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Exposure to microplastics decreases swimming competence in larval zebrafish (*Danio rerio*)

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ABSTRACT

Microplastics have been frequently detected in both marine and freshwater ecosystems. Their impact on aquatic organisms has raised much concern. This study investigated the impact of microplastics on zebrafish embryos and larvae, with a special focus on their swimming competence. The zebrafish embryos were exposed to microplastics starting from 4 h post fertilization. Microplastics first adhered to the embryo chorion, then entered the stomach and intestinal tract of the larvae later. In the free swimming test, exposure to 1000 μg/L (around 1.91×10^7 particles/L) of microplastics led to a significant decrease in both swimming distance and speed of zebrafish larvae under the dark condition by 3.2% and 3.5% respectively. In the alternating light-to-dark photoperiod stimulation assay, exposure to 100 and 1000 μg/L (around 1.91×10^6 and 1.91×10^7 particles/L) of microplastics caused a 4.6% and 2.6% decrease in swimming distance, and reduced the active speed by 4.9% and 2.8%, possibly as a result of inhibited dark avoidance in treated zebrafish larvae. At the molecular level, exposure to microplastics induced upregulated expression of inflammation (*il1b*) and oxidative stress (*cat*) related genes. This study demonstrates that exposure to microplastics significantly decreases larvae swimming competence, which may have significant impacts on its population fitness in the aquatic environment and further ecological consequences.

1. Introduction

There is an increasing concern about the potential negative impacts of microplastics (commonly defined to be less than 5 mm) in the aquatic ecosystem (Eerkes-Medrano et al., 2015). The reported environmental concentrations of microplastics are about 0.02–102, 550 items/m^3 in marine water samples and 3.00–390.7 items/kg in marine sediments (Li et al., 2016; Sweden, 2007; Claessens et al., 2011). Microplastics have also been reported at concentrations up to around 0.028–1, 146, 418.36 items/m^3 in surface water and 1.20–616.1 items/kg in sediments of freshwater environments (Eerkes-Medrano et al., 2015; Horton et al., 2017). The environmental concentrations of microplastics in freshwater ecosystems are much higher than that in marine environments. Hence, it is important to understand the potential impacts of microplastics on freshwater organisms, especially at their early developmental stages.

Microplastics may be ingested by a wide variety of aquatic organisms. The small size of microplastics makes them available to small or lower trophic level aquatic organisms as they have limited selectivity for particles and can capture anything of appropriate size (Wright et al., 2013). Microplastics of similar shape and color to zooplankton prey may be ingested by fish larvae (Lima et al., 2015). Microplastics can be easily devoured by these centimeter-long swimmers. Juvenile fish are more likely to be exposed to high concentrations of microplastic debris in nursery habitats, where there is often an accumulation of microplastics (Chae et al., 2015). Ingestion of microplastics has been reported in various freshwater fish species. In the Gulf of Mexico, 34 freshwater fish from the same class of Osteichthyes, across 26 species and 9 families, were reported to have ingested microplastics (Phillips and Bonner, 2015). Similar has been reported for bluegill (*Lepomis macrochirus*) and longear (*Lepomis megalotis*) sunfish (Centrarchidae) in the Brazos River Basin, USA (Peters and Bratton, 2016).

Though recent studies have reported ingestion of microplastics by aquatic organisms (Browne et al., 2013; Avio et al., 2015), the physiological and behavioral impacts of exposure to microplastics on fish are poorly understood. Aquatic organisms are particularly vulnerable to water-borne pollutants owing to their limited ability to regulate their external environment. In particular, early life stages of fish are under strong selection, driven by high rates of predator-induced mortality

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(Atherton and McCormick, 2017). Environmental contaminants can affect the survival of organisms by disrupting their behaviors, and in turn affect their fitness and population dynamics (Maximino et al., 2013). Behavior of juvenile fish is a key factor for important fitness correlates, such as survival, growth and reproduction, and is an important endpoint to evaluate the ecological and toxicological effects of pollutants in water bodies (Smith and Blumstein, 2008). Swimming competence is one of the major behavioral endpoints in fish (Saint-Amant and Drapeau, 1998). Active swimming competence plays a key role in preying, mating, and defense of fish throughout their lifetime (Colwill and Creton, 2011). For larvae of aquatic organisms, swimming is a basic yet critical skill for survival, and it is an innate ability to find food and avoid predators (Lönnstedt et al., 2012).

