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Sex-specific Cognitive Effects of Environmentally Relevant Microplastic Exposure in the Western Mosquitofish

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Abstract

Microplastics (MPs) are pervasive aquatic pollutants, yet their behavioral and cognitive effects in wild fish, particularly across sexes and environmentally relevant exposures, remain poorly understood. This study examines the effects of polyethylene terephthalate (PET) microfiber exposure on cognition, behavior, and body condition in male and female western mosquitofish (*Gambusia affinis*). Individuals were exposed to either control (0 MPs/L), low (10 MPs/L), or high (1000 MPs/L) MP treatments for 14 days. Using behavioral and cognitive assays, fish were assessed in inhibitory control, spatial learning, shyness, exploration, social motivation, feeding behavior, and aquatic surface respiration. We found that MP exposure elicited sex-specific cognitive impairment without affecting behavior or condition. Cognitive sex differences were suppressed under MP exposure. Under control conditions, males outperformed females in inhibitory control while females outperformed males in spatial learning. Under MP exposure, males exhibited a reduction in inhibitory control and females exhibited a loss in spatial learning resulting in no sexually dimorphic performance in either cognitive domain. Our results demonstrate that environmentally relevant MPs selectively impaired cognitive performance in a sex- and trait-specific manner without altering behavior or condition. Our findings highlight the importance of incorporating sex, dose, and cognitive endpoints when evaluating the consequences of MP pollution.

Introduction

Over the last century, a widespread and unprecedented class of pollutants has emerged. Microplastics (MPs), defined as plastic particles < 5 mm in size, are of increasing concern for both human health and the environment [1–3]. Aquatic environments are particularly vulnerable, as the small size, low density, and relatively inert chemistry of MPs allow them to permeate through and sequester in water bodies [4,5]. There, aquatic organisms inhale and ingest MPs, facilitating bioaccumulation and biomagnification across trophic levels [3]. MPs have been documented across bodily organs and tissues of wild fish, sea birds, and marine mammals where their presence is associated with suites of physiological consequences including lesions, inflammation, and tissue damage [6].

Although research on the toxicity of MPs is nascent, several studies have identified negative effects across traits in aquatic animals [6]. Physiological studies have found that MP exposure can cause inflammation and oxidative stress altering development, reproduction, metabolism, condition, and even induce mortality across taxa [3,6]. However, fewer studies have investigated the relationship between MP exposure and behavioral or cognitive changes. Physiological, behavioral, and cognitive processes are all tightly interlinked, and determine an organism's internal state and ecological interactions. Behavioral and cognitive traits, underpin processes like resource acquisition, predator avoidance, and reproduction. These processes consequently influence energy expenditure and body condition. Many pollutants have been found to alter behavior and cognition, potentially reducing the life-time fitness of animals in the wild [7], suggesting MPs should interact similarly. Despite these theoretical connections, only recently have studies begun to examine how MP exposure might impact these traits.

As of now, studies suggest that MPs have diverse and highly context-dependent behavioral and cognitive consequences. Some behavioral responses may result directly from the physical effects of MPs, as particles can accumulate in the gills and gastrointestinal tract, potentially impairing respiration and feeding [8,9]. In several fish species, various MPs disrupt movement, activity, and exploratory behaviors, as well as shoaling and shyness behavior, although the mechanisms underlying many of these behavioral changes remain unclear [10–13]. For instance, in juvenile convict cichlids (*Archocentrus nigrofasciatus*) and European hermit crabs (*Pagurus bernhardus*), polyethylene MPs alter exploration and reduce decision-making ability [14,15]. Meanwhile, other studies have reported no effects of MP exposure on behavioral or cognitive traits, even under comparable exposure conditions [16,17].This variation suggests that the behavioral and cognitive effects of MPs are highly context-dependent and may be influenced by factors such as sex, MP exposure characteristics, and species. Further experimental studies that explicitly account for these sources of variation are therefore needed to identify when and under what conditions MPs alter behavior and cognition.

Given the nascency of the field, very few MP exposure studies have explicitly included sex as a moderator [18], or employed environmentally realistic microplastic characteristics or concentrations [19,20]. Males and females of the same species can differ in physiology, reproductive investment, and behavior, all of which can strongly influence their vulnerability and responses to environmental stressors. Sex differences in behavioral strategies and cognitive abilities [18,21–23] may translate into sex-specific susceptibility or resilience to pollution. Sex-specific responses can also obscure treatment effects when males and females respond in opposite directions, potentially causing overall treatment effects to appear weak or absent when sexes are analyzed together [18]. Additionally, many environmental pollutants produce dose-dependent responses in organisms, making it difficult to extrapolate effects from unrealistically high exposure concentrations. MP toxicity can depend on physical and chemical characteristics, including particle size, shape, polymer type [4]. Failure to include both sexes and environmentally relevant MP characteristics therefore limits our ability to predict how MPs affect animals in their natural environments.

To address these gaps, we used the western mosquitofish (*Gambusia affinis*) as a model system to test aquatic MP exposure on behavioral and cognitive traits. *G. affinis* is among the most widespread invasive fish species globally and is especially common in urban freshwater environments, where MP contamination is prevalent [24,25]. The invasive success of *G. affinis* is partly attributed to its broad physiological tolerance, which allows persistence in heavily polluted environments. The global distribution of *G. affinis* allowed us to use reported MP concentrations from freshwater systems compiled from reviews and biomonitoring studies to inform environmentally relevant exposure scenarios. As a dominant low-trophic-level consumer and a prey item for avian and aquatic predators, mosquitofish populations play a substantial role in freshwater ecosystems [24]. Thus, understanding how MPs affect behavior and cognition in *G. affinis* may provide insight into how pollution influences heterospecific and community level interactions. *G. affinis* is additionally well suited for examining sex-specific effects because it exhibits pronounced sexual dimorphism in morphology, behavior, and

cognition. Male *G. affinis* are smaller, bolder, and more socially motivated, whereas females are larger, mature more slowly, and outperform males in some spatial-learning tasks [26,27] but not others [28]. These established differences provide a basis for generating sex-specific predictions and allow multiple behavioral and cognitive assays to be used to test whether MP exposure alters these traits differently between sexes.

Here we tested whether environmentally relevant microplastic exposure alters behavioral, cognitive, and physiological traits in both sexes of the western mosquitofish (*G. affinis*). Building on evidence that MPs can modify behavioral and cognitive traits [9–12,15], we exposed mixed-sex groups of wild-caught *G. affinis* to 42 µm polyethylene terephthalate (PET) microfibers at three concentrations: 0 MPs/L (control), 10 MPs/L (low), and 1,000 MPs/L (high) for 14 days (Fig. 1). We examined whether MP exposure affected several behavioral traits, including shyness, social motivation, exploration, aquatic surface respiration, feeding behavior, as well as relative body condition (Kn). We also assessed cognitive traits, including inhibitory control and spatial learning. We hypothesized that MP exposure would impair cognitive performance, alter behavioral traits such as shyness, social motivation, and exploration, increase aquatic surface respiration and feeding activity, and reduce relative condition. We further hypothesized that effects would be sex-specific and dose-dependent. Our study addresses limitations of previous research by comparing sexes and improving the environmental relevance of MP exposure conditions, while simultaneously testing behavioral and cognitive traits in wild-caught fish.

Results

174 individuals were maintained throughout the experiment and had sufficient data collected to be included in analyses (Fig. 1). However, some observations were unavailable because of mortality, procedural errors during behavioral assays, and video file corruption, resulting in some sample size inconsistencies. As a result, the final sample sizes were n = 152 for detour reaching (assay to assess social motivation and inhibitory control), n = 148 for scototaxis (assay to assess shyness and exploration), n = 119 for route learning (assay for spatial learning), n = 142 for feeding behavior (assay for aquatic surface respiration and latency to feed), n = 144 for condition factor (standard length and wet weight measurements), and n = 107 exposed individuals were used for MP retention by treatment & sex (Table 2).

