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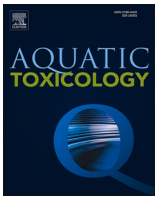

Swimming behavior affects ingestion of microplastics by fish

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ABSTRACT

Microplastics (< 5 mm) are widely found in organisms and have the potential harm to ecosystems. Despite their widespread prevalence in environments, there is high individual varation in the abundance of microplastics found in individuals of the same species. In the present study, juvenile cichlid fish (*Chindongo demasoni*) were chosen to determine the ingestion personality for microplastics in the laboratory. The visible implant fluorescent tags were used for individual recognition. The fish were fed with microplastic fiber, pellet, and food for comparison. Our results showed that the observation of the behaviors of fish could be successfully matched with subsequent measurements for each individual through the tag method in microplastic research. The difference in the abundance of fiber (0–27 items/ind.) among fish individuals was also observed in our study. Meanwhile, the abundance of fiber showed a positive correlation with the average speed and covered area of fish, which indicates the degree of activity of fish. Moreover, fish with higher speed or a front position had higher capturing times for pellet. Our results suggest that the swimming behaviors of fish affect their ingestion of microplastics, and active fish had a higher likelihood of ingesting microplastics, which might be one of the reasons for the common phenomena, i.e., great individual differences observed in microplastic studies.

1. Introduction

Microplastics, which are plastic debris smaller than 5 mm, have become a widely discussed topic in relation to environmental contamination in recent years (Thompson et al., 2004). They are widely found in water, air, sediment, and, concerningly, multiple organisms (Browne et al., 2013; Rochman, 2018; Thompson et al., 2004; Zhang et al., 2020). Fish have been one of the most studied species in microplastic pollution research, whether in field studies or laboratory experiments (Azevedo-Santos et al., 2019). Numerous studies have shown that microplastics have various adverse impacts on fish, such as growth, reproduction, and health (Barboza et al., 2018; Carlos de Sa et al., 2015).

Microplastics are considered to be an emerging pollutant that is different from traditional pollutants. The concentrations of organic pollutants in organisms usually show little individual differences due to their even distribution and accumulation in tissue (e.g., fatty tissue) (Rodrigues et al., 2019). However, differences in the characteristics of microplastics have been found among different fish species, possibly due to factors such as habitat, feeding habits and behavior (Collard et al., 2019; Jabeen et al., 2017). For instance, our previous research has shown that filter-feeding fish have no obvious feeding behaviors on microplastics, while swallow-feeding fish show chasing and capturing behavior to microplastics (Li et al., 2021).

Furthermore, individual variations in the abundance of microplastics have also been observed within the same species, both in field investigations and in laboratory exposure experiments (Avio et al., 2015; Chen et al., 2022b; Critchell and Hoogenboom, 2018; Nanninga et al., 2021; Zhu et al., 2019). The reasons for such individual variation in microplastic abundance are still unclear. Some researchers attribute it to biotic factors or experimental factors (Nanninga et al., 2021, 2019). A previous study reported that there is a correlation between the length of a fish and the number of microplastics ingested, indicating that longer fish tend to consume more microplastics. Additionally, the abundance of microplastics detected may be influenced by the volume and origin of

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the gut content analyzed (Lusher et al., 2015; Moore et al., 2020; Zantis et al., 2021). We speculate that individual differences in fish's performance on movement behaviors may also be a factor contributing to their differential ingestion of microplastics. Fish have been found to exhibit individual differences in behavior, which will lead to a variety of ecologically important outcomes including mate choice, foraging success, and fitness due to personality differences of fish (Biro and Stamps, 2008; Schuett et al., 2010; Toscano et al., 2016). While behavioral differences have previously been shown to be an important factor influencing susceptibility to other human-induced environmental changes (Reale et al., 2007), the extent to which behavioral differences among individuals within a group at the time of ingestion relate to the tendency to ingest microplastics is still poorly understood.

Here, we investigated the relationship between swimming behavior and the abundance of two shapes of microplastic (fiber and pellet) in a cichlid fish (*Chindongo demasoni*) using visible implant fluorescent tags to distinguish individual fish. Our aim was to determine whether differences in swimming activity relate to the number of microplastics ingested by individuals of the same species.

