

RESEARCH ARTICLE OPEN ACCESS

Does Microplastic Ingestion Affect Cognition and Brain Gene Expression in Zebrafish, *Danio rerio*?

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Received: 7 March 2025 | **Revised:** 10 September 2025 | **Accepted:** 9 October 2025

Editor: Redouan Bshary

Funding: This study was supported by Università degli Studi di Ferrara (FAR2022).

Keywords: aquatic pollution | cognitive plasticity | cognitive variance | conservation behaviour | fish cognition | individual differences

ABSTRACT

Cognition exhibits remarkable plasticity and therefore is potentially susceptible to non-adaptive changes caused by anthropogenic disturbances such as pollution. Among the most pervasive pollutants in aquatic environments are microplastics, which can be ingested by fish and other organisms, causing a range of physiological alterations that have been hypothesised to affect cognition. In this study, we conducted the first extended investigation of the cognitive consequences of microplastic ingestion in a teleost fish, the zebrafish *Danio rerio*. We measured brain activity, visual and spatial learning abilities and cognitive flexibility in both visual and spatial tasks across three experimental groups of zebrafish. One group was fed a diet containing microplastics from a common synthetic polymer, polyethylene (PE); one group a diet containing microplastics from a biodegradable polymer, polybutylene adipate terephthalate (PBAT); and a group received a control diet without microplastics. The treatments were continued over a medium-term period of 20–40 days. We found that PE microplastics significantly reduced brain activity in the zebrafish. However, the three experimental groups showed no differences in learning abilities and cognitive flexibility. Additionally, no detectable changes in individual differences in cognition were observed due to microplastic exposure. We conclude that microplastics derived from synthetic polymers altered brain functioning at the molecular level, although these alterations did not lead to detectable changes in the cognitive abilities in the tasks we investigated.

1 | Introduction

For most vertebrate taxa, evidence suggests that cognitive traits, such as learning and memory capacities, vary substantially among individuals (reviewed in Lucon-Xiccato and Bisazza 2017; Thornton and Lukas 2012). While part of the cognitive variance is genetically determined (e.g., Langley et al. 2020; Smith et al. 2015), a large proportion arises from adaptive plasticity triggered by environmental factors experienced by

individuals (Beldade et al. 2011; Ghalambor et al. 2007; Gotthard and Nylin 1995; Nettle and Bateson 2015; Via et al. 1995). For example, tadpoles raised in the presence of predators develop greater capacity to learn novel threats compared to those raised in environments with no predators (Ferrari 2014; Lucon-Xiccato et al. 2016). Moreover, individuals raised in structurally complex habitats develop greater learning capacities (Montalbano et al. 2022; Salvanes et al. 2013; Van Praag et al. 2000). However, in the Anthropocene, animals are increasingly exposed to

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novel environmental conditions that can trigger novel plastic responses, which are often non-adaptive. For instance, velvet geckos, *Amalosia lesueurii*, incubated under climate warming conditions develop slower learning (Dayananda and Webb 2017), and zebra finches, *Taeniopygia guttata*, subjected to disturbance by traffic noise showed decreased cognitive performance in several tasks (Osbrink et al. 2021). In aquatic species, evidence suggests various cognitive impairments due to hypoxia (Lucon-Xiccato et al. 2014), the occurrence of which is increasing due to human pollution (Diaz and Rosenberg 2008), ocean acidification (Domenici et al. 2012; Ferrari et al. 2012), anthropogenic sounds (de Souza et al. 2022) and various pollutants (Grassie et al. 2013; Moreira and Luchiaro 2022).

One of the most concerning forms of pollution is related to plastic waste. In particular, small plastic fragments (i.e., microplastics) take decades to fully degrade and are virtually ubiquitous in aquatic habitats (Li et al. 2018, 2020; Lindeque et al. 2020; Sharma et al. 2021). The tiniest microplastics (i.e., <1 mm) are similar in size to marine organisms such as fish eggs, larvae and unicellular algae (Rowenczyk et al. 2020) and can be ingested by larger organisms such as fish (Galafassi et al. 2021; Parker et al. 2021; Pinheiro et al. 2017; Silva-Cavalcanti et al. 2017). Once ingested, microplastics may cause a range of impairments in gene expression, physiology, life history and behaviour (e.g., Critchell and Hoogenboom 2018; Limonta et al. 2019; Mallik et al. 2021; Uy and Johnson 2022). Negative effects have also been reported for microplastic derived from biodegradable materials (e.g., Kedzierski et al. 2018).

The effects of microplastic ingestion on cognition in aquatic species remain largely unknown, with only two studies finding impairment of decision-making in the European hermit crab, *Pagurus bernhardus* (Crump et al. 2020) and lateralization in zebrafish, *Danio rerio* (Andrei et al. 2025). However, several lines of evidence suggest that microplastic ingestion could broadly affect cognition. First, microplastics can alter neurotransmitter systems in the brain including acetylcholine (reviewed in Xiong et al. 2023). Second, the physiological changes induced by microplastics are predicted to impact cognitive functioning (reviewed in Maille and Schradin 2017). Third, existing covariation between behaviour, physiology and cognition (De Russi, Lanzoni, et al. 2024; De Russi et al. 2025) indicates that effects on physiological or behavioural traits may lead to cognitive changes. Finally, recent work shows that microplastics, even if they do not directly reach the brain, can affect cerebral blood flow (Huang et al. 2025). Together, these findings support the hypothesis that microplastic ingestion may influence cognition. Given the importance of cognitive abilities for survival (Cauchard et al. 2013; De Russi, Lanzoni, et al. 2024; Huebner et al. 2018; Smith et al. 2015), this highlights the relevance of studying the impact of microplastics.

In this study, we exploited the zebrafish, an emerging model in research on microplastics (reviewed in Bhagat et al. 2020) and in cognitive sciences (reviewed in Meshalkina et al. 2017), to investigate the effects of microplastic ingestion over a medium-term period on several aspects of cognition. We exposed experimental groups of zebrafish to a diet with microplastics added. One group received a common commercial grade plastic widely used for flexible packaging, polyethylene (PE). Another experimental

group received a common biodegradable polymer (PBAT, polybutylene adipate terephthalate) as this type of material is also raising environmental concern (Ahsan et al. 2024; Piyathilake et al. 2024; Malafeev et al. 2023; Wang et al. 2021). After the treatment, the subjects underwent a cognitive characterisation in four experiments, which, while not comprehensive, was as broad as possible and included measures of brain activation, visual learning, spatial learning, cognitive flexibility and individual differences in these cognitive traits. In Experiment 1, we investigated subjects' brain activity at the molecular level via gene expression analysis (reviewed in Calvo and Schluessel 2021). In Experiment 2, we administered a visual colour discrimination task and once the subjects reached a predetermined learning criterion, we administered a reversal learning task by flipping the stimulus-reward association as a measure of cognitive flexibility (Lucon-Xiccato et al. 2023; Montalbano et al. 2022). In Experiment 3, we replicated the visual discrimination task albeit without imposing the learning criterion to obtain better learning curves. In Experiment 4, we administered a spatial learning T-maze task followed by the reversal learning task to measure cognitive flexibility (Lucon-Xiccato et al. 2023; De Russi, Montalbano, et al. 2024). Last, considering that prior studies revealed forms of plasticity that involved not only the average cognitive phenotype, but also individual differences (e.g., individual differences; Domenici et al. 2012; Lucon-Xiccato, Montalbano, Reddon, and Bertolucci 2022), we included this aspect of fish cognition in our analyses.