Table 2
Microplastics retained in body by treatment and sex

Treatment	Sex	Mean MP ± SD	MP-positive fish (%)	MP-positive fish (n)
High	Female	1.29 ± 1.08	75.00%	18/24
High	Male	0.64 ± 0.74	51.50%	17/33
Low	Female	0.85 ± 0.86	59.30%	16/27
Low	Male	0.83 ± 1.19	43.50%	10/23

Microplastic Retention, Feeding Behavior & Relative Condition Factor

PET microfibers stained with Nile Red were quantified for each individual sample [29]. MPs were identified in 61 exposed individuals out of 109 exposed individuals' total. Among exposed fish, individuals retained 0.76 ± 0.93 MPs per individual on average at low exposure concentrations and approximately 1.02 ± 0.88 MPs per individual on average at high exposure concentrations, with an overall mean of 0.9 ± 0.9 MPs per individual (Table 2). Nile-Red stained MPs were not detected in any control individuals.

We found that the total microplastics retained across the brain, gills, GI tract, and gonads after a 24-hour depuration was not predicted by sex, treatment, or their interaction (all $p > 0.3$), GLMM (negative binomial; n = 106). Furthermore, the inclusion of these variables did not improve the fit of the model (likelihood-ratio test: $\chi^2_4 = 9.15$, $p = 0.058$; $\Delta AIC = 1.14$). Due to low detection frequency in brain and gonad MPs, we did not perform a formal analysis to identify differences in MP retention by organ type. However, we found that MPs were retained after 24 hours of depuration in each organ type in at least one individual in the dataset. Descriptively, we found that the gill rakers (45.2% MP-positive) and GI tract (22.9% MP-positive) retained microplastics consistently (Table 1), while the brain and gonads did not (< 10% MP-positive).

Table 1
Microplastics retained in body by organ type

Organ	Mean MP ± SD	MP-positive fish (%)	MP-positive fish (n)
Brain	0.08 ± 0.31	7.0%	7/109
Gill Rakers	0.48 ± 0.56	45.2%	47/109
Gonads	0.12 ± 0.41	9.3%	9/109
GI Tract	0.23 ± 0.42	22.9%	24/109

We found no effect of MP on feeding behavior for either sex. Probability of feeding and aquatic surface respiration time did not differ between the sexes or treatments (stats, Fig. 2a,b). Relative condition factor (K_n) did not differ by sex, treatment, or their interaction (all $p > 0.1$), GLMM (Gamma family, log-link; n = 144; Fig. 2c). Consistent with these results, a likelihood-ratio comparison indicated that the full model was not a significantly better fit than the corresponding null model containing only tank as a random effect ($\chi^2_5 = 7.38$, $p = 0.194$).

Detour Reaching Task: Inhibitory Control & Social Motivation

Detour solve latency, our metric of inhibitory control, was significantly predicted by sex ($\beta = -0.635$, $p = 0.032$) and by the interaction between sex and the low treatment ($\beta = 1.216$, $p = 0.0098$) in a GLMM (Gamma family, log link; n = 152). Tank identity explained additional variation in solve latency (variance =

0.031, SD = 0.176; 33 tanks), supporting its inclusion as a random effect. The full model provided a significantly better fit to the data than a null model containing only the random effect of tank identity (likelihood ratio test: $\chi^2_5 = 11.46$, p = 0.043). Post hoc comparisons of estimated marginal means revealed that in the control treatment, females exhibited significantly longer detour solve latencies than males (ratio = 0.635, p = 0.032, Fig. 3a), indicating greater inhibitory control in males under baseline conditions. However, these sex-differences did not persist under low treatment (ratio = -0.388, p = 0.213) or high treatment (ratio = 0.484, p = 0.158). Low treatment males exhibited significantly longer detour solve latencies than high treatment males (ratio = 0.391, p = 0.015; Fig. 3a) and marginally significant longer detour solve latencies than control males (ratio = 0.483, p = 0.051)., The difference between control and low-treatment males was highly significant (ratio = 0.308, p = 0.0002) when we excluded individuals that failed to solve the detour task (non-solvers). In contrast, females did not differ in detour solve latency across treatments (all p > 0.78, Fig. 3a).

Social latency, the latency to approach the glass barrier and the conspecific reward—a proxy for social motivation, was not significantly influenced by microplastic treatment, sex, or their interaction (all p > 0.19) in a GLMM with a Gamma error distribution and log link (n = 151). Variation among tanks explained a substantial proportion of the total variance (variance = 0.188, SD = 0.433; 33 tanks), indicating that social motivation may have been influenced by the social environment or housing conditions. Post hoc comparisons of estimated marginal means revealed no significant sex differences in social latency within any treatment (control: ratio = 1.05, p = 0.82; high: ratio = 1.04, p = 0.89; low: ratio = 0.70, p = 0.12; Fig. 3b), suggesting no sexual dimorphism in social motivation. Additionally, social latency and detour solve latency were not significantly correlated (Spearman's rank correlation: $\rho = 0.07$, p = 0.35), indicating that individual differences in inhibitory control were independent of motivation to engage with the social reward.

Scototaxis Assay: Shyness & Exploration

Our measurement of shyness (total time spent in the black zone in scototaxis) revealed no differences across treatment or sex (all p > 0.09) in a Gaussian GLMM (n = 148). Similarly, residual variance, tested in a dispersion model, did not differ among treatments or between sexes (all p > 0.11). Post hoc comparisons of estimated marginal means revealed no significant differences in shyness between males and females in the control or high treatments (both p > 0.14). A significant difference was detected in the low treatment, with females exhibiting greater shyness than males (mean difference = 51.2 s, p = 0.044; Fig. 4a). However, because the interaction between sex and treatment was not significant in the overall model, this result should be cautiously interpreted. The full model including tank identity as a random effect was a significantly better fit to the data than the null model (likelihood ratio test: $\chi^2_{10} = 60.45$, p < 0.001).

We found a significant sex difference in exploration that was not affected by MP exposure. Specifically, exploration, measured as total distance moved, was significantly predicted by sex ($\beta = -401.9 \pm 171.0$, p = 0.019), but not by treatment or treatment * sex interaction (all p > 0.35). Females moved significantly

greater distances than males across treatments (EMM mean difference = 444 cm, p = 0.0001; Fig. 4b). Variation among tanks was negligible (variance < 10^{-11}). Additionally, the full model structure was significantly better than the null model containing only the random effect of tank identity (likelihood ratio test: $\chi^2_5 = 16.65$, p = 0.005).

Figure 4.

Route Learning: Spatial Learning

We observed a significant decline in participation in trial 5 of our route learning assays (participation in trial 1: 78.0%, trial 2: 83.1%, trial 3: 68.8%, trial 4: 65.3%, trial 5: 51.6%) — a common issue in poeciliid fishes [30,31]. As Trial 5 participation levels were below our threshold of participation (65%), we excluded trial 5 in our error slope analyses (Fig. 5a). Hence, spatial learning was measured as the slope of the proportion of errors made over four trials (ΔP_e) through the route learning maze. Our criteria for spatial learning required that individuals exhibited a significant reduction in errors across trials (e.g. a slope of the proportion of errors (ΔP_e) significantly lower than 0). We found that only the control female group learned the spatial maze, exhibiting a ΔP_e significantly lower than 0 ($\beta = -0.055$, p = 0.008; n = 119, GLMM Gaussian family; Fig. 5b); while females exposed to both low and high MP did not (p > 0.2). Meanwhile, males in the control, low MP, and high MP treatment did not reach our criterion for learning (p > 0.2). Treatment groups and sexes did not significantly differ from one another in ΔP_e (Wald test, holm correction).