2. Materials and methods

2.1. Husbandry of fish

Juvenile *C. demasoni* ($n = 500$) were purchased from a local farm (Yongchuan, Chongqing City, China) and housed in thermostatic tanks (1200 × 550 × 550 mm) with a water purification system for 1 month. The tanks received dechlorinated water at 25 °C, and the dissolved oxygen was higher than 90 % of saturation. The fish were held under a natural-simulated photoperiod (12: 12, light: dark) and fed ad libitum with artificial fish food particles (Tongwei group, China) once a day. To minimize the impact of differences in body size and weight, we tried to select fish that were similar in size (Table S1) and transferred groups of 10 fish to several small thermostatic tanks with a consistent temperature. Fish were acclimated to laboratory conditions for two weeks prior to the start of experiments. All fish were cared for and used in accordance with the National Animal Welfare Law of China (Zhao-20,210,625).

2.2. Preparation of microplastics

Two types of microplastics (i.e., red fibers and black pellets) were used in the exposure experiments. Both materials were purchased from a plastic store (Yuyao City, China). Red fibers (polyethylene terephthalate) were cut into tiny pieces with dissecting scissors and then filtered with a 5-mm mesh sieve using ultrapure Milli-Q water to collect appropriately sized particles in a clean jar (10 items/mL). Black pellets were the raw nylon materials used for plastic production. The basic parameters of both materials are included in Table S2.

2.3. Visible implant fluorescent tags

Visible implant fluorescent tags (VIF) (Starfish Company, Qingdao, China (http://www.starfish.cn/)) were used to track individual identification over the experiment. The fish were anesthetized for a few minutes with benzocaine (200 mg/L). After anesthesia, an ingestion syringe was used to lightly inject a 1-cm line under the skin on the side of the dorsal fin. Red, green and purple fluorochromes were used to provide each fish within a group with a unique two-color identification code (Fig. 1).

2.4. Experimental design and setup

Prior to the start of experiments, the fish were food starved for 24 h. For the fiber-exposure experiment, ten individuals were placed in a round tank (diameter- 100 cm, water depth-5 cm), which received dechlorinated water at 25 °C. To reduce the randomness and errors of behavior, we tested 10 groups (100 fish in total) to obtain the replicate

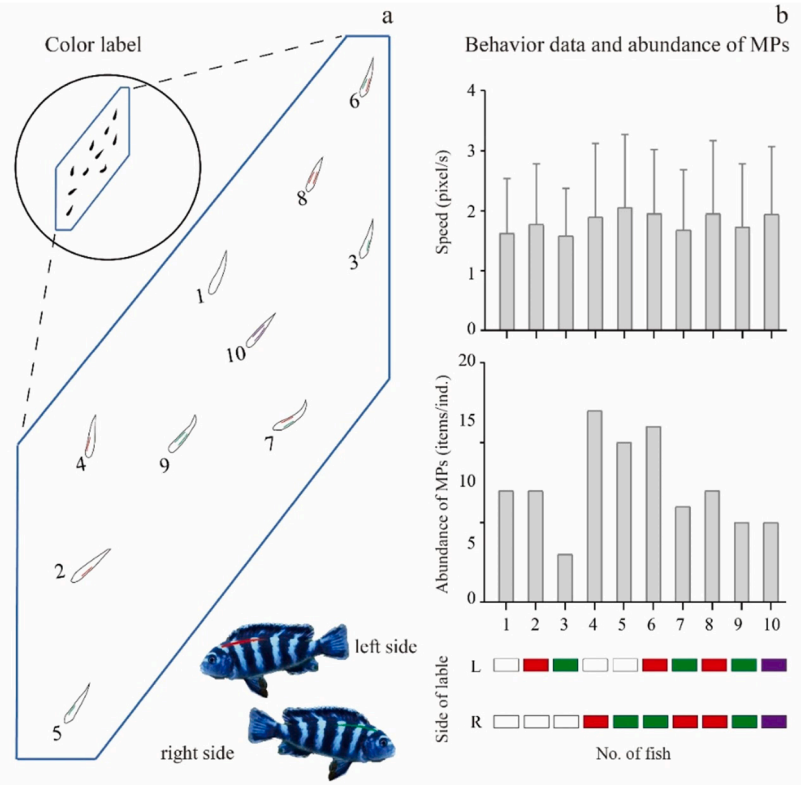

Fig. 1. The matching method on fish from video recognition and digestion. a, the color label in the fish body; b, the speed and abundance of microfibers in each fish from one sample group. Error bar is indicated by a whisker.

results. A 30-minute interval before the experiments was maintained to ensure that the fish returned to normal condition. Approximately one thousand fibers in total were then dropped into the water with a glass dropper from five positions (i.e., up, down, left, right, and central areas of the tank) and gently stirred to distribute them as evenly as possible. The behaviors of the fish were recorded for 20 min. Then, the fish were digested to measure the abundance of microplastics in the body.

