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Long-Term Pharmaceutical Contamination and Temperature Stress Disrupt Fish Behavior

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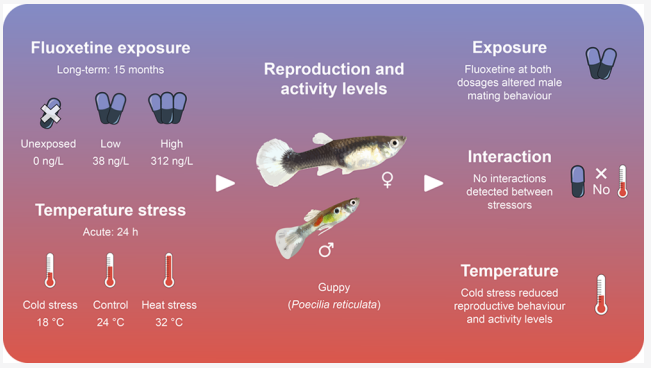
ABSTRACT: Natural environments are subject to a range of anthropogenic stressors, with pharmaceutical pollution being among the fastest-growing agents of global change. However, despite wild animals living in complex multi-stressor environments, interactions between pharmaceutical exposure and other stressors remain poorly understood. Accordingly, we investigated effects of long-term exposure to the pervasive pharmaceutical contaminant fluoxetine (Prozac) and acute temperature stress on reproductive behaviors and activity levels in the guppy (*Poecilia reticulata*). Fish were exposed to environmentally realistic fluoxetine concentrations (measured average: 38 or 312 ng/L) or a solvent control for 15 months using a mesocosm system. Additionally, fish were subjected to one of three acute (24 h) temperature treatments: cold stress (18 °C), heat stress (32 °C), or a control (24 °C). We found no evidence for interactive effects of fluoxetine exposure and temperature stress on guppy behavior. However, both stressors had independent impacts. Fluoxetine exposure resulted in increased male coercive copulatory behavior, while fish activity levels were unaffected. Under cold-temperature stress, both sexes were less active and males exhibited less frequent reproductive behaviors. Our results demonstrate that long-term exposure to a common pharmaceutical pollutant and acute temperature stress alter fundamental fitness-related behaviors in fish, potentially shifting population dynamics in contaminated ecosystems.



INTRODUCTION

The contamination of aquatic habitats by pharmaceuticals is a major environmental problem, evoking concern among scientists, health officials, and communities around the globe.[1−3] Most pharmaceuticals are incompletely metabolized when consumed, and their metabolites can remain biologically active when excreted.[4,5] Wastewater treatment plants (WWTPs) are typically not designed to remove pharmaceutical compounds from sewage,[6] and discharge of wastewater effluent into the environment is, therefore, a primary source of contamination.[7] Accordingly, pharmaceuticals such as antibiotics, painkillers, cardiovascular drugs, blood lipid regulators, and antidepressants, are frequently detected in surface and ground waters around the world.[3] The presence of these active pharmaceutical products in natural environments is problematic because of their capacity to induce a range of sublethal effects in exposed organisms.[1,8,9] Indeed, pharmaceuticals can disrupt fundamental behavioral processes, such as reproductive behavior, aggression, boldness, activity levels, and feeding rates.[1,10] Changes to such behaviors can directly impact the strength and direction of selection, fitness, and even population viability, with potential for broader ecosystem and evolutionary consequences.[11−14]

Fluoxetine (Prozac) is one of the most widely prescribed antidepressants globally,[15] used for treatment of depression and anxiety-related disorders in humans and domesticated animals.[16,17] Fluoxetine is also a relatively stable compound (half-life of 68 days in water at pH 7 under light conditions)[18] that is commonly detected in freshwater environments worldwide.[19,20] In aquatic habitats, fluoxetine concentrations range between <1 ng/L to as high as 1400 ng/L,[3,20,21] although concentrations above 350 ng/L tend to only occur in direct sewage effluent.[20] Fluoxetine inhibits the reuptake of neuronal serotonin (5-hydroxytryptamine), which acutely increases synaptic serotonin levels and, after 2−3 weeks, produces anxiolytic effects in humans.[16] The serotonergic system is conserved across all vertebrate classes[22] and, consequently, fluoxetine has the capacity to alter behavior in a wide range of species.[23]

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Continuous discharge of fluoxetine-contaminated effluent from WWTPs, coupled with the stability of dissolved fluoxetine, results in long-term ("pseudopersistent") exposure of many aquatic environments.[8] Duration-dependent effects have been observed in mussels (*Mytilus californianus*), with some physiological changes being manifested only after 6 weeks of fluoxetine exposure.[24] Yet, most studies investigating impacts of fluoxetine are conducted using short-term exposure durations (i.e., <1 month) that represent a small fraction of the model species' lifespan.[25−30] This is problematic because effects that persist after long-term exposure may have important consequences on the lifetime fitness of individuals and population dynamics, making studies that address effects of chronic exposure to pharmaceutical contaminants, such as fluoxetine, an urgent research priority.

Aquatic species are subjected to a range of environmental stressors contemporaneously.[31] It is, therefore, important to understand how species respond to pharmaceutical pollutants in the presence of other concurrent stressors, especially because joint effects of interacting stressors can be challenging to predict. In particular, effects of combined stressors can be less, or greater, than expected (i.e., antagonistic or synergistic, respectively), compared to stressors tested in isolation. For example, in the Mediterranean mussel (*Mytilus galloprovincialis*), concurrent exposure to fluoxetine and the high blood pressure medication propranolol resulted in an antagonistic interaction with regard to cell signaling,[32] with the combined effect of both pharmaceuticals being less than what would be expected if the independent effects were simply summed together.