2 | Materials and Methods

2.1 | Animal Welfare Note

The experiments of this project have been approved by the Ethical committee of University of Ferrara (OPBA) and by the Italian Ministry of University and Research (MIUR; protocol n. 446/2021-PR). Moreover, the behavioural observations were designed to avoid stress and discomfort to the subjects and followed the ASAB/ABS Guidelines for the Use of Animals in Research (<https://doi.org/10.1016/j.anbehav.2019.11.002>). Strategies to improve fish welfare are described in full in the main text. Briefly, the maintenance tanks were much larger (e.g., 5–25 L per individual) than average zebrafish housing systems (0.2 L per individual) and provided with enrichments. Fish were kept in social groups and fed with live invertebrate prey as often as possible. The health of the subjects was visually checked daily in all the phases of the study. For the tissue collection, the subjects were humanely killed by expert personnel with the overdose method recognised by EU. Subjects not involved in tissue collection were released in maintenance tanks at the end of the observations.

2.2 | Subjects

The zebrafish used in the study were adult individuals of an outbred wild-type (i.e., not genetically modified) line maintained in our facility at University of Ferrara. This zebrafish line was chosen because it has been shown to habituate to the typical testing conditions adopted in cognitive studies (Andrei et al. 2025; De Russi, Montalbano, et al. 2024; Lucon-Xiccato, Montalbano, Gatto, et al. 2022; Rovegno et al. 2025). The line

originated in 2011 from 100 zebrafish (50:50 sex ratio) obtained from a local store. The stock in the facility was maintained with periodical reproductions of fish randomly selected from different maintenance tanks. We estimate that the line was kept in our laboratory for approximately 25–30 generations, although the overall length of captivity is not known. In addition, twice per year, 50–100 new zebrafish (sex ratio 50:50) were bought and added to the stock to keep low levels of inbreeding in the population. At the time of the experiment, the stock consisted of approximately 1000 individuals.

Before the testing, the subjects were maintained in standard laboratory conditions in mixed-sex groups (sex ratio 50:50) of 15–20 individuals in 75-L glass aquaria (40×60×35 cm). The aquaria were provided with 10 coloured glass marbles and 5 artificial plants made of green plastic (approximate height of 10 cm) as enrichment. The aquaria were also provided with mechanical, chemical and biological filters. Conditions were kept as constant as possible (temperature: 27°C ± 1°C; photoperiod: 14–10 h light–dark; water conductivity: 606.7 ± 60.18 µS/cm; nitrite levels in the water: < 0.1 mg/L; and nitrate levels: < 50 mg/L). Twice per day, the zebrafish were fed ad libitum with alternate live prey (*Artemia salina* nauplii) and commercial food (Staple food Vipar, Sera, Heinsberg, Germany). The *A. salina* nauplii were obtained by incubating commercially available cysts (Ocean Nutrition, Essen, Germany) at 27°C for 24 h. The zebrafish used as subjects were approximately 6 months old.

2.3 | Experimental Treatments

The treatments consisted of feeding the experimental zebrafish with one of three diets (a control diet and two diets with microplastic). We obtained the microplastics for the treatments by milling commercially available polymer pellets. The PE pellets were purchased from DOW with trade name Dowlex 2629 as LLDPE type used for film and food packaging. The PBAT pellets were purchased from Ecoworld Biodegradable Polymer and are commonly adopted to manufacture certified biodegradable and compostable shopping bags, food packages, mulch films and garbage bags. The microplastics were prepared by using a mill (AM 200; Retsch GmbH, Haan, Germany) and then characterised by vibratory sieve machine (AC 200; Retsch GmbH, Haan, Germany) equipped with nine sieves with square apertures ranging from 25 to 1000 µm. The plastic pellets of PE and PBAT were milled as received without adding any additive. The selected milled plastic powders were separated into nine fractions and evaluated by using EasySieve software (Retch, Retsch GmbH, Haan, Germany) for calculating the average powder dimensions and their distributions. For the experiment, we selected as microplastics the powder fractions ranging from 50 to 400 µm with a mean dimension of the microplastics in the range 265–280 µm, a sample useful to demonstrate general effects of microplastics that can be found in nature.

The experimental treatments followed the procedure of an earlier study with slight modification (Limonta et al. 2019). The treatments involved overall 49 groups of zebrafish (overall $N=169$ subjects; 3–4 fish per group according to the scheme described below) randomly collected from the different maintenance tanks. For the brain gene expression analyses (Experiment 1)

and for the visual learning and reversal task (Experiment 2), we treated 22 groups of four zebrafish ($N=88$ subjects) in 2-L glass aquaria (10×10×20 cm) provided with an aerator stone. For Experiment 3 (visual learning with no criterion), we treated nine groups of three zebrafish ($N=27$ subjects) in larger tanks (25 L; 25×40×25 cm), which resembled those used for the cognitive assays. We performed this methodological change in the treatment aquaria in the attempt to habituate as much as possible the subjects to the testing aquarium before the learning assay. For the Experiment 4, we treated 18 groups of three fish ($N=54$) in the 25-L tanks. Not all the subjects exposed to the treatments were used in the following analyses due to logistic constraints (details in the description of each experiment).

The treatment aquaria were maintained in a room with temperature of 27°C ± 1°C and 14:10 h light:dark photoperiod. The water was kept with the same conditions described for the facility. New filtered water was provided daily and whenever deemed necessary by the experimenter (e.g., in case of abundant faecal and food residuals). Each group of zebrafish was randomly assigned to one of the three treatments. The three treatments consisted of a diet as follow: (1) 0.01 g of food plus 100 µg PE microplastics per fish; (2) 0.01 g of food plus 100 µg PBAT microplastics per fish and (3) control diet of 0.01 g food per fish. As food for the treatments, we used commercial dry granules (Tetra, Melle, Germany). The microplastic concentrations in the food were higher than those typically reported in water samples. This approach was chosen for two reasons: first, it is difficult to estimate exactly how much microplastic a fish would ingest under natural conditions, as this may not correlate with the amount of microplastic in the environment due to feeding behaviour; second, given the novelty of this study, we aimed to maximise potential effects to better detect any impact on cognition. The fish were fed their experimental food once per day (h 09:00–11:00). After administering the food, the experimenter visually checked that the fish consumed it entirely and after an hour, cleaned the aquaria with a syphon to eliminate eventual residuals. The treatment for the Experiment 1 and 2 lasted 20 days (Limonta et al. 2019). Instead, for the Experiment 3 and 4, the duration of the treatment was increased to 40 days with the aim to increase eventual effects. While not representing the typical chronic exposure that occurs in nature, these treatments are generally considered medium-term in duration (Gutiérrez-Rial et al. 2025; Sui et al. 2022). At the end of the treatment, inspection from the facility personnel detected no visible signs of disease in the subjects. No fish suffered mortality from this diet.