Pellet-exposure trials were conducted similarly (10 groups of 10 individuals), with some exceptions. Fish were not VIF tagged because unlike for fiber trials, ingestion of plastic pellets happened rapidly and was easily tracked without the assistance of VIF tags. Groups were tested in a smaller round tank (diameter- 50 cm). Five pellets were added to the same positions as where fibers were dropped. A 3-minute interval was chosen to record the behaviors of the fish because ingestion happened more rapidly.

2.5. Recording and trajectory tracking

Trials were recorded using a Blackmagic Pocket Cinema Camera 6 K (Blackmagic Design, Australia) with Canon EF 24–105 mm f/4 L IS USM (Canon, Japan) to shoot the entire experimental tank at 1080P, 50 frames per second (FPS) from above. Videos were analyzed using idtracker.ai (https://idtrackerai.readthedocs.io/en/latest), which is an open-source software for tracking a group of unmarked animals and giving each individual a unique identity number (ID number) from videos recorded in the laboratory conditions (Romero-Ferrero et al., 2019). The trajectory data were visually inspected for any inconsistencies or errors and manually corrected if needed. Then, Matlab R2014 and Python 3.8 were used for the calculation of the data index according to the trajectory of each fish from each frame.

2.6. Digestion of fish gastrointestinal tract for microfiber extraction

Procedures for the digestion of the fish gastrointestinal tract (GIT) were the same as in our previous study (Li et al., 2020). In brief, the dissected GIT of each fish was placed in H_2O_2 (30 %, *v/v*) solution for oscillating digestion, and the digestate was filtered through nylon membranes (20 μm). The occurrence of the fibers on membranes was measured and photographed under a Carl Zeiss Discovery V8 Stereo microscope (Micro Imaging GmbH, Göttingen, Germany). For prevention of contamination, a 100 % cotton laboratory coat was worn during all steps, and all liquid solutions, including tap water and hydrogen peroxide (H_2O_2, 30 %, *v/v*), were filtered through 5 μm Millipore filters. All containers and devices were washed with filtered water several times before use. Because the red fibers used in the experiment are likely to fade into a light red shade during the digestion process, we specifically calculated the quantity of light red fibers during the measurement process (Figure S1).

2.7. Data and statistical analysis

The speed and acceleration of each fish was measured with the *trajectorytools* python module (https://github.com/fjhheras/trajectorytools) (Heras et al., 2019). The speed (v_i) indicates the average swim speed. The covered area (P_c) is defined as the percentage area of the tank that fish swam over; equation: $P_c = \frac{A_c - A_o}{A_t}$, where A_c is the swimming distance multiplied by the width of the fish (w); A_o is the repeated area that the fish swam through; A_t means the area of the tank. The speed when moving (v_m) is the average swimming speed higher than 0.25 cm/s which is considered a threshold between movement and stillness. The proportion of time in front is the average time of the individual swimming in front of the school. The method of the calculation follows the method from Jolles et al., 2017 (Jolles et al., 2017). Firstly, we identified the mean coordinates of all fish in a group; $\mathbf{r}_c(t) = (\mathrm{x}_c(t), \mathrm{y}_c(t))$, that is, the group center, and then estimated the velocity ($\mathrm{v}_c(t)$) and direction ($\psi_c(t)$) of the group center at time t. Then for each frame, we calculated the distance of each fish to the group center; $\mathrm{CD}_i(t) = \sqrt{(\mathrm{x}_i(t) - \mathrm{x}_c(t))^2 + (\mathrm{y}_i(t) - \mathrm{y}_c(t))^2}$. To calculate the relative positions of individuals to the group, we shifted the coordinates of each fish so that the origin of the coordinate system was at the group centroid, and determined the angle between the positive y axis through the group centroid and an individual's position; $\delta_i(t) = \mathrm{atan2}((\mathrm{x}_i(t) - \mathrm{x}_c(t), (\mathrm{y}_i(t) - \mathrm{y}_c(t))$. Then, we calculated an individual's relative direction to that of the group center; $\sigma_t(t) = \delta_i(t) - \psi_c(t)$, which we then adapted to fit the Cartesian coordinate system pointing north; $\{\sigma_t(t) < -\pi\} : \sigma_t = 2\pi - |\sigma_t(t)|$ or $\{\sigma_t(t) > \pi\} : \sigma_t = -(2\pi - \sigma_t(t))$. Based on the relative direction and distance to the group center, we calculated the relative position for each fish to the group center; $(\mathrm{x}_i^{'}, \mathrm{y}_i^{'}) = \mathrm{CD}_i(t)(\sin(\sigma_t(t)), \cos(\sigma_t(t)))$.