Cognitive-Behavioral Syndromes

To assess potential cognitive–behavioral syndromes, we examined Spearman's rank correlations among cognitive and behavioral traits within each sex and treatment group. Control females displayed a negative correlation between exploration and shyness ($\rho = -0.39$, p = 0.02; Supplementary Fig. S1). However, MP- exposed females revealed no significant correlations across any traits. In contrast, control males exhibited no significant correlations across traits, while low treatment males showed a positive correlation between social latency and shyness ($\rho = 0.51$, p = 0.02), and high treatment males exhibited positive correlations between shyness and spatial learning ($\rho = 0.53$, p = 0.04) and between detour solve latency and exploration ($\rho = 0.53$, p = 0.04). However, none of these correlations remain significant after correcting for multiple tests using a false discovery rate (FDR) correction [32].

Discussion

We found that exposure to PET microfibers impaired cognitive traits including inhibitory control and spatial learning in the western mosquitofish; however, they affected each sex differently. Microplastic exposure disrupted the cognitive sex differences observed in MP-absent conditions. Specifically, the male advantage in inhibitory control under natural conditions disappeared in the MP-exposure

treatments. Conversely, the superior spatial learning abilities of females over males in MP-absent conditions was disrupted in the presence of microplastics. Notably, behavioral traits such as social motivation, shyness, exploration, feeding behavior, and aquatic surface respiration, and relative condition factor (Kn) remained unaffected by treatment in either sex. These findings suggest that even short-term exposure to environmentally relevant MPs can impair animal cognition without impacting behavior or physiology and do so in a sex-specific and domain-specific manner.

In our study, microplastic exposure impaired cognition and disrupted the baseline sex-differences exhibited between different cognitive domains. Under control conditions, males exhibited significantly greater inhibitory control than females (Fig. 3a). Yet, under low doses (10MP/L), male inhibitory control was impaired, while females were unaffected across treatments. Inhibitory control has been broadly implicated in mating, decision making, and predator avoidance[33], thus long-term impairment should have fitness consequences. Previous research in a sister taxa, *Gambusia holbrooki*, found that inhibitory control is sexually selected for in males [34], suggesting this might be a sex-specific shared trait in this genus. It would also suggest that the reduction of inhibitory control under MP-exposure presents a fitness cost for males, either through reduced mating coercive ability or female preference against poor cognitive performance. However, more research must be conducted to understand how long this effect may last, or if individuals can recover from cognitive decline through post-exposure depuration. Our findings of sexually dimorphic performance in inhibitory control under control conditions differs from earlier work in *G. affinis* [27] suggesting these results may be sensitive to differences in methodology and/or housing conditions. Interestingly, our findings confirm the results of a recent study in female *G. affinis* that found that polystyrene MP exposure did not alter risk-taking or inhibitory control [35]. The lack of effects we find here may suggest female resilience, but it may also suggest overall weakened selection for inhibitory control as baseline performance varies dramatically (Fig. 3a).

Spatial learning was also disrupted by microplastic exposure in a sex-specific manner with particularly strong consequences for females. Under control conditions, females exhibited significant spatial learning in the route-learning assay while males did not (Fig. 5b). However, this female-specific cognitive advantage was absent under any MP exposure. Females under both low and high doses failed to demonstrate spatial learning (Fig. 5b). Irrespective of treatment, males did not exhibit learning in this task. Our results fulfill our initial hypotheses that spatial learning would be impaired with sex specificity. Spatial learning enables animals to orient within their environment, recall the locations of resources and refuge, and identify predator escape routes, and thus plays a central role in survival [36]. In other poeciliids, spatial learning is important for navigating predator-rich environments [37], and exhibit high sexual dimorphism [30]. The baseline female advantage in spatial learning we find in *G. affinis* may reflect stronger selection on navigation and spatial memory in female mosquitofish. If microplastic exposure impairs spatial learning, females may be less able to learn and navigate their environment, potentially compromising predator avoidance, foraging efficiency, mate choice, and sexual coercion evasion. Such effects could have downstream consequences for lifetime fitness and sexual selection dynamics. Our finding that the sexes differ in baseline spatial learning ability is consistent with previous studies

showing female *G. affinis* exhibiting greater temporal-spatial learning abilities than males [27]. Moreover, our study is consistent with effects found in *Rattus norvegicus*, where BPA impairs spatial learning in female but not male rats [38]. Naturally, the impairment of female spatial learning suggests fitness consequences in the wild. Considering that instances of predator or male avoidance can be instantaneous, this consequence of MP exposure could reduce fitness consequences even in the short term.

Although the mechanisms underlying these sex-specific cognitive effects remain uncertain, sex differences in endocrine pathways and the idiosyncratic chemistry of PET microfibers may be at play. Males across species exhibit increased physiological susceptibility [23], and poecilid fish frequently present sex differences in cognition[21] and neuroendocrine responses to stressors [21,39]. Thus, we hypothesized and found sex-specific declines in cognition. Mechanistically, some MPs have been found to act as endocrine disruptors, which could provide a pathway for cognitive decline. For example, PET exposure has been shown to elicit behavioral impairments indicative of neurotoxicity in *D. melanogaster* [40]. PET has been hypothesized to act as an endocrine disrupting chemical (EDC), and commonly carries known EDCs like antimony trichloride and phthalates [41]. Furthermore, EDCs often produce sex-specific and non-monotonic effects by mimicking androgens like estrogen which are consistently associated with cognitive disruption [42]. If our PET microfibers are either acting as or leaching EDCs, it may explain the sex-specific cognitive decline and non-monotonic effects we find in *G. affinis*. The pronounced decline in male inhibitory control at low, but not high exposure, may reflect receptor desensitization or negative feedback in estrogen pathways, explaining the lack of effects at higher concentrations. However, an alternative mechanism may be borne of the high density and hydrophobic structure of PET microplastics. At higher concentrations, many MPs including PET flocculate and sink to the bottom of the water column, reducing waterborne exposure [43]. This could possibly explain why we found no linear dose-dependent responses, or differences in retained MPs between conditions. Physical tissue damage to the gills or GI tract is unlikely to explain our non-monotonic outcomes, as such damage should increase with concentration. Although, males of this species have far poorer baseline condition (Kn) (Supplementary Fig. S2) than females. Male *G. affinis* could experience greater tissue damage irrespective of condition, explaining some sex-specificity. While these mechanisms remain speculative, they highlight the urgent need to clarify how microplastics disrupt neuroendocrine and cognitive function.

Despite the pronounced cognitive effects of microplastic exposure, we detected no corresponding changes in the behavioral traits we measured, including shyness, exploration, feeding behavior, aquatic surface respiration, and social motivation (Fig. 2–4). Furthermore, MP treatment did not alter relative condition (Kn). These results are consistent with studies reporting no measurable effects of MP exposure on boldness or activity in Krefft's frill gobies (*Bathygobius krefftii*)[17] or sticklebacks (*Gasterosteus aculeatus*)[44], as well as previous work in *G. affinis* reporting no effects of MP exposure on female behavior or inhibitory control [35]. The absence of behavioral effects in our study may partly reflect the relatively acute exposure period and environmentally relevant concentrations used here. Behavioral

responses may require longer exposure durations or higher MP concentrations to emerge. Additionally, traits such as aquatic surface respiration may require MP exposure during behavioral assays to elicit a response, whereas feeding behavior may be more sensitive to foodborne MP exposure. The absence of behavioral effects despite cognitive impairment may also suggest that MP exposure does not produce uniform effects across neurobehavioral systems, potentially reflecting more idiosyncratic effects on neuroendocrine processes.