2.4 | Experiment 1—Gene Expression

We adopted a method based on measures of gene expression, which is increasingly used to investigate fish cognition. We focused on *c-fos* and *egr1*, two genes that are expressed in response to neuronal activity, making them reliable markers of brain activation. Measuring their expression provides an indication of levels of brain activity (reviewed in Calvo and Schluessel 2021). At the end of the treatment, 36 randomly chosen zebrafish (control treatment: $N=12$; PE: $N=12$; PBAT: $N=12$) from the 2-L treatment aquaria were humanely killed. The procedure was performed by the expert personnel authorised by the relevant Minister and involved immersion in a solution MS-222 for 10 min after cessation

of opercular movement. We extracted the whole brain under a dissection microscope and we isolated total RNA from 12 zebrafish brain pools (each made of 3 brains; $N=4$ pools per treatment) with TRIzol reagent (Thermo Fisher Scientific, USA). We measured concentration and purity using Biospec-nano (Shimadzu, Kyoto, Japan); genomic DNA was removed with a DNaseI (Sigma-Aldrich, USA) treatment performed according to the manufacturer's instructions. RNA ($1\mu\text{g}$) was reversed transcribed using iScript cDNA Synthesis Kit (Biorad, Milan, Italy).

We assessed relative gene expression by real-time quantitative PCR using SsoAdvanced Universal SYBR Green Supermix (Biorad, Milan, Italy) on a CFX Connect Real-Time PCR Detection System (Biorad, Milan, Italy) instrument. Genes of interest (*c-fos* and *egr-1*) were analysed with the primer sequences listed in Table S1. We additionally analysed housekeeping genes (*ef1a* and *18S*), which are constitutively expressed genes used to normalise gene expression levels and control for variation in RNA quantity and quality. We verified the efficiency of the primers sets by constructing standard curves and we used the dissociation curves to confirm the specificity of the amplicons. Last, we calculated the relative levels of expression with the $2^{-\Delta\Delta C_T}$ method (where C_T is the cycle number at which the signal reaches the threshold of detection) (Livak and Schmittgen 2001). Each C_T value used for the calculations is the mean of three replicates of the same reaction.

2.5 | Experiment 2—Visual Learning and Reversal

In the first learning test, we assayed 47 zebrafish (control treatment: $N=15$; PE: $N=17$; PBAT=15) that were treated in the 2-L aquaria. The initial sample consisted of 48 subjects (determined based on the availability of experimental tanks), but one subject showed no interest in the food and was excluded before data collection. During the test, the subjects had to discriminate between two colour stimuli to receive a food reward (Lucon-Xiccato, Montalbano, Gatto, et al. 2022; Montalbano et al. 2022). The stimuli to be discriminated consisted of a card with a red circle and a card with a yellow circle (size of the card: $4\times 4\text{cm}$; size of the circle: $\varnothing 1.8\text{cm}$). We kept the subjects individually in aquaria ($25\text{cm}\times 40\text{cm}$, h 25cm ; Figure 1a,b) with gravel on the bottom and an air stone (removed during testing to avoid disturbance) for the entire duration of the experiments, which varied according to when the subjects reached the criterion (see details below). The water in the aquaria was kept at $27^\circ\text{C}\pm 1^\circ\text{C}$ and was monitored and changed regularly to keep conditions as in the maintenance aquaria. Each tank was illuminated by a LED strip set with a photoperiod of 14 h of light and 10 h of dark. Along each longer wall of the aquarium, we built a trapezoidal sector with transparent plastic, containing a small aquatic plant as enrichment (Figure 1a,b). Because of these two sectors, the apparatus had the shape of an hourglass, with a central corridor and two larger sectors in correspondence of the short walls of the aquarium.

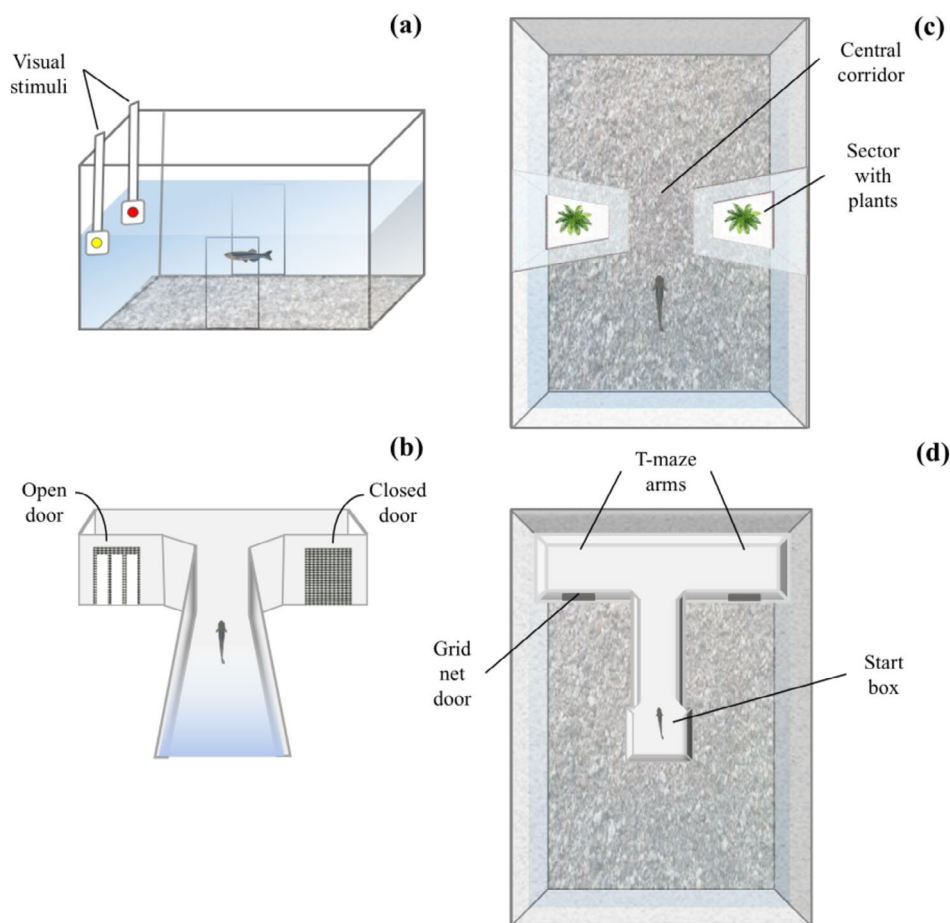


FIGURE 1 | Diagrams of the experimental apparatus used in the tests to measure visual learning, spatial learning, and cognitive flexibility. (a) Lateral view and (b) top view of the entire apparatus of the visual tasks. (c) Perspective view of the T-maze and (d) top view of the entire apparatus of the spatial tasks.

