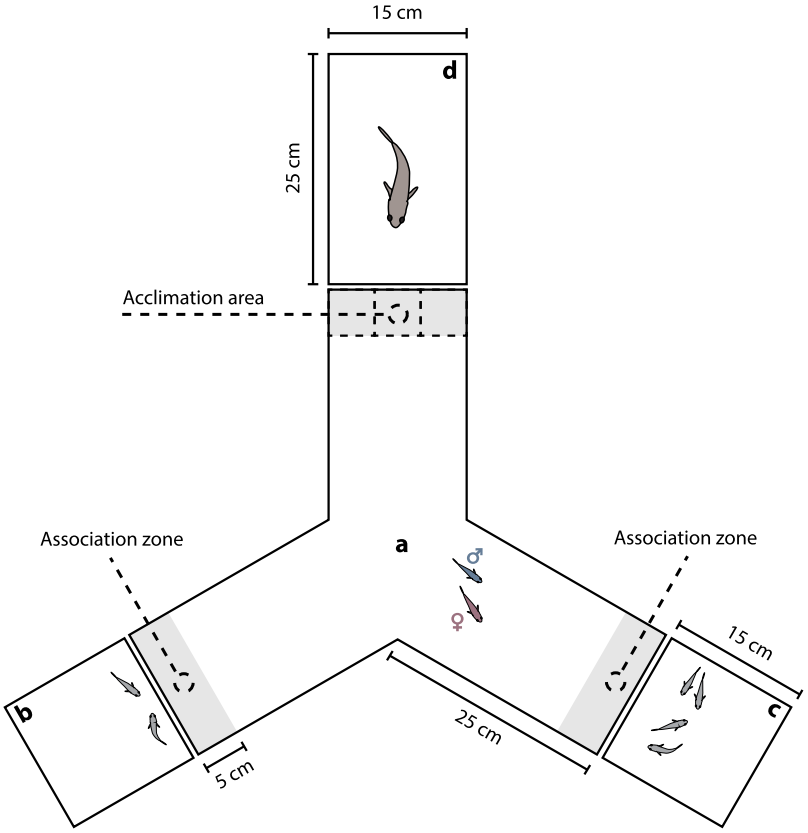

Figure 1. Shoaling assay, consisting of four primary components: (a) the central Y-maze, (b,c) two shoal compartments, and (d) a heterospecific compartment. Each Y-maze also had an acclimation area, where the focal individual or a male−female pair was placed at the start of the trial.

(*Melanotaenia splendida*) were used as a stimulus predator and nonpredator, respectively. These species were selected as their distribution overlaps with the source population of guppies used in this experiment,[50] and previous experiments have confirmed that guppies from this source population are able to distinguish between the different levels of threat posed by a predatory perch and a nonpredatory rainbowfish.[19,49] Spangled perch (mean standard length ± SD: 128.33 ± 15.06 mm; n = 6) and eastern rainbowfish (89.58 ± 16.16 mm; n = 6) were wild-caught individuals purchased from a commercial supplier (Aquagreen, Darwin). Spangled perch and eastern rainbowfish were acclimated to laboratory conditions for 5 weeks prior to the behavioral experiments (see supplementary methods for details on housing protocols).

2.3. Behavioral Experiments. Focal guppies were used in a behavioral experiment to investigate shoaling propensity and shoal choice. This was carried out by measuring the total time the focal guppies spent shoaling (i.e., shoaling propensity) and their choice to associate with conspecific groups of different sizes (i.e., shoal choice). This occurred in the presence of either a predatory (spangled perch) or nonpredatory (rainbowfish) heterospecific. Furthermore, we tested the shoaling behavior of the focal guppies twice, once individually and once in a male−female pair using a balanced repeated measures design. That is, half of the focal guppies completed the individual trial first, and the other half completed the paired trial first, with a 24 h break between each trial. Across the social contexts, the focal fish were always exposed to the same predator treatment. Guppies were isolated within their mesocosm tanks for 2 weeks prior to experiments. Food was withheld for 24 h prior to the experiments.