Temperature is an important stressor, especially in aquatic environments. Increased temperature variability represents a disproportionately greater threat to organisms than mean temperature increases,[33] and ambient environmental temperature is crucial to body temperature regulation in ectotherms.[34] In this respect, aquatic ectotherms are poorly adapted to cope with large temperature fluctuations,[35−37] especially in the context of reproduction.[38] Furthermore, temperature stress can compromise an individual's ability to respond effectively to other environmental stressors.[39] For instance, toxicity of pesticides to juvenile coho salmon (*Oncorhynchus kisutch*) was elevated at higher temperatures.[40] In zebrafish (*Danio rerio*), isolated exposures to high temperature or the endocrine disruptor progestin had a negative effect on female fecundity, whereas exposure to both of these stressors simultaneously resulted in complete reproductive failure.[41] Similarly, in the water flea (*Daphnia magna*), fluoxetine and temperature variability had an adverse synergistic effect on reproductive success and population growth.[42] More generally, however, interactive effects between temperature and exposure to realistic levels of pharmaceutical pollution have received surprisingly little attention to date. Indeed, more work is clearly needed, given the prevalence of pharmaceutical contaminants in aquatic environments and the importance of temperature to ectothermic species.

Here, we investigated how two important determinants of fitness, reproductive behavior and activity, are influenced by two stressors, chronic fluoxetine exposure and acute temperature stress, in a freshwater fish. Specifically, guppies (*Poecilia reticulata*) sourced from mesocosm populations were exposed to environmentally realistic levels of fluoxetine (nominal concentrations: 30 or 300 ng/L) or were left unexposed (i.e., solvent control only) for a period of 15 months and then underwent one of three temperature treatments. Fish were placed under cold stress (at 18 °C), placed under heat stress (at 32 °C), or maintained at a control temperature (24 °C) for 24 h prior to experimental trials. We then investigated how reproductive behaviors and activity levels of guppies were impacted by the fluoxetine and temperature treatments. Because isolated exposure to fluoxetine and cold stress have been shown to generate opposite effects on reproductive behavior in fish—i.e., increased male copulatory behavior resulting from fluoxetine exposure[25,28,43] and decreased male sexual motivation due to cold stress[44]—we hypothesized that these two stressors would act antagonistically, with the effect of one countering the effect of the other when experienced in combination. We also predicted that heat stress and fluoxetine exposure would, in turn, interact synergistically to increase levels of courtship and copulation.[45] For activity levels, we tested the generality of previous findings suggesting that fluoxetine may not significantly affect fish activity.[30,46,47] We predicted, instead, that activity increases with temperature.[48]

MATERIALS AND METHODS

Study Species. The guppy is a small poeciliid fish native to freshwaters of northern South America.[49] As a highly successful invader, the guppy is now found in tropical and subtropical regions around the world.[50] The preferred temperature range of guppies is 24−27 °C,[51−53] with females having fewer offspring per brood under heat stress (i.e., ≥32 °C)[54] and males courting less when subjected to cold stress (i.e., ≤20 °C).[44] Guppies undergo internal fertilization, with males inseminating females using a modified anal fin, the gonopodium.[49] Male guppies exhibit two alternative mating strategies, either performing courtship displays to elicit consensual copulations with choosy females or carrying out coercive "sneak" copulations that circumvent female mate choice.[55] Courtship displays involve the male positioning himself in the female's line of sight, bending his body into an s-shape and quivering (termed "sigmoid display").[49] Sneak copulation attempts involve a male chasing a female from behind and attempting to insert his gonopodium into the female's genital pore without first performing courtship.[49] Because the latter strategy is associated with lower insemination efficiency and reduced offspring quality,[56] changes in the relative use of these two strategies can impact the quality and quantity of progeny,[55] potentially altering population dynamics and size.

Mesocosm System and Fluoxetine Treatments. Guppies used in this experiment were sourced from mesocosm populations that had been maintained in a temperature-controlled greenhouse facility under natural (i.e., ambient) light conditions at Monash University, Melbourne, Australia. These mesocosm populations were founded using wild-caught guppies collected in November 2016 from Alligator Creek, a rainforest-fed stream located within Bowling Green Bay National Park, Townsville, Australia (19°23′50.3″ S, 146°56′56.5″ E; collection permit: WITK17685216). Water samples taken from this site at the time of fish collection revealed no contamination with fluoxetine (Envirolab Services; all samples under the minimum detection limit of 2 ng/L, n = 5). After collection, fish were housed in 12 stainless steel mesocosm tanks (648 L; 180 cm × 60 cm × 60 cm), each of which was established with a founding population of 300 sexually mature guppies at an equal sex ratio, with these mesocosm populations having since been utilized for a series of

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experiments, including the present study. Mesocosm tanks were filled with carbon-filtered fresh water to a depth of 30 cm and contained aquatic plants (Java moss, *Taxiphyllum barberi*) and a 3 cm layer of gravel substrate (~7 mm grain size). Commercial air pumps (Resun LP100) were used to aerate tanks, and aquarium heaters were used to maintain water temperature. The temperature and pH of all tanks were tested weekly (temperature: mean = 23.4 °C, SD = 1.0 °C, n = 720; pH: mean = 7.36, range = 5.08−9.67, n = 720). Fish were fed *ad libitum* once every 2 days with commercial food pellets (Aquasonic Nutra Xtreme C1 pellets; 0.8 mm). Once per week, 20% water changes were conducted for each tank.