Before commencing the experiment, we assigned a rewarded colour (either yellow or red) to each fish, counterbalancing across the experimental groups. The colour assigned had no significant effect on fish performance (two-samples *t* test on the number of days to reach the learning criterion: $t=1.226$, $p=0.229$). We then started the first phase of the experiment, the pre-test phase, which lasted 2 days. On Day 1 of the pre-test phase, the experimenter presented the rewarded colour to the subject by attaching the card to a transparent stick (length 20 cm) that could be blocked onto the short wall of the apparatus. When the subject was in one of the two main sectors of the apparatus, the experimenter inserted the stimulus in the water in front of the opposed short walls of the apparatus. Then, the experimenter waited motionless until the subject approached the card; when this occurred, the experimenter delivered a drop of water containing live *A. salina* in front of the card using a Pasteur pipette. To avoid satiation, the reward solution was diluted so that only 2–4 prey were delivered each time, along with chemical cues that likely also served as a positive reinforcement. This amount was also sufficient to ensure that all the prey were consumed quickly. The experimenter administered eight pre-test trials with this procedure to each subject, half in the morning and half in the afternoon, alternating the presentation of the stimulus between the two sides of the apparatus. The initial eight trials with the rewarded colour did not seem to induce a persistent colour preference, as indicated by the lack of enhanced performance in the first trials of the test phase.

On the second day of the pre-test phase, the experimenter presented the subject with both the rewarded and the non-rewarded colour stimulus. Each of the two cards was placed in correspondence of the corners of the short wall of the apparatus. The subject was therefore equally distant from the two stimuli when approaching them via the central corridor. The experimenter delivered the food if the fish approached the rewarded colour within 10 min (i.e., even if the initial choice was for the incorrect stimulus). In case of no approach, the trial was repeated. The zebrafish underwent eight trials with this procedure, four trials in the morning and four in the afternoon, with an inter-trial interval of 10 min. The left–right position of the rewarded stimulus was randomly alternated between trials.

The third day of the experiment, the fish that consumed the food in all the training trials entered the test phase. The trials of the test phase followed the procedure described above. However, in case the subject did not choose the rewarded card at first, the experimenter removed both stimuli without delivering the food reward, the trial was concluded, and the fish continued with the following trial after the 10-min interval. The subjects underwent 12 trials per day (6 in the morning and 6 in the afternoon). This number was chosen based on several earlier studies on zebrafish and other small laboratory fish, as it does not satiate the animals, which continue to participate to the experiment for the reward (e.g., De Russi, Montalbano, et al. 2024; Gatto et al. 2020; Lucon-Xiccato et al. 2019, 2023; Lucon-Xiccato, Montalbano, Gatto, et al. 2022; Lucon-Xiccato, Montalbano, Frigato, et al. 2022; Montalbano et al. 2022). The test phase continued until the subject reached a learning criterion set at 17 correct choices out of 24 trials in 2 consecutive days (70% correct choices), which corresponds to a statistically significant preference for the rewarded stimulus (Chi-squared

test: $\chi^2_1=4.167$, $p=0.041$). The criterion was necessary to allow the second phase of the experiment (the reversal learning) avoiding individual differences in overtraining (Eimas 1967; Nakagawa 1992). The specific criterion was used in previous studies because the zebrafish often showed reduced visual discrimination abilities (e.g., Gatto et al. 2020). If a subject did not pass the learning criterion within 30 days of testing, we interrupted the test. Therefore, the length of the test phase varied between subjects according to their learning speed.

The 38 subjects that reached the learning criterion in the visual learning test of Experiment 1 (control treatment: $N=11$; PE: $N=13$; PBAT=14) subsequently performed the reversal learning task to measure cognitive flexibility (De Russi et al. 2025; Montalbano et al. 2022). The reversal learning task began the day after a subject reached the learning criterion of the learning task. The subjects remained in the same tank in which they performed the learning test. The general procedure of the reversal learning resembled the one described in the test phase of the learning test. Yet, the experimenter reversed the reward contingency. Therefore, each subject had to choose the colour that was previously unrewarded. The criterion to complete the reversal learning task was set as in the previous test (70% correct choices). At the end of testing, the fish were moved back into the facility in separate aquaria for non-naïve fish. They were not used as breeders.

2.6 | Experiment 3—Visual Learning Without Criterion

In the learning test of Experiment 2, we adopted a learning criterion to perform the reversal learning task. The criterion ensured that all subjects had a comparable understanding of the discrimination learning task before facing the reversal learning. However, results of Experiment 2 showed that many fish performed relatively poorly and failed to reach this criterion. To further compare the experimental subjects, we performed a second visual learning experiment without a learning criterion (Experiment 3). All the subjects were trained with the procedure previously described up to 15 days. With this design, we aimed to more precisely characterise the learning curves and to improve group comparisons, relative to what was achieved in Experiment 2. Moreover, in this second visual learning test, we used subjects treated in large aquaria similar to the learning apparatuses in an attempt to minimise any possible source of stress. The second learning test involved 27 subjects (control treatment: $N=9$; PE: $N=9$; PBAT=9). At the end of testing, the fish were moved back into the facility in separate aquaria for non-naïve fish.

2.7 | Experiment 4—Spatial Learning and Reversal

In the spatial learning test of Experiment 4, we used 45 subjects (control treatment: $N=13$; PE: $N=15$; PBAT=17) treated in the 25-L aquaria for 40 days. An additional fish started the experiment but was discarded because it showed freezing behaviour and did not participate to the trials. At the end of the treatment, these fish were transferred to the experimental task, one subject per tank. The experimental tanks were of

the same size as those used during the treatment phase but included gravel at the bottom and a T-maze. The maze was suspended near the water surface, with approximately 1 cm of water depth inside the maze, and held in place by being glued to the wall of the tank (Figure 1c,d). The maze had a T-shape, consisting of a start box (9×6.5 cm) connected to a corridor (12×4 cm), which ended in a T-junction leading to two arms of the maze (10×4 cm), each containing an exit with two doors made of grid net. During the first 2 days of acclimation, the maze doors were closed to prevent the subjects from exploring the experimental apparatus before the beginning of the test. During these 2 days, the subjects were fed daily with the usual granulated food used during the treatment and a small amount of *A. salina* to allow gradual habituation to this second type of food.

After the acclimation period, the learning phase began. Each subject was randomly assigned a correct arm (left or right). The door of the correct arm was substituted before the trials with a fake door in which the grid net presented vertical cuts and could be lifted by the subjects pushing with the snout, allowing them to return to the main compartment of the tank. Each morning, before starting the trials, the aerators were removed from the tanks to not affect the test. Subjects were then carefully collected from the tank using a net and were placed in the start box of the maze. The trial began when the subject entered the corridor from the start box. If the subject entered the correct arm of the maze, the trial was recorded as correct; otherwise, it was considered incorrect. In case of an incorrect trial, the fish was left free to exit the apparatus via the correct arm. Regardless of the response, the subject was rewarded with *A. salina* immediately after exiting the maze.

This trial was repeated for 12 times per day, with at least a 1-h break between the first 6 trials and the final 6 trials. If a subject made 4 or fewer errors over 24 trials (i.e., 20 or more correct responses), it was considered to have reached the spatial learning criterion. This criterion was higher than in our previous experiment, as earlier works (e.g., De Russi, Lanzoni, et al. 2024; Lucon-Xiccato et al. 2023) and pilot studies with this procedure showed that the task is relatively easy for the fish. The 38 subjects that reached the learning criterion, subsequently performed the reversal spatial learning task to measure cognitive flexibility (De Russi, Lanzoni, et al. 2024). The reversal phase was performed as the learning phase, but the correct arm assigned to the subject was reversed (e.g., a subject previously assigned to the left correct arm was now assigned to the right correct arm). The same criterion used in the learning phase was applied to the reversal learning phase. Subjects that reached the criterion in this phase were removed from the experiment and moved back into the facility in separate aquaria for non-naive fish. The experiment was interrupted for subjects that did not meet the criterion within 20 days of either the learning or reversal learning.