Experiments were conducted in freshwater, using a Y-maze arena, adapted from previously established protocols.[36] Each arm of the Y-maze (25 × 15 × 15 cm) had one transparent acrylic wall to act as a viewing window, while the other walls were opaque white (Figure 1). During the trial, the focal fish were simultaneously presented with three different stimulus types: a group of two conspecifics (one male and one female), a group of four conspecifics (two males and two females), and a predatory or nonpredatory heterospecific. Conspecifics were unfamiliar to the focal fish (i.e., from different mesocosm populations) but were always from the same exposure treatment (control, low fluoxetine, or high fluoxetine). This was achieved by placing separate compartments—housing each of the different stimulus types—against a transparent window at the end of each arm (Figure 1), allowing the focal guppy/guppies visual access to the three different stimuli (but not physical or chemical interaction). The compartments containing the large and small stimulus shoals were randomized in each trial to control potential for side bias. Furthermore, each Y-maze had an acclimation area (Figure 1) at the end of one of the arms, in which the focal guppy or guppies were introduced and confined at the start of the trial. Importantly, the predatory or nonpredatory heterospecific was always placed at the end of the maze arm with the acclimation area such that the focal guppy had visual access to the heterospecific at the start of the experiment. Prior to behavioral recording, the focal guppy/guppies were confined to the acclimation area for 5 min. After 5 min, the gate to the acclimation area was opened remotely, and the focal fish was left to freely explore the Y-maze over a 20 min trial.

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All trials were filmed from above (Panasonic HC-V180), and behavior was analyzed blind to treatment from video footage, using the key-logging software BORIS (version 7.7.3[51]). More specifically, the time spent associating with the small and large conspecific group was scored as the time spent within 5 cm (i.e., less than two body lengths) of each shoal (Figure 1). In addition, the time spent in the "neutral zone" between these two shoaling zones and the initial choice of shoal zone (i.e., the first shoal with which the focal fish associated) were recorded. The choice of initial shoal was not measured as an endpoint in and of itself but was instead measured for use in statistical models to account for the order in which focal fish visited each stimulus group. In addition, in paired trials, we recorded the male and female pair association time using the time spent within two body lengths (5 cm) of each other. After the conclusion of both behavioral trials, morphometric measurements were completed for all focal and stimulus fish (i.e., focal and shoal guppies, perch, and rainbowfish). The weight and length of all fish were recorded with digital callipers (±0.01 mm) and a digital scale (±0.0001 g).

2.4. Statistical Analysis. Data analyses were performed using R version 4.0.0.[52] Where necessary, data were transformed to approximate a Gaussian distribution (see Tables S1−S6 for descriptions). Across all models, continuous covariates were mean-centered to improve the interpretability of main effects. Interaction terms that did not improve model fit—as assessed using the Akaike information criterion (AIC)—were removed. For all models, type-III Wald's *F*-tests with Kenward-Roger degrees of freedom approximation were used to calculate *p*-values of fixed effects. Where a significant main effect of exposure treatment was detected, Tukey's honestly significant difference tests were used to investigate pairwise comparisons.

We first constructed a linear mixed effect model (LME: *lmer* function and *lme4* package[53]) for shoaling propensity (i.e., the total time within the association zone of either stimulus shoal) using the full factorial design, which included a total of 215 fish (*n*: unexposed = 72, low = 73, and high = 70; see Table S7 for detailed sample size summary). This model included exposure treatment, social context, predator treatment, trial order, fish sex, and their interaction terms, as fixed effects. In addition, the fish identity was included as a random intercept to account for repeated measures across social context. This model revealed a significant two-way interaction between social context and exposure treatment (see the Results for details). Therefore, to aid the interpretation of results, to simplify the random structure of models, and to focus on the comparisons of interest, effects of exposure treatment and predator treatment were assessed in separate models for each social context. This was carried out using analyses of covariance (ANCOVAs) and LME models for the individual and paired trials, respectively (see Tables S2−S6 for details). For the trials in which fish were tested in pairs, pair ID was included as a random intercept.

