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Neuroactive Pollution Disrupts Cognition in Fish by Causing Sex-Specific Effects on Spatial Learning

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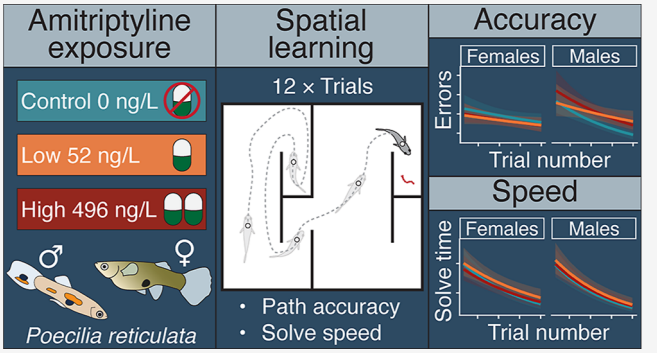
ABSTRACT: Cognition underpins how animals perceive, navigate, and respond to their environment, yet these fundamental processes are increasingly threatened by environmental pollutants. Neuroactive pharmaceuticals are now routinely detected in aquatic ecosystems, raising concern about their potential to disrupt key cognitive functions in wildlife. Here, we tested whether exposure to the antidepressant amitriptyline, a widespread pharmaceutical pollutant, impairs spatial learning in wild-caught guppies (*Poecilia reticulata*). Using a repeated-trial maze assay, we quantified learning performance across 12 trials following an 11-d exposure to either a freshwater control (0 ng/L) or ecologically relevant low (52 ng/L) or high (496 ng/L) concentrations of amitriptyline. We found strong evidence of spatial learning across all treatment groups, with maze solve times and navigational errors declining over trials. However, males exposed to low and high concentrations of amitriptyline made 26% and 34% more errors, respectively, compared to control males. Female learning, by contrast, was unaffected by amitriptyline exposure, revealing sex-specific cognitive effects. Control males were more accurate than females, yet this advantage was lost under exposure and ultimately reversed at high concentrations, where males performed worse than females. These results emphasize the need to consider sex differences in cognitive responses when assessing the ecological impacts of environmental contaminants.



KEYWORDS: *contaminant, memory, navigation, neurobehavioral, pharmaceutical, Poecilia reticulata, amitriptyline, antidepressant*

INTRODUCTION

Cognitive processes shape the ways in which animals behave and interact with their environment. An individual's ability to acquire, store, and apply information about their surroundings can directly influence their survival and reproductive success.[1−3] For example, quickly learning to associate a novel species as a predator,[4,5] or learning new foraging techniques,[6] can confer survival benefits. Among cognitive domains, spatial cognition—the capacity to map and remember the layout of an environment—is fundamental to many fitness-related behaviors. Animals, for instance, are known to rely on spatial learning to locate food[7] and mates,[8] and to maintain territories.[9] Indeed, poor spatial memory has been linked to increased mortality from predation[9] and reduced survival,[10,11] as well as lower reproductive output[12,13] (although, see ref 14). Thus, the proper functioning of spatial cognitive processes is essential and disruptions to spatial cognition are, therefore, expected to compromise survival and fitness.[15] Yet, the integrity of these processes is increasingly challenged by human-induced environmental change.

A growing body of research indicates that exposure to environmental contaminants—including pharmaceuticals—can induce subtle yet profound effects on animal behavior[16,17] and neurobiology.[18,19] Unlike lethal toxicity, these sublethal effects are often cryptic, impairing key behavioral functions in ways that can ultimately reduce survival and reproductive success.[20−22] Notably, a recent global monitoring campaign revealed that seven of the ten most-pervasive pharmaceutical contaminants are neuroactive.[23] These drugs are specifically designed to alter human behavior and mood, and do so by modulating neurophysiological pathways such as the serotonergic, noradrenergic, and dopaminergic systems.[24−27] These pathways are highly conserved across vertebrate taxa[28] and, as

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a result, neuroactive pharmaceuticals can elicit biological responses in exposed wildlife, affecting a wide range of behaviors, from predator evasion[29] and mate attraction[30] to social interaction[31] and migratory movement.[32] However, while the behavioral effects of neuroactive pollutants are increasingly recognized, their impacts on higher-order cognitive processes—such as learning and memory—remain largely unexplored.[33]

Amitriptyline, a tricyclic antidepressant, exemplifies such concerns. This drug is now routinely detected in surface waters globally[23] and has been found to bioaccumulate in the brain tissue of exposed fish.[34] By increasing synaptic levels of serotonin and norepinephrine, amitriptyline alters neural signaling in humans.[35] This is especially relevant given that serotonin acts as a fundamental neuromodulator of behavior and cognition across both invertebrates and vertebrates, regulating processes including learning, memory, and behavioral flexibility.[36] Disruption of serotonergic signaling by amitriptyline exposure may therefore extend beyond neurochemical alterations to impair these higher-order cognitive functions in nontarget wildlife. Moreover, amitriptyline exposure downregulates genes involved in central nervous system development[37] and can alter brain neurotransmitter levels and transcriptional profiles in nontarget aquatic animals (e.g., zebrafish, *Danio rerio*[38]). Despite this mechanistic evidence suggesting the potential for amitriptyline to impair the cognitive functioning of wildlife, empirical verification of its potential to disrupt cognition at field-observed concentrations remains to be tested.