Behavior, physiology, and cognition are often assumed to be tightly integrated, such that changes in one component of organismal performance are expected to manifest as changes in the others. Our results challenge this assumption by demonstrating that cognitive impairment can occur in the absence of detectable changes in several ecologically relevant behaviors or relative body condition. To explain this, we suggest that cognitive endpoints may reveal latent effects of MP exposure that may not be apparent from behavioral or physiological measures. While cognition, behavior, and physiology are integrated, they are not totally overlapping in cause or effect. Overall, incorporating cognitive assays more routinely into ecotoxicological studies may therefore provide a more comprehensive assessment of contaminant effects. We do not find support in our data for the hypothesis that MP exposure alters behavioral traits and relative condition. Our results differ from previous research that found zebrafish exhibit behavioral responses to MP exposure including: increased anxiety and reduced exploration [10,12], increased shyness [12], and interactions between microplastic exposure and initial boldness [45]. Furthermore, in African catfish (*Clarias gariepinus*), polyethylene MPs impact respiratory rates, while feeding behavior (proxied by latency to feed) was altered in Crucian carp (*Carassius Carassius*) [9,11]. Importantly, our findings do not preclude effects on non-cognitive traits, as our assays are not completely exhaustive of an animal's ethogram. Nevertheless, the detection of cognitive impairment in the absence of detectable changes in the other traits suggests that cognitive traits should be more widely assayed and investigated in ecotoxicology and neuroscience. Expanding the range of traits and their mechanisms used to evaluate contaminant effects may reveal ecologically relevant consequences that would otherwise remain undetected.

While we did not find treatment effects manifesting in individual behavioral or physiological traits, we did identify some initial patterns indicating that microplastics alter the relationship across behavioral and cognitive traits (e.g. behavioral syndromes). Specifically, we found correlations between behavioral traits that were present in the female control group but absent in MP-exposed treatments, while finding the opposite trends in males (Supplementary Fig. S1). In zebrafish, individual personality traits and correlations among behavioral traits have also been shown to influence both vulnerability to microplastic exposure and the magnitude of its behavioral effects [45]. However, in both the present study and [45], the correlations did not remain statistically significant after correcting for multiple comparisons so interpretations should be made cautiously.

Although this study provides important insight into the cognitive and behavioral consequences of microplastic exposure, several caveats should be considered when interpreting our results. To ensure consistency in MP size and shape, and allow for Nile red staining, we used virgin MPs. Weathered MPs

however, are far more prevalent and retain biofilms and co-pollutants that can substantially increase their toxicity [43]. We determined MP exposure by particle count rather than mass (as is common in with other pollutants) as this is the preferred unit in environmental MP studies. This may limit our ability to make direct comparisons with studies that report mass-based concentrations. In general, the use of particle count alone obscures the MP size and density variation found in natural environments. Furthermore, our exposure duration, while longer than many acute studies [46], is still short (14 days) compared to real world exposure durations. Chronic exposure would likely elicit stronger or qualitatively different cognitive and behavioral responses. Alternatively, individuals developing under polluted conditions could find ways to compensate for deficits [7]. Our results may in some way underestimate the effects of microplastics under natural conditions. Together, these limitations emphasize the need for future studies that incorporate weathered particles, longer exposure durations, alternative concentration metrics, and multi-stressor experimental designs to better approximate environmentally realistic exposure scenarios.

Our study underscores the importance of assessing both males and females and testing environmentally relevant concentrations when evaluating the impacts of pollutants. Furthermore, cognitive outcomes of MP exposure warrant study regardless of behavioral or physiological effects. Longer-term studies are needed to determine whether such cognitive impairments translate into fitness costs in the wild and to assess the potential for adaptive or maladaptive evolutionary responses to chronic microplastic exposure. Finally, mechanistic studies linking cognitive impairment to physiological or neural endpoints would be invaluable for identifying the pathways underlying these sex-specific responses.

Materials and Methods

Animal Care Facilities & Ethics

We collected 174 adult western mosquitofish (*G. affinis*) from a semi-natural artificial pond at the University of Texas at Austin, Brackenridge Field Laboratory (BFL). All fish were collected under the guidelines of our scientific research permit granted by Texas Park & Wildlife (permit #SPR-0725-059). The pond experiences ambient weather conditions year-round and contains stocked *Lepomis macrochirus.*, *G. affinis*, and *Poecilia latippina*. All collected *G. affinis* (male n = 88, female n = 86, n = 174) were housed in mixed-sex shoal groups in laboratory at J.T. Patterson Labs Building, for seven days under standard laboratory conditions prior to MP exposure.

All methods, housing conditions, and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin (Protocol #: AUP20252200224) and performed in accordance with the relevant guidelines and regulations. Methods were additionally carried out in accordance with relevant ARRIVE guidelines.

Microplastic Source Population Testing

We quantified prior microplastic exposure within the population to ensure that individuals were subsequently exposed to novel microplastic types and concentrations in our experimental design. Microplastic (MP) concentration, polymer composition, and particle characteristics at the source site were quantified following previously established methods [47]. 3-liter water samples were collected and sieved through a 500 µm mesh to remove large debris, chemically digested with 30% H_2O_2, and processed using microfluidic chips. Water samples were injected at 0.02 mL/h for 8 h using a syringe pump (Chemyx Fusion 200 two-channel). Microfluidic chips were dried and MPs were identified with Raman spectroscopy (WITec alpha 300 R, Oxford Instruments Inc) at the University of Rhode Island. Polymer types were determined using OpenSpecy [48], and particle abundance, size, and shape were quantified via microscopy. Across all samples, 67 particles were recovered, including 28 plastic particles comprising PDMS, polyethylene (PE), polypropylene (PP), polyvinyl alcohol (PVA), ethylene-vinyl acetate (EVA), cellulose acetate, polystyrene/styrene copolymers, and polyvinyl chloride (PVC). However, no microfibers or polyethylene terephthalate (PET) were detected, possibly due to size-dependent sampling bias, preferential loss during pre-processing steps, and false negatives caused by reduced Raman spectral quality due to environmental weathering and eco-corona coverage. As a result, all recovered MPs were microfragments with a mean diameter of ~ 30 µm, corresponding to an estimated average concentration of 2.8 MPs/L at the source site, though concentrations likely vary with precipitation and atmospheric deposition.

Plastic Sourcing & Microplastic Production

Our findings at the BFL source population were consistent with previous studies reporting microplastic concentrations in the range of 0 to 1000 MPs/L [5,49,50], which are substantially lower than microplastic concentrations commonly used in laboratory exposure studies [19,20]. We selected MPs that were environmentally relevant yet distinct from those in the source population (Brackenridge Field Laboratory) to ensure uniform exposure to a novel stressor. Polyethylene terephthalate (PET) is among the most abundant polymer types detected in aquatic environments [1,50] and commonly occurs as microfibers, which are especially pervasive in freshwater and marine habitats [50]. In addition, smaller MPs (< 500 µm) are more prevalent in environmental samples and are less detectable than larger particles (> 500 µm) [51]. Despite their abundance, PET microfibers remain underrepresented in experimental work. Together, these considerations motivated our use of small PET microfibers at environmentally relevant concentrations. Specifically, we used 42 µm PET microfibers at doses of 0 MPs/L (Control), 10 MPs/L (Low), and 1000 MPs/L (High).

To generate microplastics for exposure trials, we followed methods developed by [52]. We purchased 0.014mm filament diameter PET thread (15 monofilaments) from Goodfellow (product no. ES305710). The PET thread was then wound with a wooden spindle into a unidirectional plane [52]. The thread was fixed in paraffin wax and cut into 42 µm fibers using a Spencer rotary hand microtome. We calculated the total quantity of MP particles produced by multiplying the number of threads fixed in paraffin by the number of monofilaments within a single thread (15 monofilaments), then multiplying that by the

number of 42 µm slices made on the microtome as in [52]. The MPs were then suspended in the appropriate volume of water to reach the desired concentrations. Paraffin slices were deparaffinized and rehydrated following standard histological methods using 100% Xylene, 100% Ethanol, and 70% Ethanol. Following methods from [29], the MPs were stained with Nile Red and then stored in 70% ethanol for later use. MP concentrations were determined to be environmentally relevant using systematic reviews and meta-analyses of estimated concentrations in freshwater globally [1,43,49,53,54]. The low dosage (10MPs/L) approximates concentrations detected across most studies, while the high dosage (1000MPs/L) approximates the highest concentrations found in freshwater environments [19,49].