The duration of capturing is the time interval from the pellet dropping in the tank to the fish capturing it. The times of capturing is the frequency of fish capturing the pellets during the recording time.

Statistical analysis of the data was made using SPSS 23.0. The coefficient of variation (C_v), the ratio between the standard deviation and the mean of a data set, is a statistical indicator of the degree of variability in a set of data reflecting the spread or dispersion of the data. The normality of the data set was examined using the Kolmogorov-Smirnov test. The Spearman correlation test was used to examine the correlations between different groups. Two significance levels of $P<0.05$ and $P<0.01$ were chosen.

3. Results and discussion

3.1. The reliability of VIF tags to distinguish individual fish in microplastic study

In the present study, we used three colors (red, green, and purple) to label each individual fish under the skin of either side of the dorsal fin (Fig. 1a). The behavioral data (Fig. 1b) were obtained through analysis of the videos and calculations based on the trajectories and formulations. The abundance of microfibers in the GIT of each fish was obtained after digestion and microscopic observation. Because VIF tags were visible both in the video and during the digestion process, we were able to match the activity data to the microfiber abundance data.

Generally, we tended to observe the feeding and swimming behaviors of each individual so as to figure out each fish's behavioral response to insoluble pollutants. However, it is difficult to match the pollution levels of microplastic with the behaviors of each individual for two reasons. Firstly, we cannot distinguish different individuals with the naked eyes because the morphologies of individuals from the same species are similar. Additionally, it is challenging to calculate how many microfibers the fish ingested through the camera due to the tiny size of microplastic fibers. To our knowledge, this is the first time VIF tags have been used to study in microplastic ingestion although it has previously been used in many studies of fish behavior (Im et al., 2017). It has previously been showed that this method does not affect fish feeding and metabolic rates and does not influence the accuracy rates of video tracking (Finstad et al., 2007). Our results suggest that this method is a useful tool for investigating the relationship between the behavioral activity of fish and the ingestion of microplastics in the eco-toxicological study of microplastics.

3.2. The relationships between movement behaviors of fish and ingested fibers by fish

We found that there were significant differences in the average microplastic ingestion of juvenile fish among different experimental groups, with the highest average abundance of 10.9 ± 6.0 items/ind. observed in the 6th experimental group, and the lowest average abundance of only 4.4 ± 2.9 items/ind. observed in the 2nd experimental

group (Fig. 2a). There were significant differences among the experimental groups, with significant differences observed between the 6th experimental group and most of the groups ($P<0.05$), and between the 4th experimental group and the 2nd and 9th experimental groups ($P<0.05$).

Not only did we observe differences in the average microfiber abundance of ingested juvenile fish among different experimental groups, but we also found that even within the same experimental group, there were obvious differences between individual fish (Fig. 2b). For example, in the 6th experimental group, the fish that ingested the most fibers was fish number 5, which ingested 27 items, while the fish that ingested the fewest fibers was fish number 6, which only ingested 5 items. We also analyzed the C_v of the number of microplastics ingested by different individual juvenile fish within each group (Table S3). The average C_v for all groups was 67.5 ± 18.3 %, with the highest value observed in the 3rd group at 99.0 %, and the lowest C_v observed in the 7th experimental group at 39.3 %. A higher C_v value indicates greater variability or dispersion in the data, and thus less consistency across repeated measurements (Shechtman, 2013). In our results, the C_v values of 90 % of the experimental groups are above 50 %, which implies that there are variations in the levels of microplastics detected among the individuals in each group.