Sex-specific susceptibility to cognitive disruption by neuroactive pollution is an important consideration. Males and females often differ in cognitive abilities and neural architecture, often reflecting divergent ecological roles.[39] Such differences are shaped by hormonal, neurological, and ecological factors, and may influence vulnerability to pollutants.[40] Despite this, most ecotoxicological studies focus on general behavioral or physiological impacts and seldom consider the role of sex in moderating responses to exposure.[33] Understanding these differences is critical, as sex-specific effects may have far-reaching implications for population composition and ecosystem dynamics.[41]

Here, we tested whether exposure to amitriptyline altered spatial learning in wild guppies (*Poecilia reticulata*), a species with well-characterized cognitive abilities[42,43] and pronounced sexual dimorphism.[44] Specifically, using a repeated-trial maze assay, we quantified spatial learning performance in male and female guppies following exposure to low and high environmentally realistic concentrations of amitriptyline. We hypothesized that exposure would impair learning in a concentration-dependent manner, based on amitriptyline's serotonergic activity[35] and the reported cognitive effects of serotonergic disruption in other species.[45] Based on prior studies indicating that male guppies often outperform females in spatial tasks,[46] we expected males in the control treatment to demonstrate more rapid spatial learning by solving the maze faster and more accurately with successive trials. However, given the lack of prior research on this topic, we did not make a directional prediction regarding whether and how amitriptyline exposure would differentially affect female and male guppies.

MATERIALS AND METHODS

We report our Materials and Methods using the "Method Reporting with Initials for Transparency" (MeRIT) framework, which enhances reproducibility by attributing specific methodological tasks to individual contributors.[47]

Study Species

Wild, sexually mature guppies were collected from Alligator Creek, Queensland, Australia (19°23′48.0″ S, 146°56′57.0″ E) by JLM, ERM, and BBMW. Collection followed the Recreational Fishing Rules and procedures outlined by the Queensland Department of Agriculture and Fisheries and was conducted with Monash University animal ethics approval (Project number: 37219). This river system runs through the pristine Bowling Green Bay National Park and has been used in previous studies as an uncontaminated source population based on the absence of other commonly prescribed pharmaceuticals that are known to be neuroactive (e.g., fluoxetine[48]). The fish were transported to Monash University, Melbourne, Australia, by JLM and ERM. In total, 180 individuals (90 females, 90 males) were used in this experiment and were individually housed in 1 L glass tanks, arranged so that each individual had visual access to at least one male and one female conspecific, preserving social visual cues while ensuring each fish constituted a statistically independent replicate. These tanks contained 300 g of gravel substrate (~4 mm in diameter), an artificial plant, and a silicone air-hose. Housing conditions consisted of a 12:12 h light/dark cycle, with the room temperature being maintained between 23−24 °C (measured daily). Fish were fed commercial fish food (FKC Aqua Community Bites Tropical Floating Pellets; 0.8 mm), *ad libitum*, every second day. Fish were acclimated to laboratory conditions for at least 14 d prior to exposure.

Amitriptyline Exposure

Housing tanks were assigned to one of three treatments: control, low amitriptyline, and high amitriptyline. The nominal amitriptyline concentrations for these treatments were 0 ng/L, 30 ng/L, and 300 ng/L, respectively. The low and high amitriptyline exposure concentrations were selected to reflect environmentally relevant levels documented in wastewater-impacted aquatic systems. Specifically, amitriptyline has been detected in surface waters at concentrations ranging from 1−102 ng/L,[49] and at levels up to 335 ng/L in treated wastewater discharged into rivers.[50] Based on these data, our low nominal concentration (30 ng/L) represents a conservative, yet ecologically realistic, level commonly detected in surface waters. The high nominal concentration (300 ng/L), on the other hand, represents concentrations measured in environments receiving direct wastewater inputs, such as near outflows or in effluent-dominated streams.

Fish were individually exposed to their respective treatments in their housing tanks. To achieve the nominal concentrations, we employed a semistatic dosing protocol, where a stock solution was first prepared by dissolving 612.24 μg of amitriptyline hydrochloride (98%; Sigma-Aldrich, CAS: 549-18-8; prepared by JLM) in 1 L of reverse osmosis (RO) water and stored in an opaque glass bottle at 2 °C to minimize degradation. An additional bottle was prepared containing only RO water and stored at 2 °C to act as a freshwater control. Tanks in the high-exposure group received 5 mL of stock solution, while those in the low-exposure group received 0.5 mL, and control fish received 5 mL of RO water (control). On day 6 of the exposure period, the tanks were redosed with 1 mL of stock solution (high), 0.1 mL of stock solution (low), or 1 mL of RO water (control) to offset an estimated 20% loss of amitriptyline due to sorption to the tank and bioaccumulation in the fish. The total length of the exposure period was 11 d, although fish performed the behavioral assays in the same exposure water, meaning that they experienced a total of 15 d of exposure. This dosing schedule was informed by prior work showing that amitriptyline concentrations in static systems can fall an order of magnitude below nominal levels due to sorptive losses.[51] To verify exposure concentrations, 89 water samples (control: $n_{samples}$ = 17, n_{tanks} = 6; low: $n_{samples}$ = 36, n_{tanks} = 12; high: $n_{samples}$ = 36, n_{tanks} = 12) were collected for chemical analysis on days 3, 6, and 9 of the exposure period. On each of these days, 50 mL of water was drawn from all tanks using a 50 mL serological pipet to ensure consistent water volume changes, with only the designated 89