2.8 | Statistical Analysis

We performed the statistical analysis in R version 4.0.1 (The R Foundation for Statistical Computing, Vienna, Austria,

<http://www.r-project.org>). Significance level was set at $p=0.05$ and statistical tests were two-tailed if not stated otherwise. To examine changes in brain gene expression (Experiment 1) among the three experimental groups, we analysed the relative gene expression (log transformed due to right-skewed distribution) of *c-fos* and *egr-1* using one-way ANOVAs and Tukey post hoc test corrected for multiple comparisons.

For the Experiments 2 and 4, we analysed three dependent variables with three different models fitted with treatment as fixed effect. First, we checked for differences between treatments in the number of subjects that reached the criterion. We assigned the value '1' to subjects that reached the criterion and the value '0' to subjects that did not reach the criterion. Given the binomial distribution of this dependent variable, we analysed it with a generalised linear model with binomial error distribution. Second, we compared the number of days necessary to reach the criterion with ANOVAs. To include the subjects that did not reach the learning criterion in learning test 1 and in the reversal learning test, we assigned them the maximum possible value (e.g., 30 days in Experiment 2 and 20 days in Experiment 4). Last, we analysed the number of correct choices made by each subject that solved the task on each day of the test phase. This dependent variable had a repeated measures structure and different number of observations per each subject (due to the different number of days taken to reach the criterion). We therefore used linear mixed-effects models that can handle this type of data. In this last model, we additionally fitted individual ID as random effect to account for the repeated measurements and day as a fixed effect. We kept the treatment by day interaction in the model because, if significant, would indicate different learning rates between treatments.

In the Experiment 3, due to the absence of the criterion, we analysed only the number of errors across the days of training. This analysis was performed with a linear mixed-effects model as previously described.

Because we did not detect significant effects of the treatment in the learning and reversal learning tasks, we used a Bayesian approach to provide a more robust evidence of performance similarity between fish from the different treatments. From the models described above, we obtained an approximate Bayes factor (BF) following Wagenmakers (2007). Unlike effect sizes, which measure the magnitude of an effect and are tied to traditional hypothesis testing, the BF allows us to quantify evidence supporting similarity between groups (Dienes 2014).

To study individual differences in the different experimental groups, we followed a two-step approach. First, we used Bartlett tests to compare variance in the main performance scores of Experiments 2 and 4 (number of days to criterion). Then, we looked for correlation between individuals' performance in different tasks. Using Spearman rank correlation tests, we analysed the relationship between the performance in the visual learning test and the visual reversal learning task and between the spatial learning and spatial reversal learning task. The correlation analysis was conducted separately for each treatment.

3 | Results

3.1 | Experiment 1—Brain Gene Expression

The expression of *c-fos* was significantly affected by the treatment (ANOVA: $F_{2,9}=4.560$, $p=0.043$; $\eta^2=0.50$, 95% CI [0.02, 1.00], Figure 2a). The Tukey post hoc test revealed that the fish exposed to PE displayed downregulation of *c-fos* compared to control subjects ($t=-2.985$, $p=0.037$), while the remaining contrasts were not significant (PBAT treatment versus control: $t=-1.891$, $p=0.197$; PE treatment versus PBAT treatment: $t=-1.094$, $p=0.541$). The expression of *egr-1* was not significantly affected by the treatment ($F_{2,9}=0.367$, $p=0.703$; Figure 2b).

3.2 | Experiment 2—Visual Learning

Most of the subjects (38 out of 47; 80.85%) learned the colour discrimination task according to the established criterion. The number of learner subjects varied between treatments as follows: control=11 out of 15 (73.33%); PE treatment=13 out of 17 (76.47%); PBAT treatment=14 out of 15 (93.33%). There was no significant difference in the likelihood to solve the colour discrimination task due to the treatment (Table S2; $BF=13$). The ANOVA on the number of days necessary to reach the learning criterion indicated no difference due to the treatment (Table S2; $BF=9$; Figure 3a). The analysis on the daily number of correct choices of the learner subjects indicated an increase across days

of testing (Table S2), as expected due to learning. However, the analysis found no significant effect of the treatment (Table S2; $BF=46,373$; Figure 3b) and no significant treatment by day interaction (Table S2).

3.3 | Experiment 2—Visual Cognitive Flexibility

Twenty-six out of 38 subjects (68.42%) that underwent the visual reversal learning task reached the criterion. The number of subjects from the different treatments that solved the reversal learning task was as follows: control=8 out of 11 (72.72%); PE treatment=9 out of 13 (69.23%); PBAT treatment=9 out of 14 (64.29%). There was no significant effect of the treatment on the likelihood to solve the reversal learning task (Table S3; $BF=34$). Similarly, the ANOVA on the number of days to the criterion indicated no effect due to the treatment (Table S3; $BF=38$; Figure 3c). The analysis on the daily number of correct choices of the learner subjects indicated no significant change across days of testing (Table S3), no significant effect of the treatment (Table S3; $BF=9428$; Figure 3d) and no significant treatment by day interaction (Table S3).

3.4 | Experiment 3—Visual Learning Without Criterion

The analyses on the data of the second learning test revealed a significant increase of the correct choices across days of training