Mesocosm tanks were randomly allocated to one of three fluoxetine exposure regimes, a low-fluoxetine treatment (nominal concentration: 30 ng/L, n = 4 tanks), a high-fluoxetine treatment (nominal concentration: 300 ng/L, n = 4 tanks), or an unexposed treatment (i.e., solvent control, n = 4 tanks) from April 2017. The low-fluoxetine treatment is representative of concentrations commonly found in surface waters, whereas the high-fluoxetine treatment represents levels detected in effluent-dominated systems.[3,20,21] A population survey conducted in the month following behavioral experiments (August 2018) showed that adult densities within the 12 mesocosms were similar across the treatments (mean ± SD: 78 ± 57, 66 ± 39, and 62 ± 26, for the control, low, and high treatments, respectively).

To maintain the desired fluoxetine water concentrations, dosing solutions were added to the low- and high-exposed tanks twice weekly. This involved fluoxetine hydrochloride (Sigma-Aldrich; product number: F132, CAS: 56296−78−7) being dissolved in methanol to form two separate 100 mL stock solutions (20 and 200 mg/L for the low- and high-fluoxetine treatments, respectively), which were then used to create dosing solutions twice weekly. Dosing solutions were prepared by diluting 1 mL of either stock solution in 1 L of reverse-osmosis water. To eliminate any potential for solvent effects[57] and to ensure consistency in the level of handling and disturbances across treatments, a solvent solution (1 mL of methanol in 1 L of reverse-osmosis water) was added to all control tanks twice weekly (equates to 0.0006% methanol by volume).

Analytical Verification of Fluoxetine Treatment Levels. Throughout the experiment, water samples (40 mL) were drawn approximately once per month from each of the low- and high-fluoxetine treatment mesocosm tanks to determine the concentrations of fluoxetine and norfluoxetine (the major metabolite of fluoxetine)[19] using gas chromatography−tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware, USA; minimum detection limit: 2 ng/L). Control tanks were also sampled every second month using the same method to ensure the absence of fluoxetine contamination. Water analyses were performed by Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025) within 4 days of collection. A detailed description of the water analysis protocol is provided in Bertram et al.[25] Mean measured fluoxetine concentrations in the low- and high-fluoxetine mesocosm tanks were 38 ng/L (SD = 24, n = 60) and 312 ng/L (SD = 214, n = 60), respectively. Fluoxetine was not detected in any of the control tanks (all samples under the minimum detection limit, n = 30). Norfluoxetine was not observed in any of the tested samples. Fluoxetine readily sorbs to sediment in water/sediment systems.[19,58] Hence, while the gravel substrate used in the mesocosm system was important in simulating more natural environmental conditions, it likely contributed to the variability in measured fluoxetine concentrations observed.

Experimental Procedure and Temperature Treatments. The goal of the study was to investigate behavioral effects of long-term fluoxetine exposure under ecologically realistic conditions comprising multiple overlapping and interacting generations, which is reflective of guppy populations in nature.[49,59,60] Trials were conducted in July 2018, resulting in a 15-month exposure protocol. Given an approximate generation time of 4 months in guppies, up to 4 generations were present within the mesocosm system at the time of trials.[59,60] One week prior to experimental trials, sexually mature fish were caught and separated by sex into fine-mesh cylinders (35 cm × 32 cm, diameter × height, water depth: 30 cm) within their respective mesocosm tanks. To ensure that fish were sexually mature, we selected females that were over 15 mm in standard length and males that displayed nuptial coloration and a fully developed gonopodium.[49] Fish were then sourced from these cylinders for use in trials, ensuring individuals were only tested once. Twenty-four hours prior to experimentation, fish underwent temperature manipulations in 1 L cylindrical glass tanks (10 cm × 30 cm, diameter × height, water depth: 20 cm, maximum 3 fish per tank), with males and females housed in separate tanks. Three temperature treatments were employed: heat stress (32 °C), cold stress (18 °C), and a control temperature treatment (no change; 24 °C). The control treatment was chosen to represent the long-term average temperature observed across mesocosm tanks (mean = 23.4 °C, SD = 1.0 °C, n = 720). The heat stress treatment involved an 8 °C increase over the average mesocosm tank temperature. This temperature increase was selected to simulate heat stress but, importantly, was still below the critical thermal maximum of guppies (i.e., 38 °C).[61] The cold stress treatment involved a 6 °C reduction from the average mesocosm tank temperature, which was chosen because guppies are more vulnerable to rapid decreases in temperature than to rapid increases in temperature.[62] The temperature changes of +8 °C and −6 °C used in these experiments are plausible in an environmental context, with daily fluctuations of this scale having been observed within the guppy's native range.[53] To avoid shock caused by instantaneous temperature changes, temperature alterations occurred over a period of 6 h, which is common practice in temperature manipulation experiments involving fish.[63,64] The tanks remained at the new temperatures for the subsequent 18 h before fish were tested in behavioral trials.