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Environ. Sci. Technol. 2026, 60, 10640−10650

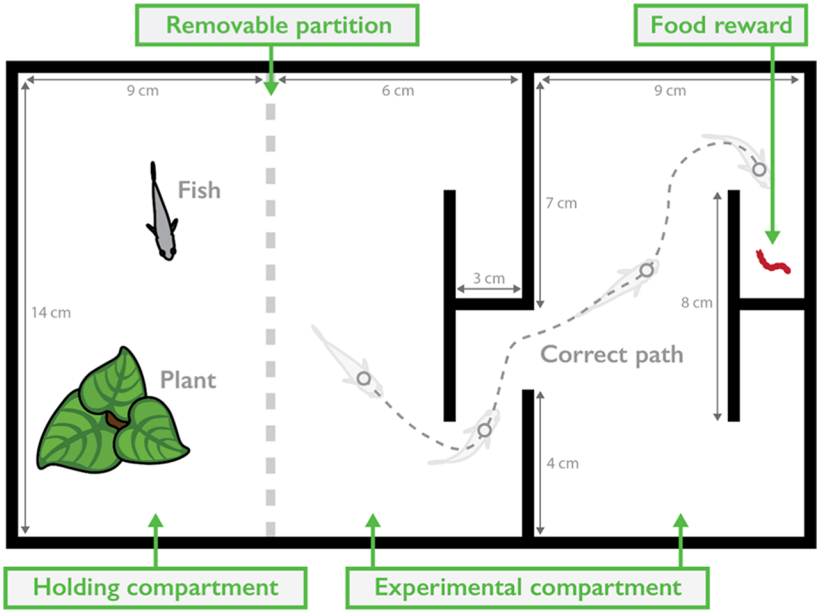

Figure 1. A top-down view of the spatial learning experiment assay. The tank was divided into two compartments using a removable partition: a holding compartment and an experimental compartment. The holding compartment was where the fish resided when not performing experimental trials and contained an artificial plant as enrichment, while the experimental compartment constituted the maze with one-third of a chironomid larva at the end as a food reward.

samples being retained for analysis. Sample selection was balanced across treatment groups, sex, shelving positions, and exposure days. Retained samples were stored at −20 °C in 50 mL conical centrifuge tubes until analysis.

Water samples were analyzed by the commercial environmental testing company Envirolab Services (MPL Laboratories, Perth; NATA accreditation: 2901; ISO/IEC 17025 compliant) using liquid chromatography tandem mass spectrometry (LC−MS/MS) with a limit of quantification (LOQ) of 10 ng/L (see Supporting Information for detailed methodology).

Spatial Learning Task

Following the exposure period, fish were transferred—along with their exposure water, air-hose, and plant—to custom-designed experimental tanks (L × W × H: 24 cm × 14 cm × 14 cm, 3D-printed from PET-G; the design of which is available for download from the associated repositories under Data Availability). These tanks were comprised of two compartments: a "holding" section and an "experimental" section, separated by a manually operated partition (Figure 1). This maze design was adapted from ref 46, where it was successfully applied to assess spatial learning performance in both male and female guppies. Fish remained in the holding compartment for the duration of the 4-d assay, except during spatial learning trials. Fish did not begin the cognitive trials until the next day to allow them to acclimate overnight.

To assess spatial learning performance, guppies were required to navigate a two-choice maze to reach a food reward. This assay tested learning capability over repeated trials, which was quantified as improvements in completion time (the time taken to successfully navigate to the end of the maze) and reduction in the number of errors (the number of times the fish entered an incorrect arm in the maze). The plant and air-hose were removed from the holding compartment 30 min before the beginning of the trial and one-third of a chironomid larva was placed at the end of the maze as a food reward. Throughout the 4-d experimental period, guppies received no food other than these chironomid larvae. Additionally, three drops of chironomid-infused water (prepared by thawing 3 g of frozen chironomid larvae in 40 mL of water before straining out the particulate matter) were dispersed throughout the tank. This was done to homogenize the olfactory cues throughout the tank and ensured that guppies relied on memory rather than scent to solve the maze.

After a 30 min acclimation period, video recording commenced (using Panasonic HC-V180 HD and Sony FDR-AX33 camcorders) and the partitions were removed, allowing access to the experimental compartment (i.e., the maze). The maze contained two decision points, each requiring a choice between a left or right passage. Successful navigation led to the food reward, reinforcing the correct path (Figure 1). Guppies were allowed 15 min to solve the maze, but individuals that failed to reach the food reward within this period were gently guided (using a clear plastic ruler) to the end of the maze, ensuring consistent information regarding the food location and level of satiation.[52] Across the experiment, 10 individuals failed to complete at least one of the 12 trials, with 16 incomplete trials in total. Each fish underwent this trial three times per day (at 9:00, 13:00, and 17:00) over four consecutive days and all trials were run by JLM and ERM. For the final trial, on day four, no food reward was provided in order to further verify that the fish relied on spatial memory and not scent-tracking. To avoid potential side biases, two mirror-image configurations of the maze were used and balanced across treatments. Following the end of the final trial, all fish were humanely killed using MS-222, and their standard length measured.