Exposure & Housing

From June 8th, 2024 to March 3rd, 2025, we ran weekly triads of subjects in control, low, and high MP-exposure treatment groups. Each week, 18 mosquitofish were assigned to one of three groups of 6 (3 males, 3 females) and placed into experimental-exposure tanks with varying levels of PET microfibers: Control (0 MPs/L), Low (10MPs/L), or High (1000MPs/L) (Fig. 1A). Each group was treated once at the beginning of the 14-day exposure with MPs suspended in 15ml water. Aquarium wavemakers were installed in each tank to ensure constant suspension of MPs. In total, we ran 36 replicate groups over 8 months including (n = 12 Control, n = 11 Low, and n = 13 High). On the final day of exposure, individuals were initially tested in the detour reaching assay, followed by a two-hour interval, and then tested in scototaxis assays and subsequently housed in 0MP/L isolation tanks overnight (Fig. 1A). The following day, all individuals were tested in the route learning assay, then euthanized, weighted, measured, and dissected.

Behavioral & Cognitive Assays

Feeding Assay: Throughout the two-week exposure period, each aquarium containing six fish was provided with 0.35 g of fish food daily at 08:00 to acclimate individuals to a standardized feeding time and quantity prior to the feeding assay. For the assay, individual fish were isolated in small tanks overnight to acclimate to the assay environment. The following day, between 08:00 and 09:00, each individual was video recorded for 10 min from a lateral viewing angle immediately after 0.05 g of fish food was introduced at the surface of the water. Two behavioral metrics were quantified from each recording: latency to feed and aquatic surface respiration. Latency to feed was defined as the time (s) elapsed between food introduction and the initiation of feeding and was used as a measure of feeding motivation and feeding behavior [55]. Aquatic surface respiration was quantified as the total duration (s) an individual spent at the water surface performing surface respiration, a behavior associated with hypoxic stress and altered respiratory function [56]. Video recordings were scored using BORIS [57] independently by two observers who were double-blinded to treatment and sex.

Detour Reaching Task

We used a detour reaching task to measure inhibitory control and social motivation (Fig. 1B) following methods from [58,59]. In this task, individuals are measured in their ability to maneuver around a

transparent glass barrier to approach a social reward (a conspecific female). A conspecific female is placed in a glass tube in the social reward area. The focal individual is then placed in a habituation chamber for five minutes to acclimate to the aquarium. After the habituation barrier is lifted, each subject is allowed ten minutes to complete the detour reaching task. Social latency, our proxy for social motivation, was measured as the time individuals took to leave the habituation chamber and approach the glass barrier. Solve latency, our proxy for inhibitory control, was measured as the time it took individuals to maneuver around the transparent glass barrier, reaching the reward compartment, after their first interaction with the glass barrier. If individuals did not complete the task in the allotted time, they were marked as non-solvers and their solve latency was assigned to 600 seconds. This task was video recorded overhead and scored by three double-blind observers.

Scototaxis Assay: To measure shyness and exploration, we used the scototaxis assay, following methods from [31,58,59]. The scototaxis, experimental aquarium is coated in 25 cm^2 of black felt and 25 cm^2 of white felt on either side (Fig. 1C). At the start of the assay individuals are placed in a habituation chamber in the center of the scototaxis aquarium for 5 minutes to acclimate. After the habituation chamber is removed, individuals are recorded for 10 minutes in the full tank environment for 10 minutes under infrared (IR) light using a Basler Pylon IR camera. Shyness was measured as the cumulative duration of time individuals spent in the black zone. Exploration is measured as the total distance individuals moved over the 10-minute duration of the assay. After the habituation chamber is removed, individuals are recorded for 10 minutes in the full tank environment for 10 minutes. Assays are recorded using an infrared camera (IR-Capable Monochrome GigE) with a IR light board underneath the tank. Recordings are behaviorally coded using Noldus Ethovision XT (RRID:SCR_000441).

Route Learning Task

The route learning task, following methods from [31], was used to measure spatial learning. The experimental aquarium is comprised of 3 zones (Fig. 1D). Zone 1 is an empty white area where individuals are placed at the start of each trial to habituate. A central transparent barrier and two potential pathways line the border between each zone. Zone 2 is another empty white area before zone 3. Zone 3 contains two social rewards in a transparent glass tube and a pathway to a dark green area with more social rewards visible in an adjacent aquarium. At the start of each trial, individuals are placed in a transparent glass tube in zone 1 for 5 minutes to habituate. Once the transparent tube is removed, individuals are allowed 5 minutes to solve the task. Directly following habituation, individuals must choose the two correct paths, leading to the social reward conspecifics in zone 3 and green area beyond. All trials were video recorded from above. For each trial, we counted the number of correct and incorrect door choices made by subjects. Our criterion for spatial learning required individuals to exhibit a significant reduction in errors made over successive trials. We calculated the rate of error reduction across trials as the proportion of incorrect door choices out of all door choices made in each trial.

To minimize the influence of task habituation on route-learning performance, we established a participation threshold for each trial, as done in [30,31]. Our participation criterion required engagement

(entering the second zone of the route learning assay) by at least 65% of individuals for the trial to be included in the final dataset (Supplementary Table 1).

Microplastic Retention & Relative Condition Factor

Between assays, individuals were held in isolation tanks containing no microplastics, which allowed for a 24-hour depuration period after the two weeks of exposure. Following completion of all behavioral and cognitive assays, fish were anesthetized in an ice bath and euthanized by rapid decapitation. Individuals were immediately weighed and photographed, and standard length was measured from digital images using ImageJ[60]. Within 5 min of euthanasia, the brain, gills, gastrointestinal tract, and gonads (testes or ovaries) were dissected and weighed separately. All tissues were then desiccated under an incandescent lamp for approximately 15 min before being stored in a cool, dry environment until microplastic analysis.

Microplastic retention was quantified in four key organs, the brain, gill rakers, gastro-intestinal tract, and gonads separately. Individual tissues were digested in 1 mol L^{-1} NaOH following the protocol of (Roch et al. 2020)[61]. Samples were incubated in a thermal dry bath for 1–5 min with periodic mechanical agitation using a vortex mixer until complete tissue digestion was achieved. Digested samples were vacuum filtered through 0.45 µm cellulose acetate filter paper, and filters were air-dried before examination under a light microscope illuminated with a 365 nm ultraviolet light source. Microplastic particles were manually identified and counted by a single treatment-blinded observer. The sum of MPs retained across organs was used as the total MPs retained for statistical analyses.

Relative condition factor (K_n) was calculated using sex-specific length–weight relationships [62]. Males and female condition factors were calculated separately because of their sex-specific differences in the length-weight relationship as females continue to grow beyond sexual maturity [63]. The relationship between wet body mass (W) and standard length (L) was described by the allometric equation ($W = aL^b$), where (a) is the intercept and (b) is the allometric growth coefficient. The fitted equations were then used to estimate the expected weight (W_e) of each individual based on its standard length. Relative condition factor was calculated as ($K_n = W_o/W_e$), where W_o is the observed wet body mass. Relative condition factor was subsequently used as the response variable in linear models testing the effects of MP treatment, sex, and their interaction.

Statistical Analyses

All statistical analyses were conducted in R[64] (vers. 4.6.1) using the packages *glmmTMB*, *emmeans*, *lme4*, *rstatix* and *DHARMa*. We used generalized linear mixed models (GLMMs) to test for the effects of microplastic treatment, sex, and their interaction on behavioral and cognitive responses, including detour solve latency, detour social latency, scototaxis duration of time spent in the black zone, scototaxis total distance moved, and route learning change in error rate. To analyze the latency to feed in the foraging assay, we used a cox model, a form of survival analysis. Initial housing group and individual identity were included as a random intercept in some models to account for repeated measurements and non-independence among observations from the same individual or those from the same housing group

during the two-week exposure. Detour solve latency and detour social latency were analyzed using a Gamma error distribution with a log link function. The total distance moved and the duration of time spent in the black zone were analyzed using a Gaussian error distribution with an identity link without transformation. For the response variables: detour solve latency, social latency, total time spent in the black zone, total distance moved, relative condition factor, and total MPs retained, the full model structure was:

$$\text{Response} \sim \ \text{Treatment} \times \ \text{Sex} + (1 \mid \ \text{group}\backslash\text{:ID})$$

where treatment and sex were included as fixed effects and their interaction tested for sex-specific treatment responses. We adopted a confirmatory modeling approach in which full models were specified *a priori*. Consequently, we did not select models based on minimum AIC values but instead evaluated the significance and effect sizes of predictors within the full model.