In this study, zebrafish embryos were used as a model to assess the potential behavioral impacts of microplastics on fish in freshwater ecosystems. The developmental impacts on zebrafish embryos upon exposure to microplastics and subsequent uptake were investigated. The swimming competence of the fish larvae was examined in a free swimming test and an alternating light-to-dark photoperiod stimulation assay. This study aims to understand the impacts of microplastics on freshwater fish's survival and fitness at their early life stages.

2. Materials and methods

2.1. Microplastics characterization

Green fluorescent (468/508 nm) polystyrene microspheres (i.e., Fluoro-Max Dyed Green Aqueous Fluorescent Particles, G0100) with a diameter of 1 μm and density of 1.05 g/cm^3 were purchased from Thermo Fisher Scientific (USA). Morphology and fluorescence of polystyrene microplastics was examined and photographed under a fluorescence microscope (DP80, Olympus, Japan) with microplastics at a concentration of 10 μg/L. The hydrodynamic diameter of microplastics in embryo medium (2 mM CaCl2·2H2O, 0.5 mM MgSO4·7H2O, 0.75 mM NaHCO3 and 0.08 mM KCl) was determined using dynamic light scattering technique with the help of a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd, Malvern, UK). The experiment was performed in 12-well cell culture plates with embryo medium containing 10 mg/L of microplastics (around 1.91×10^8 particles/L). The measurements were carried out once every 24 h from 0 to 120 h. Three wells were set up at each time point and used for analysis (n = 3 wells).

2.2. Fish husbandry, embryo collection, and exposure design

Wild-type (AB strain) zebrafish were picked and maintained in laboratory aquaria, at 28 °C with a 14:10 h light: dark cycle and a recirculation system. The fish were fed twice daily with dry flake food and brine shrimp. Before egg collection, each tank contained six adult zebrafish with a female/male ratio of 1:2. Spawning was induced in the morning when the lights came on after the 10 h dark period. At 4 h post fertilization (hpf), the embryos that developed normally and reached the blastula stage were selected for subsequent experiments under a dissection microscope (SMZ168, Motic, Xiamen, China). Twenty embryos were randomly selected and transferred into 12-well cell culture plates containing 3 ml of embryo medium per well. Microplastics (1 μm) in the medium were prepared at 0 (control), 100, and 1000 μg/L (0, around 1.91×10^6 and 1.91×10^7 particles/L). The solutions were replaced once every 24 h and all exposure experiments were conducted at 28 ± 0.5 °C.

2.3. Distribution of microplastics in zebrafish

Two exposure experiments for distribution of microplastics in zebrafish were performed in 12-well cell culture plates; three wells were set up for each concentration (0, 100 and 1000 μg/L), with each well consisting of 20 embryos exposed from 4 to 24 hpf in the first experiment and from 4 to 96 hpf in the second. At 24 and 96 hpf, five embryos and larvae from each well were randomly selected for observation and imaging. A total of 15 embryos/larvae for each treatment group were used for analysis (n = 15 embryos/larvae). The selected embryos and larvae were rinsed with Milli-Q water (Millipore, 18.2 MΩ cm) three times, and then anesthetized with 0.024% tricaine (Sigma-Aldrich, USA). Observations were conducted under an Olympus BX53 florescent microscope (Olympus Optical Co., Ltd, Tokyo, Japan) at 40× and 100× magnification, and images were taken with an Olympus DP 80 camera.

2.4. Quantification of microplastics in zebrafish larvae

The exposure experiment for uptake of microplastics in zebrafish larvae was performed in 12-well cell culture plates; three wells were set up for each concentration (0, 10, 100 and 1000 μg/L), with each well consisting of 30 embryos with exposure from 4 to 96 hpf. The quantification of microplastics in zebrafish larvae was determined according to previous methods with modifications (Lu et al., 2016; Chen et al., 2017). At 96 hpf, all 30 fish larvae from each well were rinsed with Milli-Q water (Millipore, 18.2 MΩ cm) three times and then pooled as one sample for analysis. The fish were checked under an Olympus BX53 florescent microscope (Olympus Optical Co., Ltd, Tokyo, Japan) after rinsing to make sure no microplastics adhered to their surface. A total of 3 pools for each treatment group were used for these assays (n = 3 pools/samples). After lyophilization (GT20, SRK SYSTEMS ASIA, Germany) for 72 h, the larvae were weighed and then digested in 30% H_2O_2 (1 mL) at 70 °C for 2 h, and then diluted with Milli-Q water (Millipore, 18.2 MΩ cm) to a final volume of 1 mL. The microplastic concentrations in larvae were measured using a fluorescence spectrophotometer (SpectraMax M5/M5e, Molecular Devices, USA) with excitation at 468 nm and emission at 508 nm. The background fluorescence of the unexposed larvae was subtracted from that of the microplastics treated samples (0, 10, 100 and 1000 μg/L). The recovery rate of fluorescently labeled microplastics was between 71.1% and 111.9%.