Due to logistical constraints and, in particular, the need to accommodate a large number of fish, the experiment was conducted in three staggered batches. While one batch underwent exposure, another concurrently completed the cognitive assays. This approach ensured that all fish within a batch were tested on the same day at consistent times, with treatment and sex combinations balanced across batches to maintain equal representation. Although the batches were temporally staggered, they all followed identical exposure protocols, and potential batch effects were accounted for in the statistical analyses.

Statistical Analysis

All videos were tracked by JLM using *idtracker.ai*,[53] after which the trajectories were smoothed with a Savitzky−Golay filter (third-order polynomial with a window size of 13 frames) and cleaned of tracking artifacts using *cleanRfish* (v. 2.0.0).[54] Maze arms and decision zones were defined in R using the sf package (v. 1.1.0),[55] and fish trajectories were assigned to these zones to determine maze position and choices over time. This approach was used to identify incorrect

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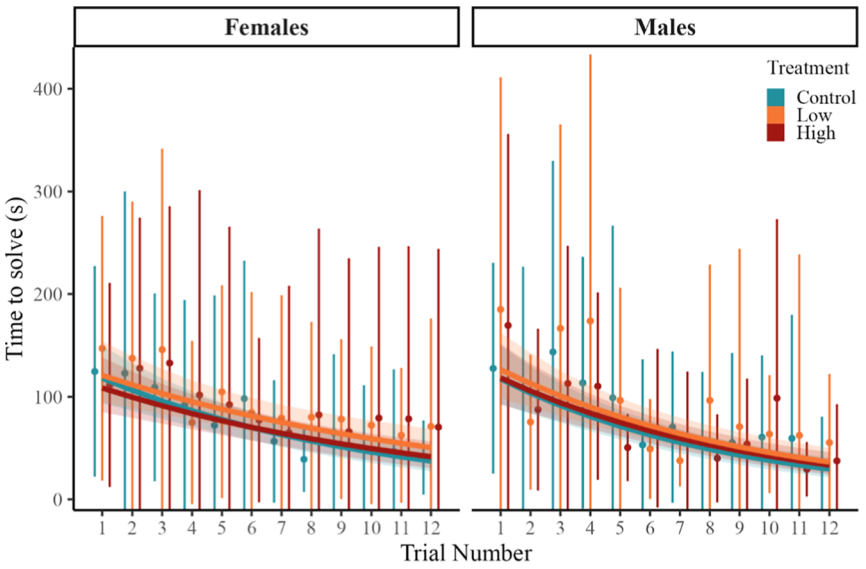

Figure 2. Learning curves illustrating mean solve times across successive maze trials in male and female guppies exposed to control (0 ng/L), low (52 ng/L), or high (496 ng/L) concentrations of amitriptyline. Solid lines represent model-estimated means with shaded ribbons denoting 95% credible intervals. The points and error bars show the raw data means and standard deviations. All groups showed meaningful improvements in solve time across trials, which is indicative of learning. Amitriptyline exposure did not affect learning rates or overall solve times in either sex. Sample sizes: control males = 29, control females = 29; low males = 26, low females = 29; high males = 28, high females = 28.

arm entries and extract decision sequences for each trial. Code for maze construction and spatial classification is available in the associated repositories (see Data Availability). Final trajectories were overlaid on the original videos, and each track was manually verified. For logistical reasons, experimenters were not blind to treatment during exposure and cognitive assays, but automated tracking minimized observer bias.

Statistical analyses were conducted in *R* (v. 4.3.2; R Core Team, 2023) using *RStudio* (v. 2023.09.1; Posit Software, PBC) by JLM. Bayesian statistical models were fitted using the *brms* package (v. 2.22.0[56]) with the *CmdStanR* (v. 0.9.0[57]) backend. Two primary outcomes were analyzed: (1) maze solve time (sec), and (2) the number of incorrect maze-arm entries prior to solving the maze (hereafter, "accuracy"). Solve time was modeled using a generalized linear mixed-effects model (GLMM) with a log-normal distribution. Accuracy was modeled using a negative binomial GLMM to capture overdispersion.

Fixed effects for both models included trial number (the sequential attempt by each fish to solve the maze: 1−12), treatment (control, low, or high amitriptyline), sex, and all of their interactions, as well as maze orientation (left or right solve path), batch (1−3), and fish standard length (center-scaled within sex). Individual identity was included as a random intercept with random slopes by trial to account for the repeated measures of the same individual across all trials. To assess changes in behavioral consistency across trials and treatment groups, we modeled the dispersion (variance) structure of the negative binomial distribution via its shape parameter (sigma). Treatment, sex, their two-way interaction, and trial number were included as fixed effects, while individual identity was included as a random intercept. In this context, higher shape values correspond to lower between-individual variance in the number of errors. We used posterior draws from the model to evaluate how this parameter varied across successive trials, between sexes, and treatments.