Experimental Trials. Behavioral trials (n = 162) were conducted on the day after fish were exposed to temperature manipulations in glass tanks (60 cm × 30 cm × 30 cm, water depth: 15 cm) filled with carbon-filtered water maintained at 18 °C (mean: 18.2 °C, range: 17.8−18.5 °C), 24 °C (mean: 24.3 °C, range: 24.0−24.5 °C), or 32 °C (mean: 32.3 °C, range: 31.8−32.5 °C). The male and female used in each trial had been subjected to the same fluoxetine and temperature treatment (solvent control treatment [i.e., 0 ng/L fluoxetine]: control temperature: n = 20, low temperature: n = 17, high temperature: n = 17; low-fluoxetine treatment: control temperature: n = 16, low temperature: n = 20, high temperature: n = 19; high-fluoxetine treatment: control temperature: n = 19, low temperature: n = 18, high temperature: n = 16). Fish tested in each trial were sourced

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from different mesocosm tanks to ensure that male−female experimental pairs were novel to each other. This was done to control for familiarity, which is known to influence mate choice in guppies.[65] Each trial involved the male and female being placed into separate acclimation chambers (opaque cylinders; 7.5 cm × 20 cm, diameter × height, water depth: 15 cm) in an experimental tank matching the desired temperature used in the temperature manipulation. Fish were acclimated for 5 min, after which the acclimation chambers were removed so that the two fish were free to explore the trial tank and interact for 40 min. Tank water was replaced between each trial to prevent any potential for chemical cues to influence fish in subsequent trials.[66]

Throughout behavioral trials, tanks were video-recorded from above (Panasonic HC-V180), with male and female behavior subsequently scored from recordings, blind to treatment, using behavioral observation key-logging software (BORIS v. 6.3).[67] Specifically, the time taken for the male to first attempt a sneak copulation and the number of attempted sneak copulations were scored for each trial. The time until the first courtship display and the number of courtship displays performed by each male were also recorded. Additionally, activity levels of the male and female were evaluated using 5 cm grid squares marked on the base of each experimental tank. We counted the number of 5 cm grid squares crossed by each fish for 1 min every 5 min over the 40 min trial, resulting in a total of 8 min of observations for each fish. We then estimated activity as movement/time (cm/sec). After each trial, both of the fish were measured for body mass (±0.0001 g) and standard length (i.e., body length excluding tail; ± 0.01 mm). The fish were then returned to their respective mesocosm source tanks, where they were isolated from untested fish.

Statistical Analyses. Statistical analyses were performed in R 3.5.1.[68] As a proxy for body condition, we calculated a scaled mass index, which was done separately for males and females.[69] Specifically, we performed a standard major axis regression on the log of body mass and standard length of fish (*sma* function, *smatr* package), and calculated a sex-specific beta coefficient, which was then used (with mean standard length) to obtain the scaled mass for each fish. These scaled mass indices for males and females were initially included in all models but were later removed as they did not significantly improve their fit, as tested by Akaike information criterion comparisons.

Generalized linear mixed models (GLMMs) were used to test the effects of fluoxetine treatment, temperature treatment, and the interaction between them, for both sneak copulations and courtship displays, separately. For sneak copulations, a negative binomial distribution (NB GLMM; *nb.glmer* function, *MASS* package) was selected to account for overdispersion. For courtship displays, a binomial distribution was selected over a Poisson distribution because an insufficient number of fish conducted the behavior for it to be analyzed as a count variable (i.e., 19.8% across all groups). To account for possible mesocosm tank effects, the source tank IDs of male and female fish, as well as the combination of male and female source tank IDs, were included as random effects in both GLMM models (see SI tables S1−S4 for random effects results).

To analyze potential effects of the fluoxetine and temperature treatments, and their interaction, on the time taken for males to perform their first sneak copulation and courtship display, we applied separate Cox's mixed effect (COXME) proportional hazard models (*coxme* function, *survival* package) for the two response variables (i.e., sneak attempts and courtship displays). Both models met the assumption of proportionality, as determined by examining the interaction between Schoenfeld residuals and log time (*cox.zph* function, *survival* package).

Data on fish activity levels were square-root transformed so that assumptions of normality and homogeneity of variance were satisfied (Shapiro-Wilk test, *shapiro.test* function and Bartlett test, *bartlett.test* function). We then analyzed activity levels of fish using linear mixed effects models (LME; *lmer* function, *lme4* package). Fluoxetine and temperature treatments, the interaction between the two, and sex, were included as fixed effects, and mesocosm source tank ID was added as a random effect.

Where relevant, general linear hypothesis tests (GLHTs) with Tukey's posthoc *p*-adjustments were used to generate pairwise comparisons (*glht* function, *multcomp* package).

Effects of fluoxetine exposure on morphology (weight, standard length, and scaled mass index) were investigated for each sex, using Kruskal−Wallis tests to account for non-normal distributions in data (KWT; *kruskal.test* function). Dunn's tests were used with Bonferroni corrections for pairwise comparisons (*dunnTest* function, *FSA* package).

RESULTS

Reproductive Behaviors. No significant interaction was detected between fluoxetine treatment and temperature treatment for the number of sneak copulations performed by males (NB GLMM: χ^2 = 3.885, *df* = 4, *p* = 0.43). We did, however, find a significant difference between the number of sneak copulations conducted by males in different fluoxetine treatments (NB GLMM: χ^2 = 7.843, *df* = 2, *p* = 0.019; Figure 1a), with unexposed fish performing fewer sneaks than males in the low- and high-fluoxetine treatments (NB GLMM: *z* = −2.455, *p* = 0.037, and *z* = −3.129, *p* = 0.005, respectively). The low- and high-fluoxetine treatments did not differ significantly (NB GLMM; *z* = 0.3595, *p* = 0.63; Figure 1a).