2.5. Early developmental effects of microplastics: hatching, body length and yolk sac absorption

The exposure experiment for embryos hatching was performed in 12-well cell culture plates; four wells were set up for each concentration (0, 100 and 1000 μg/L) of microplastics, and each well consisted of 20 embryos exposed from 4 to 72 hpf. A total of 4 wells for each treatment group were used for these assays (n = 4 wells). The hatching of zebrafish embryos was examined from 48 to 60 hpf under a dissection microscope (SMZ168, Motic, Xiamen, China). The exposure experiment for larvae development was performed in 12-well cell culture plates; three wells were set up for each concentration (0, 100 and 1000 μg/L) of microplastics, with each well consisting of 20 embryos exposed from 4 to 72 hpf. At 72 hpf, the embryos were anesthetized with 0.024% tricaine (Sigma-Aldrich, USA) for the examination of the body length and yolk sac area under a Zeiss stereomicroscope (V8, Göttingen, Germany) equipped with AxiocamICc3 photomicrography system. Ten embryos per well from each treatment (100 and 1000 μg/L microplastics) and control (0 μg/L microplastics) group were randomly selected for photography. A total of 30 larvae for each treatment group were used for these assays (n = 30 larvae). The body length and yolk sac area of the exposed embryos were determined using ImageJ2x software (National Institutes of Health, Bethesda, MD, USA).

2.6. Examination of free swimming competence in the free swimming test

In the free swimming test, zebrafish embryos were exposed to 0, 100 and 1000 μg/L of microplastics from 4 to 120 hpf in 12-well cell culture plates; four wells were set up for each concentration with each well

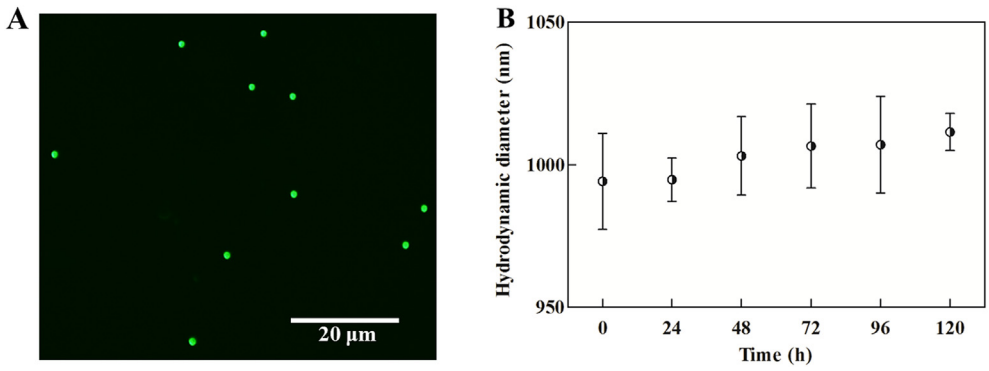

Fig. 1. Fluorescence microscope image (A), and hydrodynamic particle size in embryo medium at different time points (B) (n = 3 wells) of microplastics used in this study. Error bars represent the mean ± standard error. (One-way ANOVA followed by a Dunnett post hoc test, no significant differences in the hydrodynamic diameter of microplastics were observed).

consisting of 20 embryos. At 120 hpf, eight larvae were randomly selected from each well and gently transferred to a 96-well plate, with one larva per well. A total of 32 larvae for each treatment group were used for these assays (n = 32 larvae). The 96-well plate contained 200 μL of the respective microplastic solutions and was kept at a constant temperature of 28 °C via the water jacket circulation feature on the ZebraLab stage. As described in a previous study (Wang et al., 2013), larvae were allowed to adapt for 10 min in the dark condition (infrared light) before their swimming speed and distance were monitored. The free swimming test was assessed in a Zebralab Video-Track system (Videotrack, version 3.5, View Point Life Science, France), and all recording hardware were linked to the computer control program and kept insulated from the lab environment in the Zebrabox. The data (frequency of movements, distances travelled and total durations of movements) in the following 20 min dark period (infrared light) were collected once every 2 min in the behavioral test system.