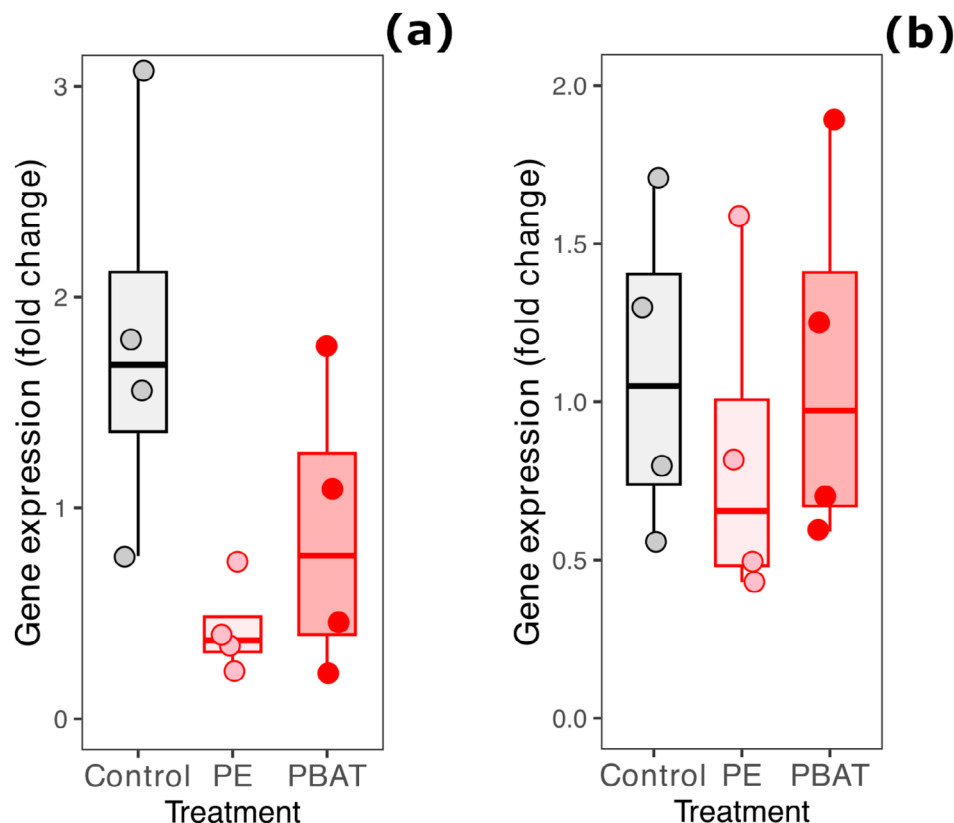


FIGURE 2 | Results of brain gene expression (Experiment 1). (a) *c-fos* and (b) *egr-1* expression in the brain of zebrafish from the three treatments; boxes represent the interquartile range (IQR), horizontal lines the median, whiskers extend to $1.5 \times IQR$, and points are individual subjects' scores. Control = zebrafish fed with normal food; PBAT = zebrafish fed with food supplemented with microplastics of a biodegradable polymer; PE = zebrafish fed with food supplemented with microplastics of a synthetic polymer.

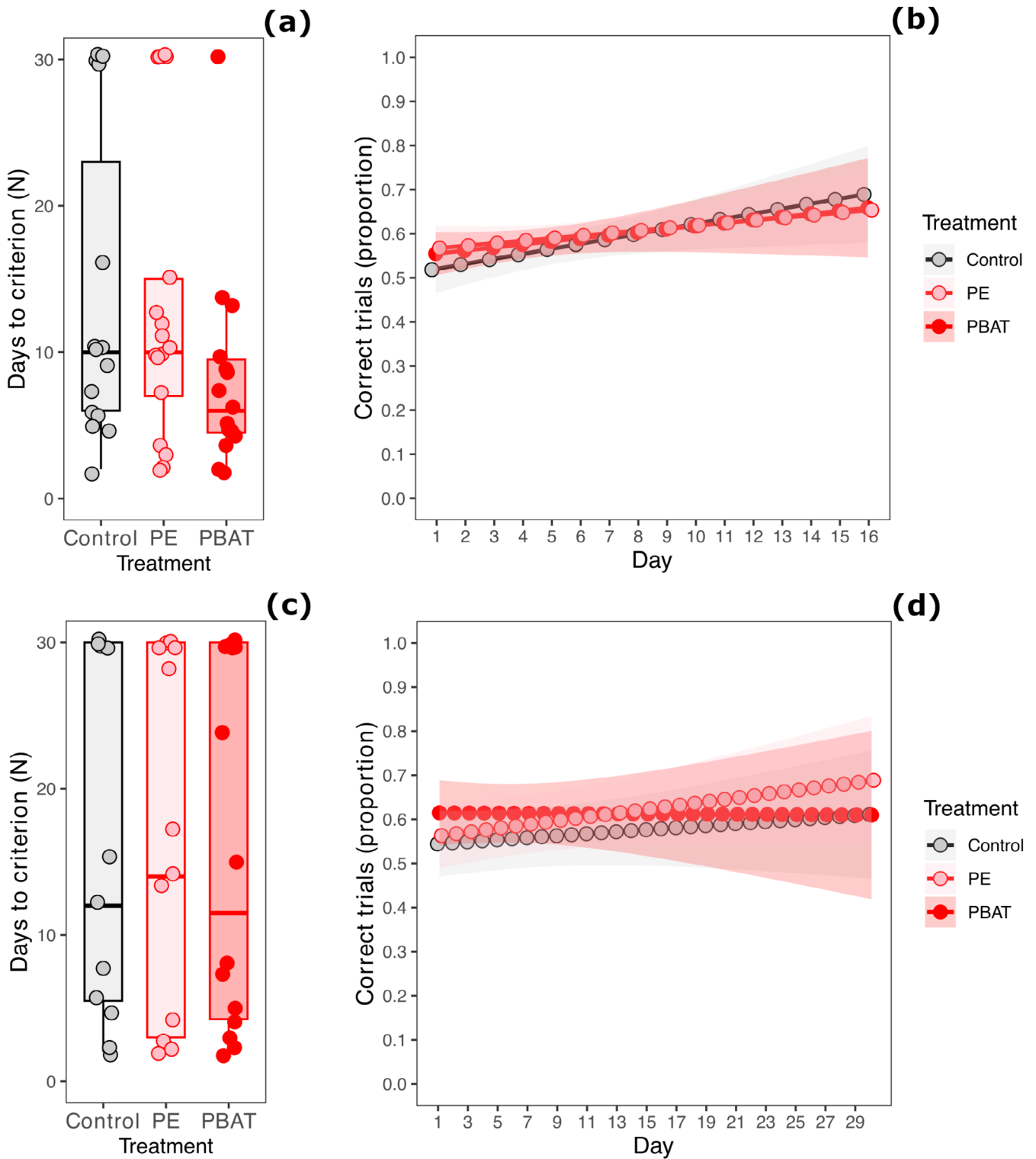


FIGURE 3 | Results of the visual tasks of Experiment 2. Number of days to reach the criterion of all the subjects including those that did not reach the criterion (value assigned: 30days) in the (a) learning and the (c) reversal learning task; boxes represent the interquartile range (IQR), horizontal lines the median, whiskers extend to $1.5 \times \text{IQR}$, and points are individual subjects' scores. Proportion of correct trials in each day of testing of subjects that reached the criterion in the (b) learning and the (d) reversal learning task; points represent means estimated by the model, and shaded areas represent estimated CI. In both panels, Control = zebrafish fed with normal food; PBAT = zebrafish fed with food supplemented with microplastics of a biodegradable polymer; PE = zebrafish fed with food supplemented with microplastics of a synthetic polymer.