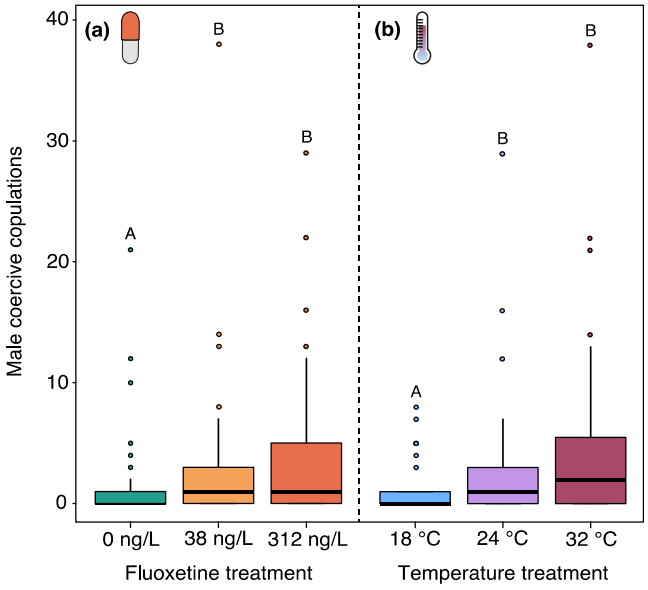

Figure 1. Number of male coercive "sneak" copulations performed in the (a) unexposed (0 ng/L; *n* = 54), low-exposed (38 ng/L; *n* = 55), and high-exposed (312 ng/L; *n* = 53) fluoxetine treatments, and in the (b) low (18 °C; *n* = 54), control (24 °C; *n* = 56), and high (32 °C; *n* = 52) temperature treatments.

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Temperature treatment also significantly affected the number of sneak copulations performed by males (NB GLMM; χ^2 = 20.33, df = 2, p < 0.001; Figure 1b). Males under low-temperature stress performed fewer sneak copulations than those in the heat stress and control treatments (NB GLMM: z = 4.745, p < 0.001, and z = 3.370, p = 0.002, respectively; Figure 1b). No significant difference was detected between the control and heat-stress treatments (NB GLMM; z = 1.728, p = 0.19; Figure 1b).

We found no significant interactive effect between fluoxetine exposure and temperature treatment in terms of time taken to the first male sneak copulation (COXME: χ^2 = 1.491, df = 4, p = 0.83). Furthermore, fluoxetine treatment did not affect the time elapsed before males attempted a sneak copulation (COXME: χ^2 = 3.783, df = 2, p = 0.15; Figure S1) but temperature treatment did (COXME: χ^2 = 13.17, df = 2, p = 0.001; Figure 2). Specifically, males at 18 °C were significantly

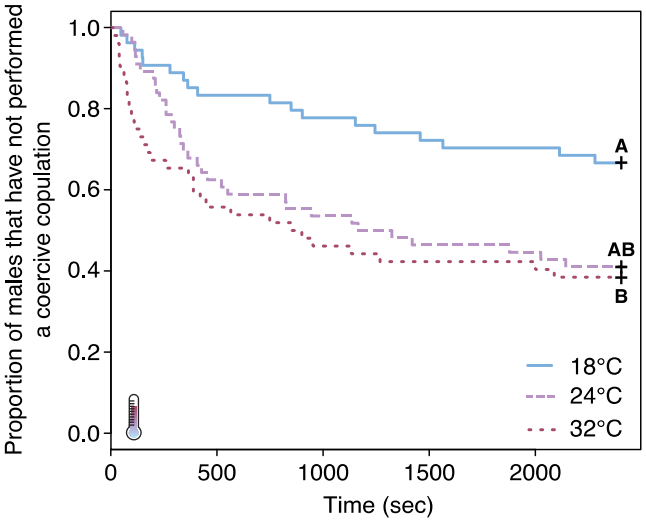

Figure 2. Time taken to first coercive "sneak" copulation for males by temperature treatment, right-censored at 2400 s. The solid line represents the low-temperature treatment (18 °C; n = 54), the dashed line represents the control temperature treatment (24 °C; n = 56), and the dotted line represents the high-temperature treatment (32 °C; n = 52).

delayed in performing their first sneak relative to males at 32 °C (GLHT: z = 2.387, p = 0.043). Males in the 24 °C control temperature treatment did not differ significantly from those at 18 °C (GLHT; z = 2.045, p = 0.098) or 32 °C (z = 0.5680, p = 0.83).

There was no significant interaction between fluoxetine and temperature treatments regarding the proportion of males that performed courting behaviors (GLMM: χ^2 = 2.559, df = 4, p = 0.63) nor was there a significant effect of fluoxetine treatment (GLMM: χ^2 = 1.906, df = 2, p = 0.39), with 14.8% of males in the control treatment, 16.4% in the low-fluoxetine treatment, and 28.3% in the high-fluoxetine treatment performing at least one courtship display. Temperature, however, did significantly influence the proportion of males that performed courtship displays (GLMM: χ^2 = 7.284, df = 2, p = 0.026). A significantly lower proportion of males in the low-temperature treatment performed at least one courtship display (3.6%), relative to those in the control treatment (30.9%; GLMM: z = 3.138, p = 0.002) and the high-temperature treatment (25.0%; GLMM: z = 2.791, p = 0.005). No significant difference between the control and high-temperature stress treatments was observed for courting behavior (GLMM: z = 0.6820, p = 0.52).