Second, to investigate the effects of fluoxetine and predator treatment on shoal choice (i.e., the choice of a large or small conspecific shoal), we calculated the proportion of time fish spent associating with the larger group over the smaller group (i.e., time with large shoal ÷ [time with large shoal + time with small shoal]). To ensure that fish were aware of the presence of both conspecific groups—and therefore were able to make an active choice—only fish that associated with both conspecific groups were included in the analysis. For individual trials, 151 fish entered both zones (*n*: unexposed = 46, low fluoxetine = 56, and high fluoxetine = 49), and for paired trials, 177 entered both zones (*n*: unexposed = 54, low fluoxetine = 58, and high fluoxetine = 65). For both trial types, models included the following predictors: exposure treatment, predator treatment, visit order, and fish sex, as well as their interaction terms (see Tables S5 and S6 for details). For the paired trials, pair ID was included as a random intercept.

Finally, to investigate the potential effects of exposure treatment and predator treatment on male and female pair association time (time within two body lengths of each other), we used linear regression, with exposure treatment, predator treatment, and their interaction term as predictors.

3. RESULTS

The full factorial model indicated a significant two-way interaction between exposure treatment and social context on shoaling propensity ($F_{2,205}$ = 3.40 and p = 0.035; Table S1). This indicates that the effects of fluoxetine exposure were mediated by social context (Figure 2). As a result of this interaction, the data were split by the social trial type (i.e., individual trials or male−female paired trials) to further investigate the effects of exposure and predator treatment on shoaling behavior.

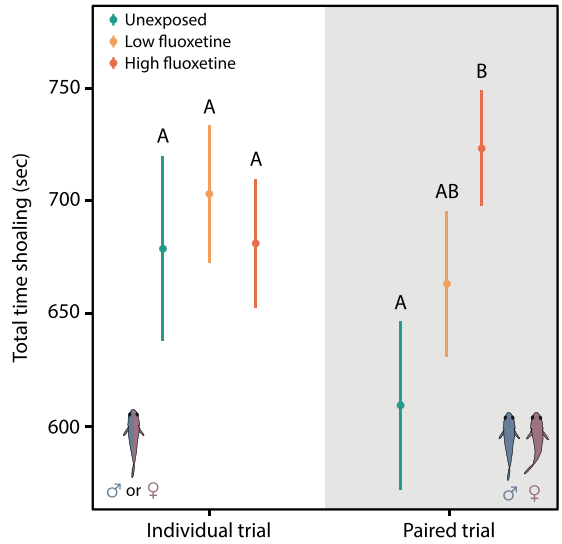

Figure 2. Mean (±SE) total time shoaling (i.e., time shoaling with the large or small group of conspecifics) for fish from the unexposed (green, *n* = 72), low-fluoxetine (yellow, *n* = 73), and high-fluoxetine (orange, *n* = 70) treatments, plotted across individual (white shaded area) and paired trials (gray shaded area). Statistical comparisons are made within each social context, not across social contexts. Groups that share a capital letter are not significantly different from one another.

For individual trials, there was no significant effect of exposure treatment on shoaling propensity ($F_{2,200}$ = 0.97 and p = 0.380, Figure 2) nor was there a significant interaction between fluoxetine exposure and predator treatment ($F_{1,200}$ = 2.00 and p = 0.130). There was a significant two-way interaction between predator treatment and fish sex ($F_{1,200}$ = 10.00 and p < 0.001, Figure S1) and an overall effect of predator treatment ($F_{1,200}$ = 23.00 and p < 0.001). For details on sex-specific responses to predator treatment, see Supporting Information (supplementary results). Regarding shoal choice in individual trials, fish preferentially associated with the larger

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Environ. Sci. Technol. 2021, 55, 13024−13032

group of conspecifics over the smaller group (t = 6.13, df = 150, and p < 0.001). The strength of this preference (i.e., the relative proportion of time fish spent with the larger group) was not influenced by exposure treatment ($F_{2,140}$ = 0.57 and p = 0.570, Figure S2) or predator treatment ($F_{1,140}$ = 3.10 and p = 0.080).