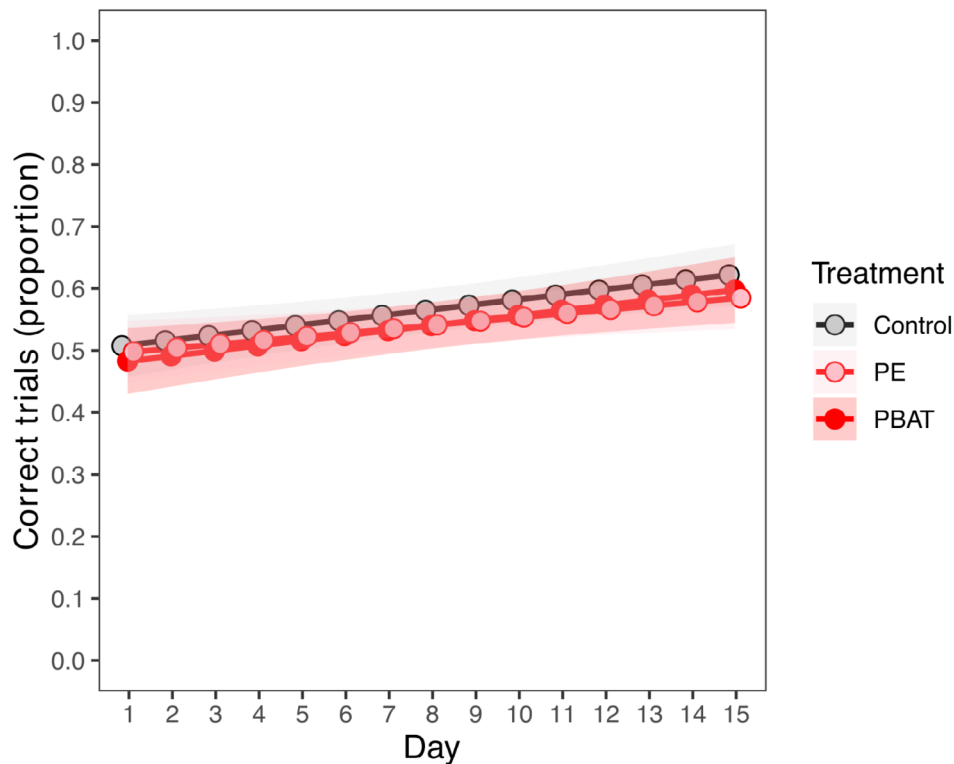


FIGURE 4 | Results of the visual learning task without criterion of Experiment 3. Proportion of correct trials in each day of testing; points represent means estimated by the model, and shaded areas represent estimated CI. Control=zebrafish fed with normal food; PBAT=zebrafish fed with food supplemented with microplastics of a biodegradable polymer; PE=zebrafish fed with food supplemented with microplastics of a synthetic polymer.

($F_{1,361}=24.532$, $p<0.001$). However, the effect of treatment ($F_{2,23}=0.588$, $p=0.566$) and the treatment by day interaction ($F_{2,361}=0.199$, $p=0.828$) were not significant (Figure 4).

3.5 | Experiment 4—Spatial Learning

Thirty-eight out of 45 subjects (84.44%) that underwent the spatial learning task reached the criterion. The number of subjects from the different treatments that solved the reversal learning task was as follows: control=12 out of 13 (92.31%); PE treatment=12 out of 15 (80.00%); PBAT treatment=14 out of 17 (82.35%). There was no significant effect of the treatment on the likelihood to solve the reversal learning task (Table S4; BF=27). Similarly, the ANOVA on the number of days to the criterion indicated no difference due to the treatment (Table S4; BF=28; Figure 5a). The analysis on the daily number of correct choices of the learner subjects indicated a significant increase across days of testing (Table S4), but no significant effect of the treatment (Table S4; BF=7922; Figure 5b) and no significant treatment by day interaction (Table S4).

3.6 | Experiment 4—Spatial Cognitive Flexibility

All subjects that underwent the spatial reversal learning task reached the criterion. There was no significant effect of the treatment on the likelihood to solve the reversal learning task

(Table S3; BF=37). Similarly, the ANOVA on the number of days to the criterion indicated no effect due to the treatment (Table S5; BF=22; Figure 5c). The analysis on the daily number of correct choices of the learner subjects indicated a significant increase across days of testing (Table S5), but no significant effect of the treatment (Table S5; BF=9205; Figure 5d) and no significant treatment by day interaction (Table S5).

3.7 | Cognitive Individual Differences

The variance in number of days to reach the criterion did not differ among the three experimental groups considering visual learning (Bartlett $K^2_2=2.617$, $p=0.270$), visual reversal learning ($K^2_2=0.007$, $p=0.996$), spatial learning (Bartlett $K^2_2=1.250$, $p=0.535$) and spatial reversal learning ($K^2_2=2.665$, $p=0.264$). Moreover, there was no significant correlation between learning and reversal learning performance in any of the experimental groups in the visual (control: Spearman rank correlation $\rho=-0.066$, $p=0.847$; PE: $\rho=-0.279$, $p=0.356$; PBAT: $\rho=-0.266$, $p=0.377$) and the spatial task (control: $\rho=-0.321$, $p=0.309$; PE: $\rho=-0.237$, $p=0.459$; PBAT: $\rho=-0.314$, $p=0.274$).

4 | Discussion

We investigated whether the ingestion of microplastics derived from synthetic or biodegradable polymers over a medium-term

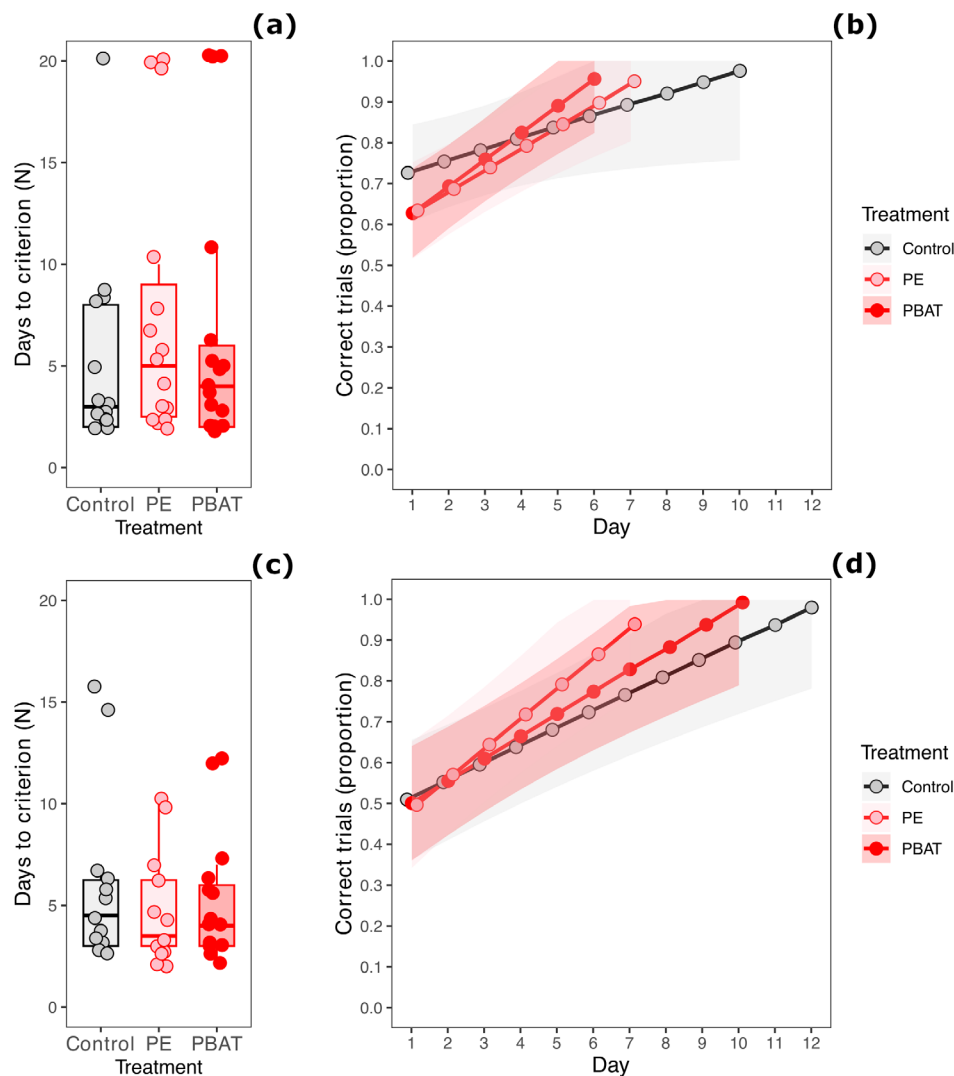


FIGURE 5 | Results of the spatial tasks of Experiment 4. Number of days to reach the criterion of all the subjects, including those that did not reach the criterion (value assigned: 30 days) in the (a) learning and the (c) reversal learning task; boxes represent the interquartile range (IQR), horizontal lines the median, whiskers extend to $1.5 \times \text{IQR}$, and points are individual subjects' scores. Proportion of correct trials in each day of testing of the subjects that reached the criterion in the (b) learning and the (d) reversal learning task; points represent means estimated by the model, and shaded areas represent estimated CI. In both panels, Control = zebrafish fed with normal food; PBAT = zebrafish fed with food supplemented with microplastics of a biodegradable polymer; PE = zebrafish fed with food supplemented with microplastics of a synthetic polymer.