Priors for regression coefficients were weakly informative and biologically grounded. For fixed effects, Gaussian priors (mean = 0, SD = 2) were used, ensuring plausible ranges of fold-changes in solve times and error rates. Furthermore, the final models chosen for analysis were rerun with flat (noninformative) priors and resulted in no meaningful changes in posterior distributions or mean estimates. Posterior predictive checks confirmed adequate model fits and model selection was also informed by leave-one-out cross-validation (LOO-CV).[58]

Posterior estimates were summarized as estimated marginal means using the *emmeans* (v. 1.11.2[59]) and *tidybayes* (v. 3.0.7[60]) packages. Pairwise contrasts between treatment groups and sexes were computed and the 95% credible intervals (CrIs) of these contrasts were used to evaluate the strength and direction of the differences. We interpreted contrasts as providing strong evidence of meaningful differences when the 95% CrIs did not overlap zero, and as suggesting weaker evidence when CrIs only marginally overlapped with zero.[61]

We began with 180 fish, of which ten died during the acclimation and exposure periods (control: n = 2; low amitriptyline: n = 5; high amitriptyline: n = 3), leaving 170 fish for behavioral testing. Trials were excluded a priori if a fish "misstarted" by squeezing under the partition and entering the maze before the scheduled start (i.e., before video recording). One individual misstarted in all 12 trials and was excluded from the analysis. The analyzed data set therefore comprised 169 fish and 1938 trials. Of these fish, 86 were female (mean standard length = 18.78 mm, SD = 3.47) and 83 were male (mean standard length = 15.86 mm, SD = 1.26). Of the included trials, 16 were right-censored at 15 min because the fish did not reach the reward within the allotted time.

RESULTS

Chemical Analysis

Measured water concentrations of amitriptyline confirmed clear separation among the treatments. The low treatment had a mean amitriptyline concentration of 52.47 ng/L (SD = 35.30, n = 36), while the high treatment had a mean of 496.44 ng/L (SD = 249.93, n = 36). No amitriptyline was detected in any of the control tanks (detection limit: 10 ng/L; n = 17).

Solve Time

Across all treatment groups, guppies exhibited clear learning, with solve times decreasing across successive trials (Figure 2; Table S2). The global learning rate was estimated at −6.88 s per trial [95% CrI: −7.90 to −5.88]. In other words, with each successive trial, fish solved the maze ∼7 s faster than their previous attempt. The global average solve time declined from 119.07 s [CrI: 107.90, 132.38] in the first trial to 38.37 s [CrI: 33.66, 43.77] in the final trial. There was no strong evidence

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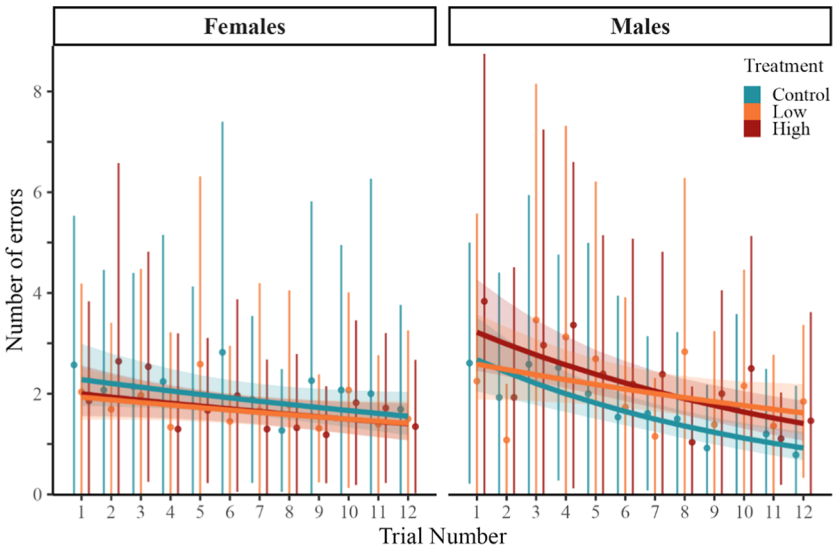

Figure 3. Learning curves illustrating the mean number of errors across successive maze trials in male and female guppies exposed to control (0 ng/L), low (52 ng/L), or high (496 ng/L) concentrations of amitriptyline. Solid lines represent model-estimated means with shaded ribbons denoting 95% credible intervals. The points and error bars show raw data means and standard deviations. Males in the control treatment showed marginally greater improvements in accuracy across trials than females, resulting in meaningfully fewer errors from trial eight onward. However, male learning rates were reduced by amitriptyline exposure in a dose-dependent manner, with high-exposed males ultimately performing worse than females. Conversely, female accuracy was not strongly affected by amitriptyline exposure. Sample sizes: control males = 29, control females = 29; low males = 26, low females = 29; high males = 28, high females = 28.

that amitriptyline exposure influenced learning rate or absolute solve times, and learning trajectories were comparable across the treatment groups (Figure 2; Tables S3 and S5). Similarly, no sex differences were observed in either learning rate or absolute solve time (Figure 2; Tables S3 and S5). Maze orientation, batch, and fish standard length also did not meaningfully influence solve time (Table S6).

Maze-Navigation Accuracy

Guppies improved in accuracy with experience, making fewer incorrect choices with successive trials (Figure 3). Moreover, treatment and sex interacted strongly with accuracy outcomes.