For paired trials, there was a significant effect of exposure treatment on the total time spent shoaling (LME: $F_{2,110}$ = 3.40 and p = 0.038). Specifically, there was a significant increase in the shoaling propensity of high-fluoxetine-exposed fish compared to unexposed fish (Tukey's HSD: t = 2.59 and p = 0.029, Figure 2). There was no significant difference between the low-fluoxetine and unexposed groups or the low-fluoxetine and high-fluoxetine groups (t = 1.12 and p = 0.504 and t = −1.49 and p = 0.302, respectively; Figure 2). In addition, there was a significant effect of predator treatment ($F_{1,110}$ = 19.00 and p < 0.001), with fish spending significantly more time shoaling in trials with predators. There were no significant interactions between any experimental treatments for the paired trials (see Table S4). In regard to the shoal choice in paired trials, we detected a preference for fish to associate with the larger group of conspecifics over the smaller group (paired t-test; t = 2.31, df = 176, and p = 0.022). The strength of the preference to associate with the larger group was not influenced by exposure or predator treatment (Figure S4; Table S5).

The time each fish pair spent within two body lengths of each other was not affected by exposure treatment (ANCOVA: $F_{2,96}$ = 1.49 and p = 0.230, Figure S5) but was significantly affected by the predator treatment (ANCOVA: $F_{1,96}$ = 7.30 and p = 0.008). Pairs in the presence of a predator spent more time within two body lengths than those in the nonpredator treatment (Figure S6).

Finally, in an effort to understand what was potentially driving this difference in response between individual and paired trials, a post hoc model was constructed to investigate how within-individual shoaling behavior of males and females changed across the two social contexts (i.e., individual and paired). Specifically, a linear model was used, which included the total shoaling time in individual trials as the dependent variable and the total shoaling time in the pair trial, fish sex, exposure treatment, and their interaction terms as predictors (Table S9). This post hoc model indicated that there was a statistically significant three-way interaction between the shoaling time in the pair trial, fish sex, and exposure treatment ($F_{2,160}$ = 5.00 and p = 0.007). This suggests that the relationship between behavior in the individual and paired trial was dependent on the fluoxetine exposure treatment and sex of the fish. To identify sex and exposure-specific effects, this model was centered at each sex and treatment, and the relationship between the total shoaling time in individual trials and the total shoaling time in paired trials was interpreted (essentially, correlations within each sex and treatment group; see Table S10). A Bonferroni correction was applied to the p-values to adjust for the total number of comparisons being made. We found that, for females, there was a significant positive relationship between the total shoaling time in individual trials and the total shoaling time in paired trials for control and low-fluoxetine-exposed females (t = 3.984 and p < 0.001 and t = 3.342 and p = 0.006, respectively, Figure S3), while there was no significant relationship in high-fluoxetine-exposed females (t = 0.484 and p = 0.999, Figure S3). For males, on the other hand, we saw the opposite effect of exposure treatment. Specifically, for control and low-fluoxetine males, there was no significant relationship between the total shoaling time in individual trials and the total shoaling time in paired trials (t = 1.906 and p = 0.384 and t = −0.548 and p = 0.999, respectively; Figure S3), whereas for high-fluoxetine males, there was a significant positive relationship (t = 2.777 and p = 0.036; Figure S3).

4. DISCUSSION

Here, we report evidence that long-term exposure to field-detected concentrations of the globally pervasive pharmaceutical pollutant fluoxetine alters shoaling propensity in fish. However, these impacts were contingent on social context, with effects of fluoxetine detected in paired trials (i.e., male and female pairs) but not in individual trials.