Here, we found that the abundance of fibers in the fish body was closely related to the swimming behavior of fish. The average speed of fish showed the highest positive correlation with the abundance of fibers ($r = 0.512$, $P<0.01$), followed by acceleration ($r = 0.402$, $P<0.01$). However, there was no correlation between the abundance of fibers and the speed when moving (Fig. 3). Only one fish species (*Chindongo demasoni*) with similar sizes in the present study, so the individual differences in ingestion of microplastics could not be explained using factors such as size, habit, or feeding types of fish, as suggested in previous studies (Critchell and Hoogenboom, 2018). In our study, although the fish did not show feeding behaviors on microfibers, we detected microfibers in the GIT of the fish. Meanwhile, the positive correlations between the abundance of microplastics and speed, and acceleration indicated that fish with higher values of behavioral activity ingested more microplastics. It has been demonstrated that more active fish have higher swimming performance (i.e., higher acceleration) (Laskowski et al., 2021). Our previous study suggested that fish ingest microfiber passively (Li et al., 2021). Therefore, the active fish are supposed to have higher average speeds and swim longer distances in the tank, which makes them more susceptible to encountering and passively ingesting the microfibers. A previous study showed that swimming activity is also influenced by the personality of fish. The bold zebrafish have higher feeding activities on pellets than shy ones (Chen et al., 2022a).

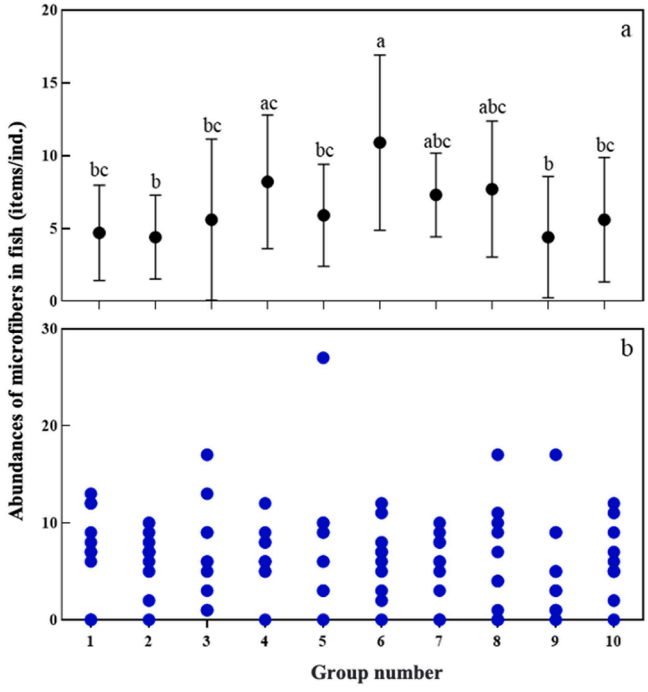

Fig. 2. The average abundance of fibers in each group (a), and the abundance of fibers retained in each individual fish (b). Letters a, b, and c indicate significant differences between different groups ($P < 0.05$). Error bar is indicated by a whisker.

The correlation index between the abundance of fibers and the covered area was 0.301 ($P<0.01$) (Fig. 3). However, there was no correlation between the abundance of fibers and the position of the fish in schools.

Fish that swim in the front position in a school have higher feeding rates than those in the rear position (Krause, 1993; McLean et al., 2018). In the present study, however, the fish that swam at the front of the school did not ingest more fibers. We speculate that the fish might not consider the fibers as food particles due to their different shapes, colors, and odours (Ory et al., 2017; Savoca et al., 2017). Fish did not show active feeding behaviors on fibers rather than sucked passively the fibers when they swam in the tank. Therefore, each individual was supposed to have the same opportunity to ingest fibers whether in front or back of school. Galloway et al. pointed out that the bioavailability of microplastics is completely different from that of conventional soluble pollutants (Galloway and Lewis, 2016). We need to pay more attention to the specific method of the microplastic-exposure experiment to assess its real toxic effects and ecological risks rather than directly using the methods for the soluble pollutants.

3.3. The relationships between movement behaviors of fish and their feeding behaviors on pellets

In the pellet-exposure experiments, we found a different phenomenon from that in fiber-exposure experiments. Some fish in front of the school always captured the pellet preferentially. One of the videos was used as an example to show this phenomenon, in which each fish has been identified and labeled with the ID number (Fig. 4). All of the frames were derived from the video. When the first pellet was dropped in the tank, the fish with ID 2 swam at the forefront and was the first one to capture the pellet (Fig. 4a). Moreover, during the other pellets were dropped, the fish with ID 2 was also the first to reach the locations where the pellets were dropped (Fig. 4b-d). Due to the small size of the fiber and the fact that the shape is quite different from the food of *Chindongo demasoni*, they did not exhibit significant feeding behavior on the fibers. However, the pellets were similar to the food in terms of size, shape, and color, and they would recognize the microplastic pellets as food and actively capture them. Therefore, characteristics such as the shape and size of microplastics might be one of the reasons why *Chindongo demasoni* showed different feeding behaviors.