To quantify learning performance in the route-learning task, we first calculated the proportion of errors (p_e) made per trial for each individual as:

$$\text{Pe} = \frac{\text{Total}\backslash\text{:incorrect}\backslash\text{:door}\backslash\text{:choices}}{\text{Total}\backslash\text{:door}\backslash\text{:choices}}$$

the proportion of errors made per trial (p_e) was then fit in a linear model as:

$$\text{Pe} \ \sim \ \text{trial}\backslash\text{:number}$$

to obtain the slope (or rate of change) of the proportion of errors made across trials (hereafter, ΔP_e). ΔP_e was then analyzed using a gaussian GLMM. The initial model included a Treatment × Sex interaction; however, this model was simplified to include only main effects due to lack of model support and to improve model parsimony. The final model structure was:

$$\Delta \ \text{Pe} \sim \ \text{Treatment} + \text{Sex} + (1 \mid \ \text{Individual}\backslash\text{:ID})$$

For the total time spent in the black zone in the scototaxis assay and ΔP_e, residual variance was allowed to vary among experimental groups by fitting dispersion submodels in *glmmTMB*. For shyness, dispersion was modeled as a function of the treatment × sex interaction, whereas for route-learning mean error rate dispersion was modeled as a function of treatment + sex. Dispersion models were fitted using a log link function.

Model assumptions and fit were evaluated using the *DHARMa* package. Statistical significance of fixed effects was assessed from model summaries at $p <= 0.05$. Post hoc pairwise comparisons were conducted using estimated marginal means implemented in the *emmeans* package, with p-values adjusted using Holm's method to control for multiple comparisons. Model-estimated means are reported ± standard error unless otherwise stated and estimates from models with non-Gaussian error distributions were back-transformed to the response scale for visualization and interpretation. Sample

sizes varied among treatments due to variable mortality during the two-week exposure period; final sample sizes are reported in the results and figure captions.

To test for cognitive–behavioral syndromes, we calculated Spearman's rank correlations among all behavioral and cognitive traits separately within each sex and treatment group. Correlation coefficients were converted to Fisher's z scores to allow statistical comparison among groups, and pairwise t-tests were used to assess differences in correlation strength between sexes and treatments. P-values were adjusted using a FDR correction [32].

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Declarations

Declaration of Competing Interests

The authors have no competing interests to declare.

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Author Contributions

Maxximus Ramsaroop: Conceptualization, Methodology, Formal analysis, Investigation, Writing, Visualization, Project administration, Funding acquisition. **Jingping Graber**: Methodology, Investigation, Visualization. **Adrienne Lihou**: Methodology, Investigation. **Liyuan Gong**: Validation, Resources, Investigation, Writing - Review & Editing. **Rivers Hartzell**: Methodology, Investigation. **Yang Lin**:

Resources, Writing - Review & Editing. **Molly Cummings**: Conceptualization, Methodology, Supervision, Writing - Review & Editing.

Data Availability

The datasets generated during and/or analyzed during the current study are available in the Github repository, https://github.com/MaxxRAM/mps_g_affinis_24-26_ch1

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Figures

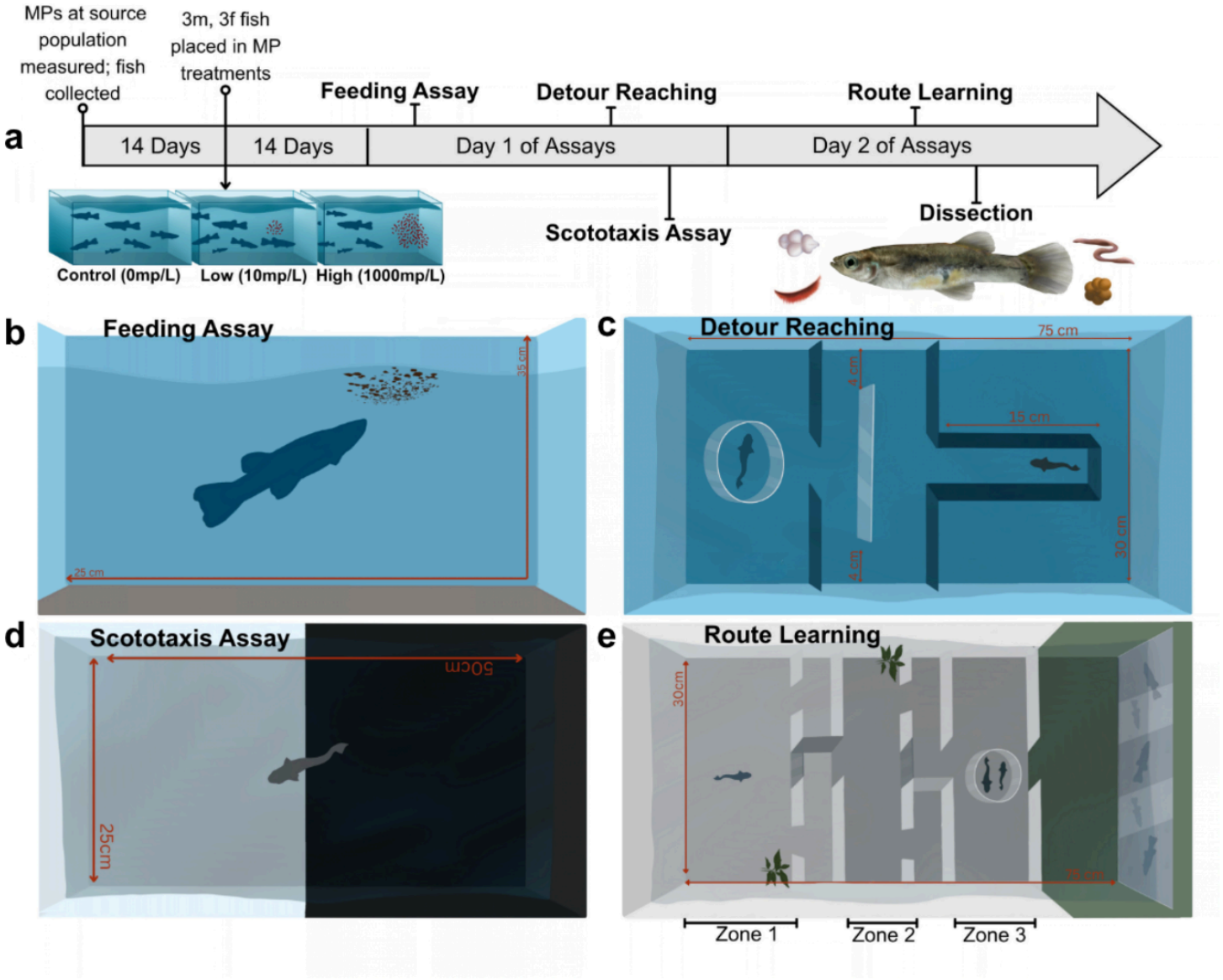


Figure 1

Experimental timeline and experimental aquarium schematics. **(a) Experimental Timeline:** All *G. affinis* were collected 14 days before the start of the experiment and then placed in one of three treatments (0 MPs/L, 10 MPs/L, or 1000 MPs/L) for 14 days. Over two days following exposure, individuals were tested in the feeding assay, detour reaching task, scototaxis assay, route learning task, and then

dissected. **(b) Feeding Assay:** Fish were fed 0.05g of dry food in an isolated tank. Latency to feed and aquatic surface respiration were scored in a 10-minute recording after food was dispensed. **(c) Detour reaching task:** Fish were placed at the back of a corridor with a social reward (conspecific female) in a glass cylinder at the other end of the tank and recorded for 10 minutes. Social latency was recorded as the time at which individuals approach the transparent barrier. Solve latency (our measure of inhibitory control) was calculated as the time from the focal individual reaching the transparent barrier to navigate around the transparent barrier to reach the social reward. **(d) Scototaxis assay:** Individuals were placed in a novel half-black, half-white aquaria for 10 minutes. We recorded the total duration of time individuals spent in the black area (Shyness), and the total distance individuals moved over the duration of the assay (Exploration). **(e) Route learning task:**Individuals were placed in zone 1 at the start of each trial with social reward females in zone 3 and zone 4. Central transparent barriers along the midline of the tank allow focal fish to observe the social rewards. At each partition between zone 1 and 2 there are two doors that lead to either successful access to reward or an enclosed chamber. To solve the task, individuals must pass through the correct doors between zone 1 and zone 2, reaching the social reward females. Individuals repeat this process for a total of 5 trials. Evidence for spatial learning was determined as a significantly negative slope in the proportion of errors made over successive trials.