period affects cognition in a teleost fish species, measuring the expression of two gene markers of brain activity, as well as learning and cognitive flexibility. Our results showed that synthetic microplastics reduced the expression of *c-fos*, a marker of neural activation, in the brain. Notably, *c-fos* expression in the brains of control zebrafish was more than double that of zebrafish exposed to PE microplastics. This finding is consistent with previous studies reporting altered basal *c-fos* expression in fish (Baraban et al. 2005; Salierno et al. 2006, 2007; Topal et al. 2015) and mammals (Hidaka et al. 2011; Ryabinin et al. 1997; Zhang et al. 2002) exposed to stressors such as pollutants and parasites. Interestingly, the second immediate early gene examined, *egr-1*, did not show downregulation in response to microplastic exposure. This likely reflects the distinct roles of these two genes (Calvo and Schluessel 2021). For example, *egr-1* is also constitutively expressed (Worley et al. 1991), and this baseline expression might obscure the detection of changes due to treatment.

Considering the cognitive abilities investigated, namely visual and spatial learning and visual and spatial cognitive flexibility, we found no significant alterations due to microplastic ingestion. Single cognitive experiments pose some interpretative challenges when considered in isolation. For instance, in Experiment 2, zebrafish demonstrated reduced learning performance, consistent with previous findings in similar visual discrimination tasks (e.g., Gatto et al. 2020). This lack of learning may have prevented detection of significant differences between groups. However, the overall results from our comprehensive cognitive assessment based on multiple procedures, including the spatial tasks where learning performance was stronger, consistently indicate a lack of treatment effect. The approximate Bayes factors indicated that models excluding the effect of treatment were several times more likely to explain our data, providing positive to decisive evidence in favour of similarity between the experimental groups. Consistent with our findings, ingested PE microplastics did not impair learning and memory in honeybees

(Balzani et al. 2022) and mosquitofish performance in a detour test (Irwin et al. 2024). Additionally, another study in a teleost fish reported that microplastics did not affect individual differences behaviour (activity and exploration; Tosetto et al. 2017), like in our study microplastics did not impact individual differences in cognition.

From our data, we can conclude that microplastics somehow affected a marker of brain functional activity, yet without determining measurable changes in cognitive abilities. This contrasting pattern of results might seem counterintuitive. For example, in rodent models, experimental reduction of the *c-fos* pathway via mutational and pharmacological interventions decreases cognitive performance across several tasks (Clements et al. 2005; Fleischmann et al. 2003; Grimm et al. 1997). A potential explanation is that the molecular alterations affect behaviours that we did not measure in this study. Alternatively, it is worth noting that *c-fos* is expressed not only during stimulation (Bosch et al. 1995; Morgan and Curran 1989), but also during the early phases of neuronal plasticity (Kaczmarek 1993; Minatohara et al. 2016) and to determine neuronal survival (Zhang et al. 2002). Since we measured *c-fos* RNA across the entire brain without specific stimulation, our molecular data may generally reflect the action of *c-fos* on neuronal plasticity and survival. Therefore, we propose that microplastics might impact these two actions of *c-fos*, potentially affecting brain circuitry in the long term, rather than causing immediate effects that can be detected through analyses of cognitive abilities. It is possible that longer exposure to microplastics would increase the chances of observing effects on cognitive abilities.

Another general explanation for the lack of effects on learning and cognitive flexibility is that the impact of our treatment could be relatively small and difficult to measure. The average size of the microplastics used in our study was too large to cross the blood–brain barrier (Campanale et al. 2020). Therefore, the effects of microplastics on cognition were likely indirect, arising as by-products of physiological responses known to influence cognition, such as stress hormone production (Lindau et al. 2016; Lupien et al. 2007), prolonged inflammatory responses (Sun et al. 2017), and disruptions of intestinal homeostasis (Alemohammad et al. 2022; Davidson et al. 2018). Because these effects are indirect, they may result in relatively small effect sizes that are difficult to detect with learning and flexibility assays, even if they remain evident at the molecular level.

In conclusion, our study demonstrated alterations in a proxy of brain activity, but not in learning abilities and cognitive flexibility in zebrafish exposed to microplastics. The key question for future research is whether the observed molecular changes in the brain may lead to fitness impairments in natural environments, where animals are chronically exposed to microplastic pollution, either by affecting behaviours we did not measure or through alterations in brain processes discussed above such as neural plasticity and survival. Additionally, future research should also explore the effects of microplastics collected from natural environments. Microplastics can absorb chemical pollutants from the water, some of which are known to somehow affect cognitive processes (e.g., heavy metals: Brennecke et al. 2016; Hildebrandt et al. 2021; Strungaru et al. 2018). Therefore, in nature, fish ingesting microplastics

may experience greater effects than those measured in our treatment with virgin microplastics, due to additive or multiplicative interactions with pollutants.

Author Contributions

Tyrone Lucon-Xiccato: conceptualization, funding acquisition, formal analysis, writing – original draft, writing – review and editing. **Giulia Montalbano:** conceptualization, investigation, formal analysis, writing – review and editing. **Georgiana Andrei:** conceptualization, investigation, formal analysis, writing – review and editing. **Elena Frigato:** conceptualization, investigation, formal analysis, writing – review and editing. **Marco Scoponi:** conceptualization, writing – review and editing. **Luigi Abelli:** conceptualization, writing – review and editing. **Annalaura Mancina:** conceptualization, writing – review and editing. **Cristiano Bertolucci:** conceptualization, funding acquisition, writing – review and editing.

Acknowledgements

We have no competing interests. We are thankful to Andrea Margutti for help in building the apparatuses and to Alessandro Colombani, Simona Sandrin, Claudia Corbino and Ludovica Modesto for help in testing the subjects. Funding was provided by University of Ferrara (FAR2022 grant to T.L.-X. and C.B.). Open access publishing facilitated by Università degli Studi di Ferrara, as part of the Wiley - CRUI-CARE agreement.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets supporting this study have been uploaded as [Supporting Information](#).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** eth70029-sup-0001-TablesS1-S5.pdf. **Table S2:** eth70029-sup-0001-TablesS1-S5.pdf. **Table S3:** eth70029-sup-0001-TablesS1-S5.pdf. **Table S4:** eth70029-sup-0001-TablesS1-S5.pdf. **Table S5:** eth70029-sup-0001-TablesS1-S5.pdf.