In control fish, both sexes made a similar number of errors in early trials. However, from trial eight onward, males made meaningfully fewer errors than females (Figure 3). By the final trial, control males averaged 0.92 errors [CrI: 0.66, 1.20] compared to 1.56 errors [CrI: 1.18, 1.99] in control females—a difference of 0.63 [CrI: 0.15, 1.13]. There was weak evidence to suggest that males improved their accuracy at a greater rate than females. Control females marginally improved by −0.07 errors with each trial [CrI: −0.14 to 0.01], while control males improved by −0.15 [CrI: −0.22 to −0.09], a between-sex difference in learning rate of 0.09 errors per trial [CrI: −0.01 to 0.18].

Among males, amitriptyline exposure concentration was associated with reduced accuracy. In the early trials, there were no meaningful differences between the treatment groups (Table S9). However, temporal patterns revealed that low-exposed males began making meaningfully more errors than control males from trial seven onward, while high-exposed males made meaningfully more errors from trial five onward (Table S9). When averaged across all trials, low-exposed males made 0.42 more errors [CrI: −0.07, 0.93] than controls, while high-exposed males made 0.55 more errors [CrI: 0.06, 1.06]. This equated to 26.35% [CrI: −3.71, 62.93] and 34.21% more errors [CrI: 3.05, 72.63] on average, respectively, though the difference between control and low-exposed males was only weakly supported. For females, however, amitriptyline exposure did not appear to affect the number of errors made in each trial or the learning rate (Figure 3; Tables S8 and S9).

The interaction between sex and treatment modulated the pattern of learning. As mentioned above, males in the control treatment were more accurate when solving the maze. However, this sex difference was absent in the low amitriptyline exposure (Table S8) and, under high exposure, males actually performed worse than females, making meaningfully more errors for the first six trials, after which the difference diminished (Figure 3; Table S8). In contrast to the control fish, low-exposed males did not learn at a faster rate than females (see Table S8 for contrast). However, high-exposed males improved at a greater rate than high-exposed females—specifically, males improved at a rate of −0.16 errors [CrI: −0.25, −0.07] with each subsequent trial, while females improved at a rate of −0.05 errors [CrI: −0.11, 0.01] with each subsequent trial. This equates to a difference in learning rate of 0.11 errors [CrI: 0.00, 0.21] with each subsequent trial. Maze orientation, batch, and fish standard length had no meaningful effect on accuracy (Table S12).

Within-Individual Variation (Behavioral Consistency)

Within-individual variation declined across successive trials, indicating that fish became more behaviorally consistent with experience. This was reflected as a positive trend in the shape parameter (increase of 0.10 per trial [95% CrI: 0.06, 0.14]), corresponding to reduced overdispersion and greater trial-to-trial stability. Contrasts of within-individual variance weakly suggested that males exposed to amitriptyline were less consistent than control males, with both low- and high-exposed males showing marginally greater variability across trials (contrasts of variance estimates: control−low = 2.80 [−0.19, 6.89], control−high = 2.59, [−0.23, 6.18]; Tables S13 and S14). In biological terms, this means that while most of the control males responded fairly consistently from trial to trial, exposed males tended to fluctuate more in their accuracy,

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though this is only partially supported. In females, there is weak evidence to suggest that the opposite is true, whereby females from the high treatment showed less variability than control females (a difference of 1.38 [CrI: −0.08, 3.40]). Between-sex comparisons revealed that males and females from the control treatment exhibited the same amount of within-individual variance (a difference of −0.47 [CrI: −2.61, 1.70]; Table S14), but both low- and high-treatment males were meaningfully more variable than females (low: 2.84 [CrI: 0.34, 7.02]; high: 3.38 [CrI: 1.24, 7.09]; Table S14).

Among-Individual Variation

Posterior distributions revealed strong individual differences in baseline accuracy, with substantial variation in mean error rates across individuals. The standard deviation of individual intercepts was 0.28 [CrI: 0.06−0.45], corresponding to approximately a 2-fold difference (estimated fold change of 2.02 [CrI: 1.16−3.15]) in the mean number of errors made between low- and high-accuracy fish. By contrast, there was little evidence for among-individual heterogeneity in the learning rate (rate of improvement in accuracy; SD = 0.03 [CrI: 0.00, 0.05] on the log error scale), and no intercept−slope correlation (correlation estimate = 0.01 [CrI: −0.75, 0.94]), indicating no systematic pattern whereby initially inaccurate individuals either caught up (compensatory learning) or fell further behind (amplifying learning). In other words, some individuals were consistently accurate, while others were more error-prone. However, the rate of improvement was comparable across individuals, conditional on sex and treatment. Overall, these results demonstrate robust personality-like variation in baseline performance, but weak or absent variation in learning capacity across individuals, after accounting for sex and treatment effects.

DISCUSSION

Here, we investigated whether environmentally relevant concentrations of the neuroactive pollutant amitriptyline impair the cognitive performance of fish in a spatial learning task, and whether males and females differed in their susceptibility to such impairment. Our findings provide strong evidence for spatial learning in guppies, as demonstrated by reduced solve times and fewer navigational errors across repeated maze trials. Improvement in solve time was comparable across sexes. However, males made fewer errors than females with increasing maze experience and improved at a marginally faster rate. Notably, cognitive impairment was sex specific. On average, males exposed to low and high concentrations of amitriptyline made approximately 26% and 34% more errors than control males, respectively. Thus, males in the low exposure group made a comparable number of errors to females, while males in the high exposure group made more errors than females—losing the accuracy advantage seen under control conditions. Individuals generally became more consistent in their behavior with successive trials. Furthermore, while control females and males showed comparable among-individual variation, males displayed more variation in their accuracy, from trial to trial, than females in both the low and high treatments.