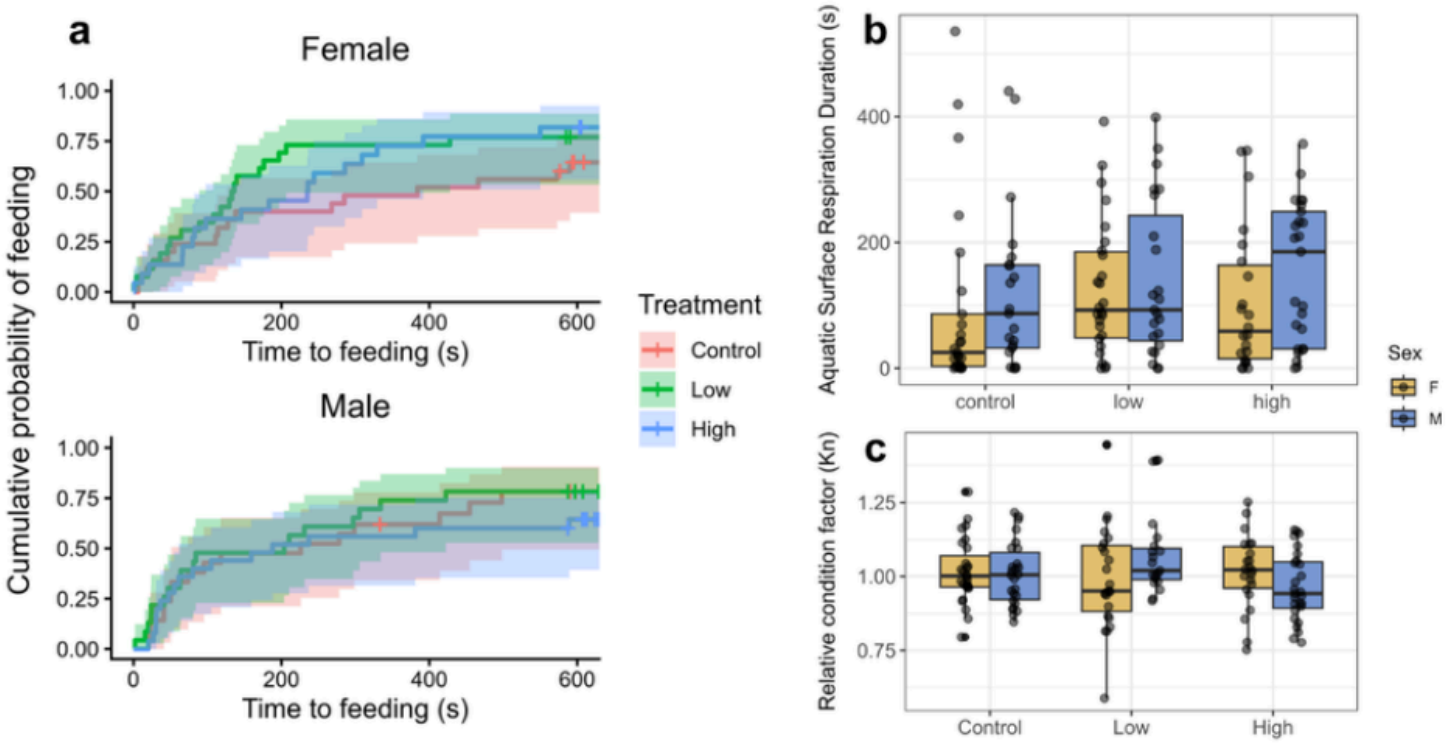


Figure 2

Feeding assay metrics & relative condition factor. **(a.)** Latency to feed. The latency to feed is a feeding behavior metric we modeled as the cumulative probability of feeding over the duration of the assay, faceted by sex. Semi-transparent shading around plot lines represent 95% confidence intervals, while vertical notches indicate right-censored individuals. **(b)** Aquatic surface respiration, measured as the total time individuals spent with their snout at the water line. **(c)** Relative condition factor calculated as

$K_n = W_o/W_e$), where W_o is the observed wet body mass is modeled with a sex-specific linear regression. Both aquatic surface respiration and relative condition factor were tested with linear models, but raw observations are plotted with medians indicated by horizontal bars, the interquartile range indicated by bar height, and 1.5 x IQR vertical bars. No effect of treatment or sex found on the latency to feed, aquatic surface respiration, or relative condition.

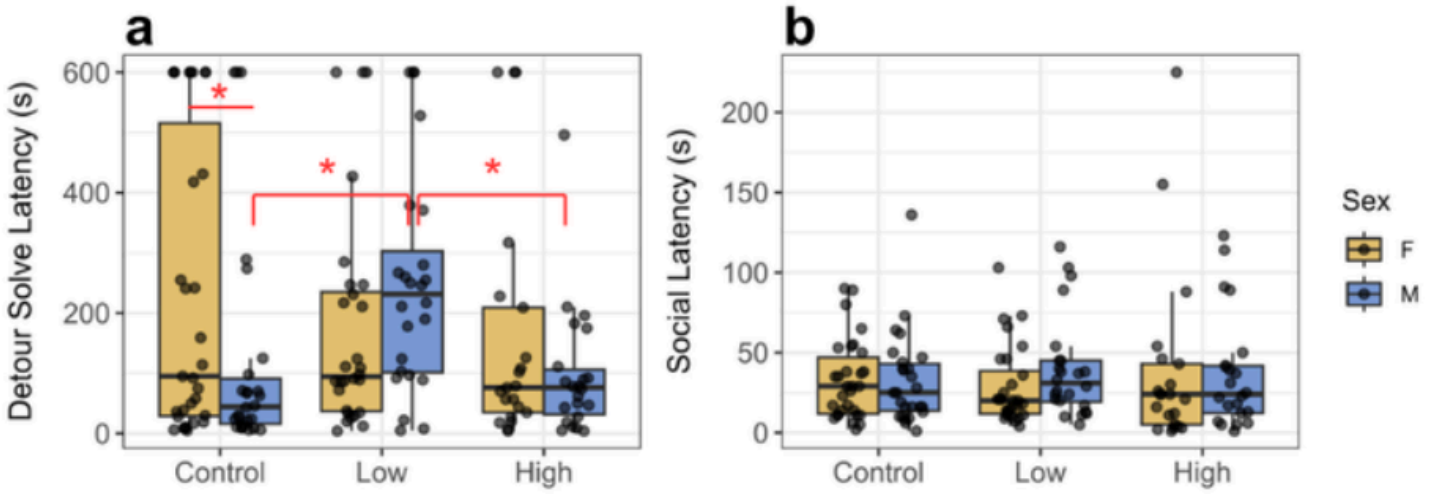


Figure 3

Detour reaching task performance and social motivation **(a)** Control males exhibited significantly lower detour solve latency (greater inhibitory control) than control females (ratio = 0.635, p = 0.032). Males in the Low MP exposure group exhibited significantly longer detour solve latency (poorer inhibitory control) than males of the high MP (ratio = 0.391, p = 0.015) or control group (ratio = 0.483, p = 0.051 and ratio = 0.308, p = 0.0002) when excluding non-solvers). **(b)** Social latency (social motivation) did not vary by treatment or sex (p > 0.19). Asterisks (*) indicate post hoc p <0.05. Boxes indicate the interquartile range (IQR), horizontal lines indicate medians, whiskers extend to 1.5 × IQR, and individual points represent raw observations.