In individual trials, there was no interaction between fluoxetine and predator treatment. In addition, there was no main effect of fluoxetine exposure on the shoaling propensity or shoal choice of fish. This result is consistent with work addressing the effects of environmentally realistic SSRI exposure (i.e., <1000 ng/L) on individual shoaling propensity and conspecific association preference in other fish species (*Betta splendens*;[54] *Neogobius melanostomus*;[55] *Gambusia holbrooki*;[14,56] and *N. furzeri*).[57] Together, these studies suggest that environmentally realistic concentrations of fluoxetine are not sufficient to alter the shoaling propensity of fish when tested in isolation (i.e., tested individually with conspecifics confined behind a barrier).

In paired trials, predatory threat did not modulate the effects of fluoxetine. However, in contrast to individual trials, there was a significant effect of fluoxetine exposure on shoaling behavior. Specifically, fish exposed to high levels of fluoxetine in the male−female paired trials spent a significantly longer time associating with stimulus shoals than did unexposed fish. We suggest that the change in shoaling propensity—seen only in the male−female paired trials—is driven by females attempting to avoid male mating harassment.[45,46] The mating system of guppies is dominated by males incessantly attempting to mate with females, even when under direct threat of predation.[58] Indeed, it has been estimated that females are subject to as much as one mating attempt per minute in the wild.[59] Work on other poeciliid fishes has shown that females being pursued by males engage in shoaling behavior to reduce targeted male mating attempts.[60,61] Importantly, environmentally realistic fluoxetine exposure has previously been shown to increase the rate of mating behavior in fish.[12,18,19,43] For example, Fursdon et al.[19] and Wiles et al.[18] report that fluoxetine exposure causes male guppies to increase their rate of coercive sneak copulations. Hence, a fluoxetine-induced increase in male sexual behaviors could explain why we saw an increase in the amount of time spent shoaling in the high-exposed fish, as female guppies may have sought to dilute the effects of male harassment. Indeed, in the present study, females from the unexposed and low-fluoxetine treatments demonstrated a consistent shoaling propensity across the individual and paired trials, although this was not the case for high-fluoxetine-exposed females. Regardless of their shoaling propensity when tested alone, high-fluoxetine-exposed females had higher shoaling propensity in paired trials. We hypothesize that this shift could be the result of females modifying their behavior in response to harassment. Interestingly, for males, we also detected a shift in the relationship between behavior in individual and paired trials at the high fluoxetine exposure

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Environ. Sci. Technol. 2021, 55, 13024−13032

treatment. For males, the effect was in the opposite direction, with the shoaling behavior of males in the unexposed and low-fluoxetine treatment being less consistent across social contexts, while males in the high-fluoxetine treatment became more consistent. It is not clear why the behavior of males in the high-fluoxetine treatment would become more consistent across the two social contexts, while females become less consistent, but it is clear that there is a shift in the shoaling motivation in both sexes. Within the current experiment, we did not have sufficient video resolution to quantify the number of reproductive attempts performed by males toward females, so we could not directly measure the level of harassment. However, it is important to highlight that increased reproductive behavior has been measured previously in fluoxetine-exposed fish,[17,19,43] including those sourced from the same mesocosm exposure populations.[18]

An increase in shoaling propensity (as seen in high-fluoxetine-exposed pairs) could have direct implications at individual and population levels. For example, in females, increased shoaling behavior to avoid male harassment can reduce foraging efficiency by up to 25%.[62,63] More generally, shoaling behavior in social species such as guppies plays an important role in many aspects of behavior, including monitoring conspecifics,[64,65] increasing accurate decision making,[36,66] increasing vigilance toward predators,[36] and allowing information transfer between individuals within groups.[67] As such, altered shoaling behavior can result in adverse changes to predation, foraging efficiency, and reproductive output.[61,68] However, it is important to highlight that the impacts seen here were at the higher dosage (359 ng/L) of fluoxetine, which represents direct effluent concentrations and the highest levels detected in surface waters, thus representing a worst-case exposure scenario for wildlife.