Males in the control treatment made fewer navigational errors than females and showed marginally greater improvements in accuracy across trials, which is indicative of enhanced spatial cognitive performance. This difference in errors is unlikely to be driven by exploratory tendencies, as both sexes made a comparable number of errors in early trials—when exploratory tendencies would be most apparent—and prior studies have found no consistent sex differences in exploration in guppies.[62] Male-biased spatial cognitive performance has been reported across a broad range of taxa, including mammals,[63] reptiles,[64] amphibians,[65] and ray-finned fish.[46] These differences are often attributed to ecological divergence between the sexes. In guppies, males and females occupy distinct behavioral niches—males independently engage in active mate searching, frequently traversing complex and variable environments on their own in pursuit of mating opportunities,[66] while females typically shoal and navigate socially.[67] This increased navigational demand may provide males with more frequent and repeated exposure to solitary spatial challenges, enhancing their task performance through learning and experience. Lucon-Xiccato and Bisazza[46] suggest that such sex differences in guppies' spatial ability may reflect adaptive specializations. However, recent comparative analyses have questioned this interpretation, suggesting that there is little consistent evidence that sex differences in spatial cognition are evolutionary adaptations.[68] Instead, they propose that performance differences may arise from nonadaptive processes such as phenotypic plasticity or sex-linked physiological variation.[68] In this context, our findings may reflect experience-driven cognitive plasticity shaped by ecological behavior, rather than fixed, evolved cognitive traits.[69] This interpretation is plausible given that the guppies used in our study were wild-caught adults and, as a result, would have accumulated substantial navigational experience in their natural environment. To disentangle the relative roles of innate versus experience-driven mechanisms, future studies could rear male and female guppies under common garden conditions, thereby isolating the genetically based adaptations from developmental plasticity.[20] Apart from the role of experience, biological mechanisms may also contribute to cognitive sex differences. Hormonal modulation, especially via androgens and estrogens, has been shown to influence spatial learning and memory in other vertebrates.[70] For instance, exogenous testosterone administration has been shown to improve spatial cognitive performance in both rats[71] and humans.[72] Therefore, although the neuroendocrine basis of cognitive sex differences in guppies is not fully understood, different hormone profiles could possibly shape the development or expression of spatial abilities across sexes.

Amitriptyline exposure impaired spatial cognition, as evidenced by reduced learning accuracy. Solve times, however, were unaffected, suggesting that exposed fish remained motivated and physically capable but committed more navigational errors—a pattern consistent with deficits in memory and decision-making.[73] A subsequent analysis of total distance traveled confirmed that high-exposed males covered greater distances before reaching the reward (see Supporting Information), ruling out hesitation or repeated back-and-forth movements at decision points as an explanation. Instead, their higher average velocity compensated for the greater distances, effectively offsetting the time cost of navigational errors and producing equivalent solve times across treatments. These impairments in learning accuracy align with our mechanistically derived hypothesis that amitriptyline would alter serotonergic and anticholinergic signaling[74]—neurochemical systems that are critical for cognitive function.[75] In fish, the telencephalon—specifically the dorsolateral telencephalon—plays a central role in spatial learning and

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Environ. Sci. Technol. 2026, 60, 10640−10650

memory.[76,77] Importantly, amitriptyline is known to bioaccumulate in fish brains,[34] with evidence of preferential accumulation in the telencephalon,[78] making this the likely site of action. Although large-scale neural damage is unlikely following short-term exposure, functional disruption of monoaminergic signaling in this region offers a potential explanation for the observed cognitive deficits. Similar impairments have been documented following exposure to other neuroactive pollutants; for example, chlorpyrifos exposure disrupted dopaminergic signaling and impaired spatial learning in zebrafish.[79] However, further work is needed to investigate the transcriptomic and neurochemical changes induced by amitriptyline exposure, which may explain the mechanistic basis of the perturbations observed here. Nonetheless, our results demonstrate that even low, environmentally relevant concentrations of neuroactive pharmaceuticals can acutely disrupt the neural systems that underpin learning and memory. Such cognitive disruption is likely to carry direct fitness consequences, as poor spatial performance in other species has been linked to higher predation risk and decreased survival.[9] Moreover, similar cognitive disruptions caused by other anthropogenic stressors—such as heat waves—have been shown to impair key behaviors, such as mate selection and antipredator responses in guppies,[80] reinforcing the idea that disruptions to spatial cognition can propagate into fitness costs.

Amitriptyline exposure impeded spatial learning in males but not females, revealing a clear sex-specific effect. Although regularly overlooked in behavioral ecotoxicology,[33] studies that do consider sex often uncover a broader pattern of sex-specific responses to contaminants. For instance, exposure to the antidepressant fluoxetine has been shown to induce sex-specific disruption of antipredator behavior in eastern mosquitofish (*Gambusia holbrooki*[29]) and inhibitory control in guppies.[81] Comparable patterns are evident in humans, where the therapeutic use of neuroactive drugs is associated with sex-specific cognitive side-effects.[82] These divergent responses likely arise from fundamental sex differences in two core toxicological domains: toxicokinetics (how contaminants are absorbed, distributed, metabolized, and excreted) and toxicodynamics (how contaminants interact with molecular targets to produce biological effects). In this regard, insights into the role of toxicokinetics and toxicodynamics in driving sex-specific responses have been reported in male and female roach (*Rutilus rutilus*) exposed to a mixture of neuroactive pharmaceutical pollutants, including amitriptyline.[78] In that study, both sexes accumulated comparable drug concentrations in the same brain regions, suggesting similar toxicokinetics, but only males exhibited significant neurochemical disruption, including altered serotonin levels and other sex-specific shifts in neurotransmitter profiles. These findings suggest that toxicodynamic differences may explain the observed sex-specific cognitive effects we found in guppies and highlight the importance of integrating sex as a critical variable in behavioral ecotoxicology.