For the time elapsed until first courtship display, there was no significant interaction between temperature and fluoxetine treatment (COXME: χ^2 = 3.877, df = 4, p = 0.42), nor was there a significant main effect of fluoxetine exposure (COXME: χ^2 = 5.066, df = 2, p = 0.41; Figure S2). A marginally nonsignificant main effect was, however, observed for temperature treatment (COXME: χ^2 = 10.88, df = 2, p = 0.054, Figure S3).

Activity Levels. We found no significant interaction between fluoxetine treatment and temperature treatment on fish activity levels (LME: χ^2 = 5.385, df = 4, p = 0.25). Fluoxetine exposure did not influence activity (LME: χ^2 = 1.217, df = 2, p = 0.54; Figure S4) but temperature did (χ^2 = 118.6, df = 2, p < 0.001; Figure 3). The cold-temperature

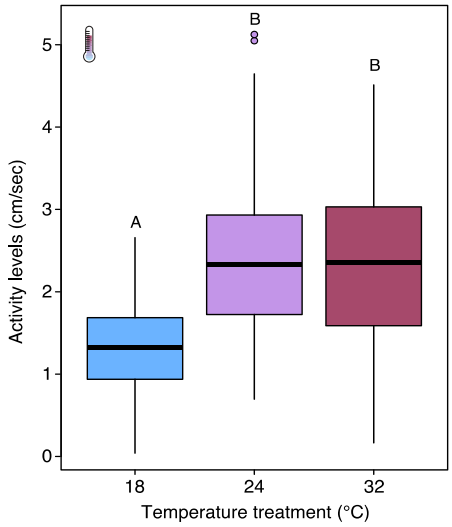

Figure 3. Activity levels of fish in the low-temperature treatment (18 °C; n = 108), the control temperature treatment (24 °C; n = 112), and the high-temperature treatment (32 °C; n = 104).

treatment resulted in a significant reduction in activity levels relative to the control and heat-stress treatments (GLHT: z = 5.902, p < 0.001, and z = 4.904, p < 0.001, respectively), but there was no significant difference between the control and heat-stress treatments with regard to activity levels (GLHT: z = −0.8040, p = 0.70). Regardless of exposure treatment, female fish were significantly less active than males (χ^2 = 7.577, df = 1, p = 0.006), and activity level correlated with a variety of reproductive behaviors. Specifically, activity level was negatively correlated with time to first courtship display (Spearman's rank correlation: ρ = −0.273, df = 160, p < 0.001) and time to first sneak copulation (Spearman's rank correlation: ρ = −0.301, df = 160, p < 0.001). In addition, activity was positively correlated with the propensity to conduct at least one courtship display (Spearman's rank correlation: ρ = 0.273, df = 160, p < 0.001) as well as the number of sneak copulations performed (Spearman's rank correlation: ρ = 0.304, df = 160, p < 0.001).

Morphology. Fluoxetine exposure did not impact weight (KWT: χ^2 = 2.527, df = 2, p = 0.28), standard length (KWT: χ^2 = 1.925, df = 2, p = 0.38), or scaled mass (KWT: χ^2 = 0.9388, df = 2, p = 0.63) in male guppies. Fluoxetine did, however, impact weight (KWT: χ^2 = 9.115, df = 2, p = 0.010) and

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standard length (KWT: χ^2 = 9.263, df = 2, p = 0.010) in females. Specifically, females in the low-fluoxetine treatment were heavier (z = 2.995, p = 0.008) and longer (z = 2.958, p = 0.009) than those in the control treatment. There was no difference between unexposed and high-exposed fish (weight: z = 1.165, p = 0.73; standard length: z = 0.8590, p = 0.39) or low- and high-exposed fish (weight: z = 1.810, p = 0.21; standard length: z = 2.081, p = 0.11). Fluoxetine exposure did not have a significant effect on female scaled mass (KWT: χ^2 = 5.539, df = 2, p = 0.063).

DISCUSSION

Contrary to predictions, we did not find an interaction between fluoxetine exposure and temperature stress on guppy reproductive behavior. We did, however, find that both stressors generated independent effects on reproductive behavior. Specifically, for fluoxetine, long-term (15-month) exposure resulted in an increase in male coercive mating behavior (i.e., sneak copulations) in both the low (38 ng/L) and high (312 ng/L) treatments but did not alter courtship behavior. With regard to the effect of temperature on reproductive behaviors, we found that acute (24 h) cold stress (i.e., 18 °C) resulted in reduced courtship by males as well as a delay in the time taken to first perform a coercive mating attempt. Activity levels, in turn, were not affected by the interaction between fluoxetine exposure and temperature stress or by fluoxetine exposure independently. There was, however, an independent effect of temperature stress on activity levels, with cold stress causing a reduction in activity in both males and females.

To date, we know of only one other study addressing impacts of fluoxetine exposure under varying temperature conditions. In contrast to the results of the present study, Barbosa et al.[42] reported that chronic (fourth-generation) fluoxetine exposure interacted synergistically with temperature variability to impair reproductive success in water fleas (*Daphnia magna*). However, that study examined temperature variability and rate of reproduction, rather than acute temperature stress and reproductive behavior, which may explain the disparity in results. We also cannot rule out differences in species-specific sensitivities to fluoxetine or temperature stress.[30,52] In this regard, it is worth noting that previous research in guppies has also failed to find interactions between fluoxetine (61 ng/L and 350 ng/L for 28 days) and another common environmental stressor, predation risk.[43] Yet, it is important to highlight that independent effects induced by two stressors can nevertheless be detrimental if an individual is exposed to both stressors simultaneously.