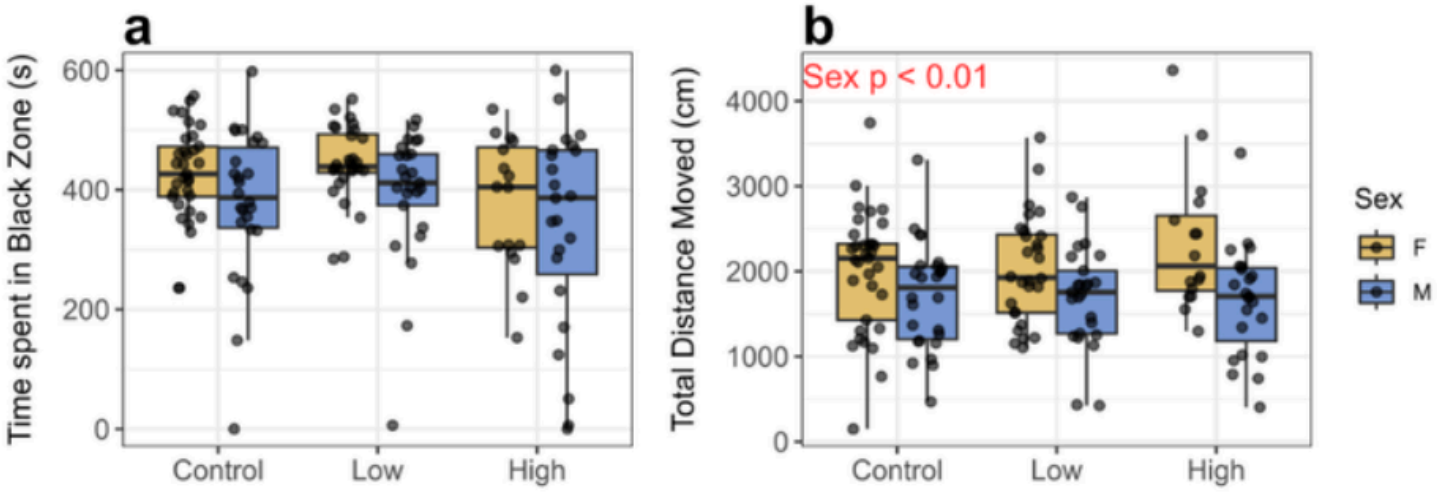


Figure 4

Shyness and exploration in the scototaxis assay. **(a)** The total time spent in the black zone (shyness) was not significantly predicted by treatment or sex (all $p > 0.35$). **(b)** We found a significant difference between males and females in exploration (total distance moved in the scototaxis assay) with females exhibiting greater distance traveled than males (mean effect = 444 cm, $p = 0.0001$). Boxes indicate the interquartile range (IQR), horizontal lines indicate medians, whiskers extend to 1.5 × IQR, and individual points represent raw observations.

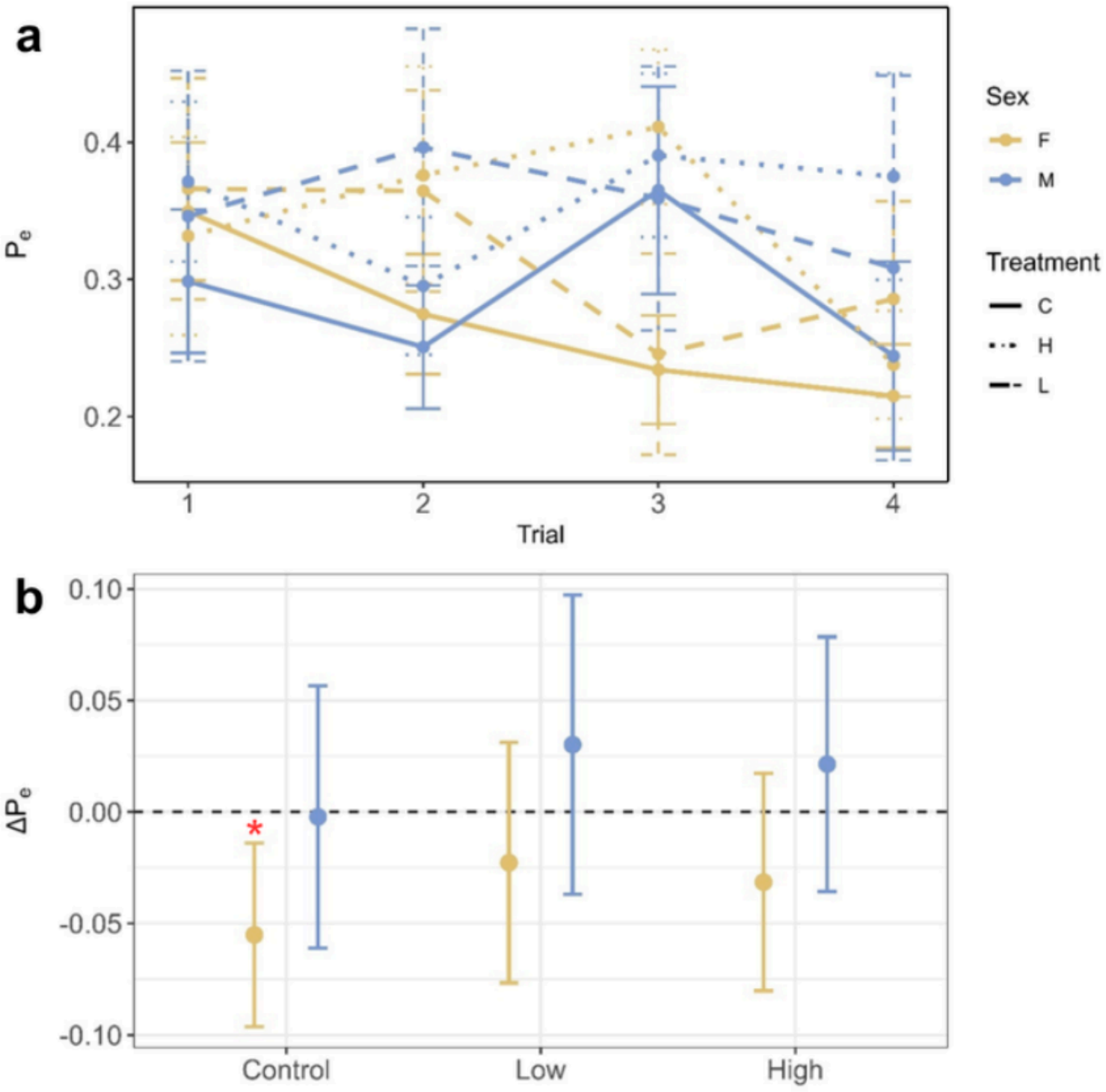


Figure 5

Route learning task performance by sex and treatment over the first four trials. **(a)** P_e of each sex and treatment over the first four trials of the route learning assay **(b)** Proportional error rate ΔP_e, over the first four trials across sexes and treatments. Control females exhibited spatial learning (a significant reduction in errors made across trials, ΔP_e, $\beta = -0.055$, $p = 0.008$)) whereas MP- exposed females and both control and MP-exposed males did not. Asterisks (*) indicate post hoc $p < 0.05$, solid circles indicate the mean ΔP_e, and vertical bars are 95% confidence intervals.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- RamsaroopetalSupplementalMaterials1012026.docx