According to the result of fish swimming and feeding behaviors on pellets, the times of capturing pellets had significant positive correlations with the proportion of time in front ($r = 0.399$, $P<0.01$) and also with the speed of fish ($r = 0.243$, $P<0.05$) when fish swam in front of the school (Fig. 5). The fish in the rear position of the fish school had little chance to capture the pellets. In the environment, the schooling behavior of fish is of great biological importance, playing a crucial role in foraging and predator avoidance (Killen et al., 2012). The fish at the forefront of the schools may be more affected by microplastic particles because they are more likely to ingest them. This result also illustrated that fish with active behavior could capture more pellets.

4. Conclusion

In the present study, we used visible implant fluorescent tags to successfully match the behavioral data from the video with the

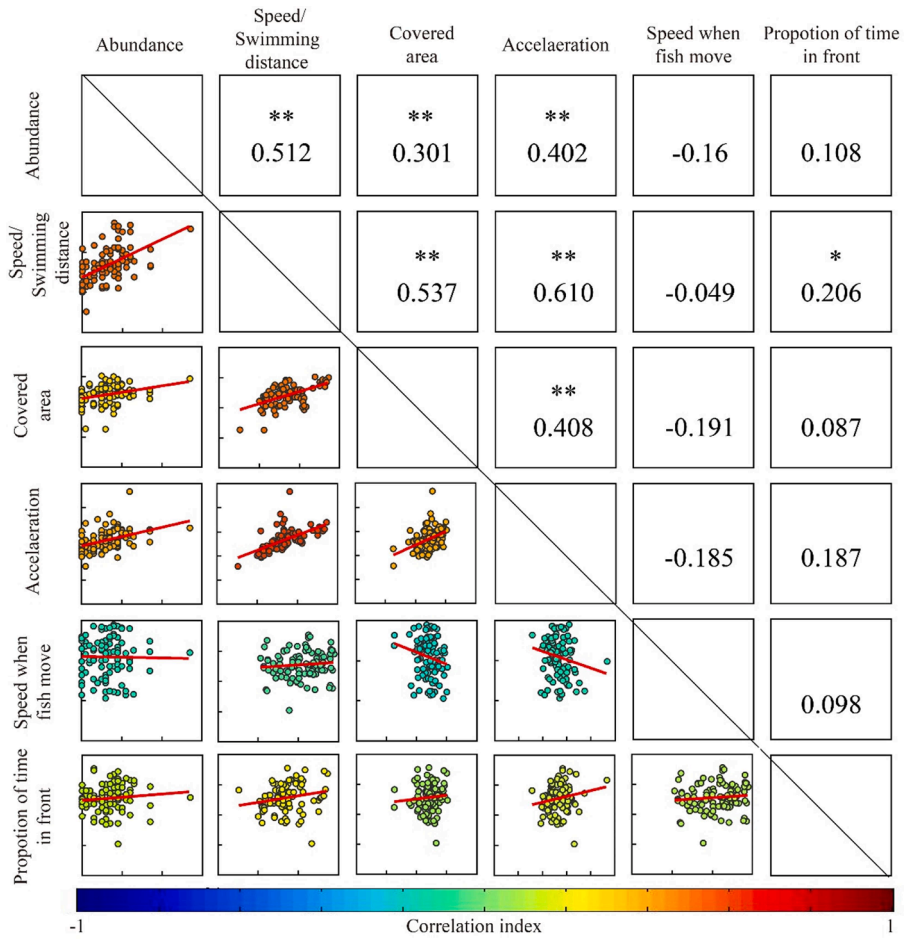

Fig. 3. The correlations among the abundance of fibers and several behavior indexes (**: $P<0.01$, *: $P<0.05$).