In individual and paired trials, fish spent significantly more time shoaling in the presence of the predatory fish (spangled perch) than the nonpredatory fish (rainbowfish). This suggests that the predatory fish was perceived as a threatening stimulus and is consistent with previous work using these two species as predatory stimulus.[19,49] Furthermore, in both individual and paired trials, the impacts of fluoxetine on the total time spent shoaling were not significantly influenced by the presence of the predatory fish, that is, the effects of fluoxetine were independent of perceived predatory threat. Fluoxetine exposure has previously been shown to reduce antipredator behavior and anxiety-related behavior in fish (e.g., ref 14 20 21 24 25, and 41). Therefore, we originally hypothesized that in the presence of a predator, fish exposed to fluoxetine would have reduced propensity to shoal relative to unexposed fish. However, there is emerging evidence to suggest that the direction and magnitude of fluoxetine-induced behavioral changes—particularly in regard to anxiety-related behavior—vary depending on the duration and dosage of exposure.[20] As a result, direct comparison across dosage ranges and dosage durations is difficult, particularly in the case of the present study, as we employed a multigenerational exposure (2−4 overlapping generations[47]). The present study presents tentative evidence that multigenerational exposure to environmentally realistic levels of fluoxetine does not significantly alter antipredator behavior. We hypothesize that offspring raised in fluoxetine-contaminated environments, as in our system, are adapting (plastically and/or genetically) to fluoxetine-induced changes in serotonin neurotransmission (i.e., other aspects of their serotonergic systems have also changed). Indeed, in mice, serotonin transport molecule knockouts (i.e., individuals without the molecular target that is blocked by fluoxetine) show associated developmental changes in their neurons, brain, and hypothalamic−pituitary−adrenal axis, including desensitization of serotonin receptors.[69,70] To our knowledge, of the studies investigating behavioral alterations in fish at environmentally realistic concentrations, only one other study has employed mutigenerational exposure (i.e., exposed parents and offspring throughout ontogeny[34]), while two have used transgenerational exposures (i.e., exposed parents but not offspring).[20,71] Of the two studies employing a transgenerational exposure, Vera-Chang et al.[71] did not report a significant change in anxiety-related behavior (*Danio rerio*, 6 day postfertilization parental exposure at 540 ng/L), whereas Al Shuraiqi et al.[20] did report transgenerational effects on anxiety-related behavior at environmentally realistic levels (*D. rerio*, 28 day parental exposure at 100 ng/L). Thoré et al.,[34] who used a mutigenerational exposure (500 ng/L), reported that fluoxetine did not affect anxiety-related behavior in the first generation but did in the second generation. The results of the present study appear to conflict with reports of Thoré et al.[34] and, to some degree, Al Shuraiqi et al.[20] It is possible that within the present study, exposure over multiple generations (2−4) has resulted in genetic adaptation or a plastic response to fluoxetine induced serotonin perturbation (although the relative contribution of the two mechanisms is unknown). It is clear from the abovementioned studies, in combination with the present study, that the mutigenerational impacts of fluoxetine on the serotonergic system warrant further investigation.

In summary, we contend that the increase in shoaling propensity of high-fluoxetine-exposed male−female pairs is likely driven by increased shoaling by females, in an attempt to avoid male harassment, consequently increasing male shoaling. Changes in shoaling behavior, such as those seen here, could have important implications for individual- and population-level fitness, as changes to predation, foraging efficiency, and/or reproductive output could occur if shoaling behavior is pushed away from its selected mean.[61,68] More broadly, these results would not have been detected if the effects of fluoxetine were assessed on individuals only, highlighting the importance of considering the social context when investigating the effects of environmental contaminants.

5. DATA ACCESSIBILITY

All data and the associated statistical *R* script are available from the Open Science Framework repository: https://osf.io/gtyxd/.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c04084.

Supplementary methods (detailing stimulus predator and nonpredator housing and exposure protocols), supplementary results, supplementary tables, and supplementary figures (PDF)

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