For fluoxetine, our study shows that irrespective of temperature, environmentally realistic exposure levels (i.e., 38 ng/L and 312 ng/L) can disrupt reproductive behaviors in fish, with exposed males increasing their use of a coercive mating strategy. In this regard, it is important to note that effects of fluoxetine on reproductive traits can vary between species. For instance, fluoxetine has been shown to increase nest defense behaviors in fathead minnows (*Pimephales promelas*; 1000 ng/L for 28 days),[70] reduce courtship displays in starlings (*Sturnus vulgaris*; 2700 ng/day for 28 weeks),[71] and increase copulatory behaviors in livebearing fish (479 ng/L for 30 days;[25] 350 ng/L for 28 days;[43] 31 ng/L for 35 days[28]), while shorter-term exposure has been shown to have no effect on reproductive behaviors in Siamese fighting fish (*Betta splendens;* 540 ng/L for 5 h).[26] The mechanisms by which selective serotonin reuptake inhibitors alter reproductive traits are not fully understood.[23,72] One possible explanation is that fluoxetine can influence circulating levels of hormones via the hypothalamic−pituitary−gonadal axis by affecting the retention of serotonin and, more generally, the serotonergic system.[23,73] For example, in fish, increases in extracellular serotonin can stimulate the release of gonadotropin-releasing hormones, gonadotropic hormones, and androgens. Such hormonal changes can, in turn, alter levels of sexual motivation[74] and potentially modify how attractive individuals are to the opposite sex by, for example, altering chemical and visual cues of sexual fitness in males and females.[75] However, pinpointing precise physiological factors and hormones affected by fluoxetine is challenging. For example, female starlings exposed to fluoxetine were courted less by males than were unexposed females, but no differences in body condition or levels of circulating testosterone or oestradiol (sex hormones) were observed between females from different exposure treatments (2700 ng/day for 28 weeks).[71] Further research targeted at identifying what physiological changes underpin observed effects of fluoxetine on reproductive behaviors would be valuable in understanding differences between species and in determining which species may be particularly susceptible to fluoxetine-mediated alterations to reproductive processes.

While the physiological processes underpinning fluoxetine's effects on reproductive traits remain unclear, this study has nonetheless shown that long-term exposure to fluoxetine generates an increase in coercive reproductive behavior in male guppies. The relative shift toward this unsolicited sneaking strategy over cooperative mating (i.e., male courtship) could impair fitness by, for example, reducing the ability of females to exercise mate choice. Female mate choice plays an important role in reproduction, and when females are unable to select males that display indicators of high fitness to mate with, the quality and quantity of offspring may be impacted.[13] Additionally, increases in male sneaking behavior often result in females spending more time actively avoiding males, with consequences for female fitness even in a nonreproductive context. For example, female guppies will alter their habitat use to areas where predation risk is high to avoid sexual harassment by males[76] and suffer reduced foraging opportunities when targeted by male sneaking behaviors.[77] Female avoidance tactics further impair male fitness by reducing interaction between the sexes and mating opportunities for males. Moreover, sneak copulations confer a lower insemination efficiency compared to postcourtship copulations.[56] Hence, although fluoxetine exposure increased the number of male copulation attempts performed, it may actually reduce overall male fitness.

Because effects of fluoxetine may be dependent on exposure duration, we employed a long-term 15-month experiment to identify effects of chronic exposure on reproductive traits. Recently, a shorter-term experiment on guppies found that 28 days of fluoxetine exposure at 350 ng/L caused males to perform more frequent sneaking behavior than unexposed fish, but this effect was not seen in males exposed at the lower concentration of 61 ng/L.[43] The latter finding contrasts with our results in that 15-month exposure to 38 ng/L of fluoxetine did increase sneaking behavior in the present study. Given that both studies were conducted in a similar fashion and on the same species, we contend that exposure duration is the most likely explanation for the different results observed, with male guppy reproductive behavior being relatively more vulnerable

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to disruption by longer-term fluoxetine exposure. Our study, therefore, provides new evidence for time-dependent effects of fluoxetine exposure on behavioral traits and underscores the importance of longer-term studies for understanding impacts of environmentally realistic pharmaceutical contamination. In this regard, it is important to note that this study was specifically designed to simulate a realistic exposure scenario, with up to four overlapping generations exposed and allowed to interact, as is reflective of natural populations. However, future studies investigating long-term effects of pharmaceutical exposure and disentangling plastic versus genetic responses to contamination will also be valuable.