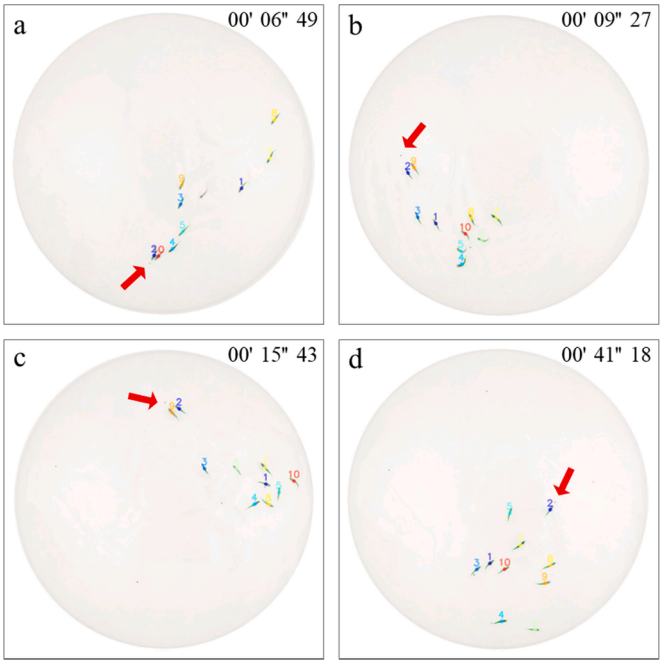

Fig. 4. Example of group foraging on pellets. Red arrays indicate pellet location; numbers in the upper right corner represent the time elapsed in the trail; colored numbers indicate the ID number of each fish after identification.

occurrence of microplastic from digestion. Such a method had not been used in most conventional contaminant exposure experiments because of their solubility. We found that the swimming behaviors of fish affect their ingestion of fibers and pellets. That might be one of the reasons leading to the great variations in the abundance of microplastics among fish individuals either in field investigations or in laboratory experiments. Our study can not only help better understand the interaction between organisms and microplastics but also provide a scientific basis for the bioavailability of microplastics for establishing methods of microplastic exposure in the future.

CRediT authorship contribution statement

Bowen Li: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Weiwenhui Liang:** Methodology, Software, Validation, Investigation, Resources, Writing – review & editing, Visualization. **Shijian Fu:** Methodology, Writing – review & editing. **Cheng Fu:** Methodology, Writing – review & editing. **Zonghui Cai:** Software. **Amelia Munson:** Writing – review & editing. **Huahong Shi:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as

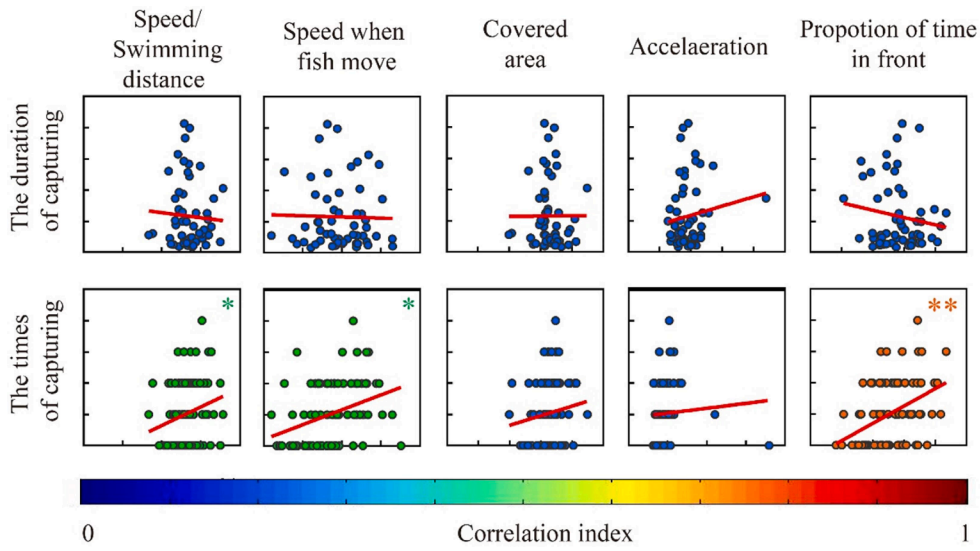

Fig. 5. The correlations among the feeding behaviors on pellets and swimming behaviors (**: $P<0.01$, *: $P<0.05$).

influencing the position presented in, or the review of, the manuscript entitled.

Data availability

Data will be made available on request.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106798.

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