Sex-specific effects can drive sex-biased selection pressures that ultimately skew population sex ratios. Such imbalances are known to reduce breeding success, hinder population growth, and erode genetic diversity, thereby compromising long-term population viability.[83,84] Moreover, disruption to normal cognitive function can impair key behaviors, such as foraging,[85] mate attraction,[86] and predator evasion.[87] These behavioral perturbations can cause disruptions to trophic interactions and, thus, can permeate through food webs.[88] Consequently, even subtle sex-specific effects at the individual level can cascade into broader shifts in community dynamics, ultimately affecting ecosystem processes. As such, ignoring sex-based variation risks overlooking significant contaminant effects and underestimating ecological and population-level impacts.

Across trials, fish became increasingly consistent in their cognitive performance, where within-individual variation declined as experience accumulated. Effects of amitriptyline exposure on behavioral consistency were weak and sex dependent. Exposed males tended to show greater trial-to-trial variability than controls, whereas females displayed the opposite trend, with individuals in the high-amitriptyline treatment appearing marginally more consistent than controls. Although evidence for these patterns was limited, their combined effects produced notable sex differences: both low- and high-exposed males were consistently more variable than females, whereas no such difference was present in controls. Our findings suggest that short-term amitriptyline exposure may weakly modulate within-individual variation, driving a divergence in the cognitive variation of male and female guppies. Comparable patterns have been observed with other neuroactive pollutants, such as fluoxetine, where multigenerational exposure altered within-individual variation in guppies.[89] Importantly, these effects were sex- and task-specific, and sometimes acted in opposing directions depending on the behavior examined.[89] Taken together, this emerging body of work indicates that neuroactive pollutants can shape cognition and behavior, influencing both mean responses and trait variation, where outcomes may be contingent on sex and the behavioral or cognitive domain under study. More broadly, our results add to growing recognition that behavioral and cognitive variation itself represents a sensitive axis of response to environmental change.

Beyond within-individual consistency, our results reveal meaningful among-individual variation in baseline cognitive performance. The approximately 2-fold difference in mean error rates between the least accurate and most accurate individuals, combined with stability of this variation across trials, is consistent with cognitive personality-like variation.[90] Notably, individual differences in absolute accuracy were not mirrored by equivalent variation in learning rate, suggesting that personality-like variation is expressed in absolute performance rather than in the capacity to improve. In other words, some fish are consistently more accurate and some more error prone, but they appear to learn at a comparable rate.

Overall, our results demonstrate that exposure to environmentally realistic levels of neuroactive pharmaceutical pollution can disrupt spatial cognitive performance in wildlife. While both male and female guppies learned to navigate a maze, males exposed to amitriptyline exhibited impaired accuracy and learning, suggesting heightened sensitivity to pharmaceutical-induced cognitive disruption. These results emphasize the need for behavioral ecotoxicology to integrate sex-specific analyses and to consider cognitive processes as a vital end point in assessing environmental contaminant impacts.

■ ASSOCIATED CONTENT

Data Availability Statement

All data and code (R script) used to produce this work are publicly available on J.L. Manera's GitHub repository (https://

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Environ. Sci. Technol. 2026, 60, 10640−10650

github.com/JLManera/Spatial_learning_Monash). They are also available on the OSF framework: https://osf.io/ph37s/?view_only=c4feed1d480c4972b613d73691d29b48.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.6c00552.

Detailed analytical chemistry methodology; supplementary analyses of treatment effects on distance traveled and swimming velocity; supplementary tables of model coefficients, marginal estimated means, marginal trends, and pairwise contrasts for all response variables (Tables S1−S15) (PDF)

EthoCRED reporting guidelines (XLSX)

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Author Contributions

#J.M.M., M.G.B. and B.B.M.W. co-senior authors. Following the Method Reporting with Initials for Transparency (MeRIT) guidelines,[47] we have reported what processes were conducted by certain members of the author team, to improve the granularity of author contributions as well as to enhance reproducibility and replicability (see Materials and Methods). Here, we report a list of each author's roles using the Contributor Role Taxonomy (CRediT[91]). Jack L. Manera: Conceptualisation, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Visualization, Writing−Original Draft Preparation, and Writing—Review & Editing. Eleanor R. Moore: Conceptualisation, Investigation, and Writing—Review & Editing. Jake M. Martin: Supervision, Visualization, and Writing—Review & Editing. Michael G. Bertram: Funding acquisition, Resources, Supervision, and Writing—Review & Editing. Bob B.M. Wong: Conceptualisation, Funding acquisition, Methodology, Project administration, Resources, Supervision, and Writing—Review & Editing.

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Notes

The authors declare no competing financial interest.

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