In contrast to reproduction, fluoxetine exposure had no effect on activity levels in male or female guppies. This is consistent with studies in zebrafish (100000 ng/L for 2 weeks),[78] killifish (*Aphanius dispar*; 300 ng/L for 7 days),[46] and mosquitofish (*Gambusia holbrooki*; 31 and 374 ng/L for 35 days)[28] in which no impacts of fluoxetine on activity were observed. In contrast, activity levels increased in mosquitofish after 28 days of exposure to a low level of fluoxetine (25 ng/L), although no change in activity was seen at a higher dosage (226 ng/L).[79] These studies highlight the potential for fluoxetine to induce nonmonotonic effects (i.e., where the slope of a dose−response curve changes direction within the range of tested doses) and generate contrasting results depending on exposure concentration and duration, and the species tested. While fluoxetine was not found to affect activity in the present study, it is important to emphasize that activity is just one aspect of spatial use. In particular, Egan et al.[78] found that fluoxetine-exposed zebrafish were quicker to enter the top half of a trial tank and spent more time in the upper portion of the water column. Fish that spend more time near the water's surface are more vulnerable to aerial predators[80] and, therefore, exposure to fluoxetine may increase vulnerability to predation. These potentially costly alterations to behavior would not have been identified if only activity levels had been measured, suggesting that future research may benefit from investigating how other aspects of swimming performance, movement, and spatial use respond to fluoxetine exposure.

Regarding independent effects of temperature, we found that cold stress leads to a reduction in reproductive-related traits in fish, whereas elevated temperatures did not affect these traits. Reproductive processes are sensitive to temperature and are often impaired when temperature falls outside of an organism's optimal range.[81,82] For example, courting behaviors are lower in guppies exposed to temperature decreases,[44] and reproductive performance is hindered in female pejerrey (*Odontesthes bonariensis*) under heat stress.[83] It may, therefore, seem counterintuitive that the heat-stress treatment employed in our experiment did not alter reproductive behavior. However, this may be because the temperature used was not sufficiently high to induce a notable stress response in guppies.

Guppies under low-temperature stress showed reduced reproductive behaviors relative to other treatments. Such temperature stress in aquatic organisms can generate responses including changes to behavior, metabolic rate, and the expression of heat shock proteins.[44,84] Under acute stress, these changes are usually temporary and are reversed when ambient temperature returns to normal.[84] The temperature manipulations used in this study were acute, indicating that the reproduction-related behavioral changes observed may not persist once temperatures return to the long-term average of 24 °C. Future research may, therefore, benefit from investigating whether such temperature-mediated short-term adjustments in mating behaviors have long-term impacts on the reproductive success of individuals and populations.

In line with our predictions, fish exposed to cold-temperature stress were significantly less active than those undergoing heat stress or maintained at an intermediate control temperature, irrespective of fluoxetine treatment. Temperature variation results in shifts to metabolic rate in aquatic ectotherms, and behavioral changes, such as adjusted activity, are a key mechanism used by animals to restore metabolic homeostasis.[85] Swimming speed increases with temperature, meaning that fish at low temperatures tend to have lower cruising speeds,[86] which is consistent with the results of the current experiment. This reduction in swimming speed may have fitness consequences because fish that are slower when encountering predators are less likely to escape and/or survive.[87]

The 15-month fluoxetine exposure resulted in sex-specific, nonmonotonic changes to fish morphology, with females in the low-fluoxetine treatment being heavier and longer than unexposed fish. This contrasts with research on goldfish (*Carassius auratus*) in which exposure resulted in decreased weight gain (540000 ng/L for 28 days)[88] and juvenile guppies which had reduced weight and standard length under fluoxetine exposure (30 and 500 ng/L for 35 days).[89] It is worth noting that fluoxetine has previously been reported to alter foraging dynamics in mosquitofish (215 ng/L) following a 28-day exposure, while no associated changes in morphological traits (i.e., weight, body length and body condition) were detected.[90] It is important to point out, however, that the earlier studies on goldfish, guppies, and mosquitofish employed relatively short-term exposures. Further research into the mechanisms causing morphological changes in long-term fluoxetine exposure would be valuable in identifying why the observed nonmonotonic, sex-specific differences arise. It is also important to highlight that fluoxetine-induced nonmonotonic effects have previously been reported in a wide range of species, especially in the context of behavioral traits.[79,91−97] The mechanism(s) driving these types of fluoxetine-induced nonmonotonic effects is/are not yet fully understood. However, a number of mechanisms that are known to drive other nonmonotonic effects[98] have the potential to apply to fluoxetine.

In summary, we found no interaction between chronic exposure to the pervasive pharmaceutical contaminant fluoxetine and acute temperature stress on reproductive behavior or activity levels in guppies. However, long-term (15-month) exposure to fluoxetine led to an increase in the frequency of coercive sneak copulations carried out by male guppies at both of the environmentally realistic dosages tested, while male courtship behavior, and activity levels in both sexes, were not affected. Regarding effects of temperature, males exposed to acute (24 h) cold stress were slower to first perform a coercive copulation (relative to males in the heat-stress treatment), performed fewer such copulations, and were less likely to perform courtship behavior. In addition, cold-temperature stress was associated with reduced activity levels in both males and females. In combination, our findings demonstrate complex independent effects of multiple stressors on ecologically important behavioral processes in fish. Despite a growing appreciation of the importance of a multistressor approach, there remains a dearth of knowledge on this topic,

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particularly for novel stressors like pharmaceutical pollutants as well as the direct and indirect effects they can generate. Such studies are clearly necessary, however, if we are to gain a more holistic understanding of the potential impacts of pharmaceutical contaminants on wildlife populations around the globe.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c01625.

Figures showing latency to first sneak for males by fluoxetine treatment (S1), latency to first courtship display for males by fluoxetine treatment (S2) and by temperature treatment (S3), activity levels of fish by fluoxetine treatment (S4); tables showing impact of fixed and random effects on behaviors (PDF)

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Notes

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