2.7. Examination of swimming competence: response to light-to-dark transition stimulation

In the behavior test of response to light-to-dark transition stimulation, the exposure experiment was performed in 12-well cell culture plates; four wells were set up for each concentration (0, 100 and 1000 μg/L), with each well consisting of 20 embryos exposed from 4 to 120 hpf. At 120 hpf, eight larvae were randomly selected from each well and gently transferred to a 96-well plate, with one larva per well. A total of 32 larvae for each treatment group were used for these assays (n = 32 larvae). The 96-well plate contained 200 μL of the respective microplastic solutions. The alternating light stimulation test monitored the swimming distance and speed of zebrafish larvae in response to a light-to-dark transition stimulus with lighting parameters of 10 min light (visible light) followed by a 10 min dark (infrared light) interval. Larvae were allowed to adapt for 10 min under the dark condition before monitoring. Swimming behavior was then monitored in response to light-to-dark transitions (10 min light-10 min dark) with three cycles. The data (distances travelled and total durations of movements) in each 10 min light/dark period were collected once every 2 min in the Zebralab Video-Track system.

2.8. Total RNA extraction and Q-PCR

For quantitative real-time PCR analysis, the treatments for each concentration (0, 100 and 1000 μg/L) of microplastics were performed in 12-well cell culture plates; four wells were set up for each concentration (0, 100 and 1000 μg/L), with each well consisting of 20 embryos exposed from 4 to 96 hpf. All 20 embryos from each well were pooled for analysis (n = 4 pools). Total RNA extraction and real-time polymerase chain reaction was performed at 96 hpf as detailed in a previous study (Zhang et al., 2015). The primers for *il1b* (interleukin 1β) (Forward primer: ATGGACTGTTCAGATCCGCTT; Reverse primer: GGATTGGGGTTTGATGTGCT-T) were designed through the PrimerBlast program provided by NCBI. The primers for *sod* (superoxide dismutase gene), *cat* (catalase gene) and *β-actin* (β-non-muscle) were used as previously described (Zhang et al., 2015).

2.9. Statistical analysis

Statistical significance was accepted at $p < 0.05$ and values were presented as means ± standard error. Significant differences between mean values were determined using one-way analysis of variance (ANOVA), and the Dunnett's test was used to determine the significant difference ($p < 0.05$) between microplastics treated and control groups. The ANOVA and the figures were performed and created using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Characterization of microplastics

Fluorescence microscope image shows that the polystyrene microplastics used in this study were uniform and spherical with green fluorescence, and well dispersed in the fish embryo medium without any aggregation (Fig. 1A). To elucidate the size-stability of microplastics over time, the hydrodynamic diameter of the microplastics in the embryo medium was examined once every 24 h from 0 to 120 h which covers the entire exposure period of this study. The result (Fig. 1B) shows that the hydrodynamic diameter of the microplastics used in this study was stable at 1 μm in the embryo medium over time, and no significant aggregation was observed in the fish embryo medium (p = 0.926, Fig. 1B) during the study period.

3.2. Uptake and distribution of microplastics in zebrafish embryos and larvae

The distribution of microplastics in zebrafish embryos and larvae was examined under a fluorescence microscope at 24 hpf and 96 hpf respectively. Microplastics were found in the chorion of zebrafish embryos in the treatment groups (100 μg/L and 1000 μg/L) at 24 hpf (Fig. 2A). The distribution of microplastics in the embryo chorion generally increased with increasing concentrations of microplastics, and were most abundant in the 1000 μg/L treatment group (Fig. 2A). As shown in Fig. 2, the plastic particles were observed in the mouths, stomachs, and intestinal tracts of the exposed zebrafish larvae at 96 hpf (Fig. 2B).

The uptake of microplastics in zebrafish larvae was quantified at 96 hpf. As shown in Fig. 3, microplastics uptake by zebrafish larvae was significant when the exposure concentrations were greater than or equal to 100 μg/L and the uptake of microplastics increased with increasing concentrations of microplastics. The detected concentrations of microplastics in the exposed larvae were 0.168 ± 0.018 and 0.179 ± 0.016 mg/g dry weight for the 100 μg/L and 1000 μg/L treatments respectively (Fig. 3), which were significantly greater

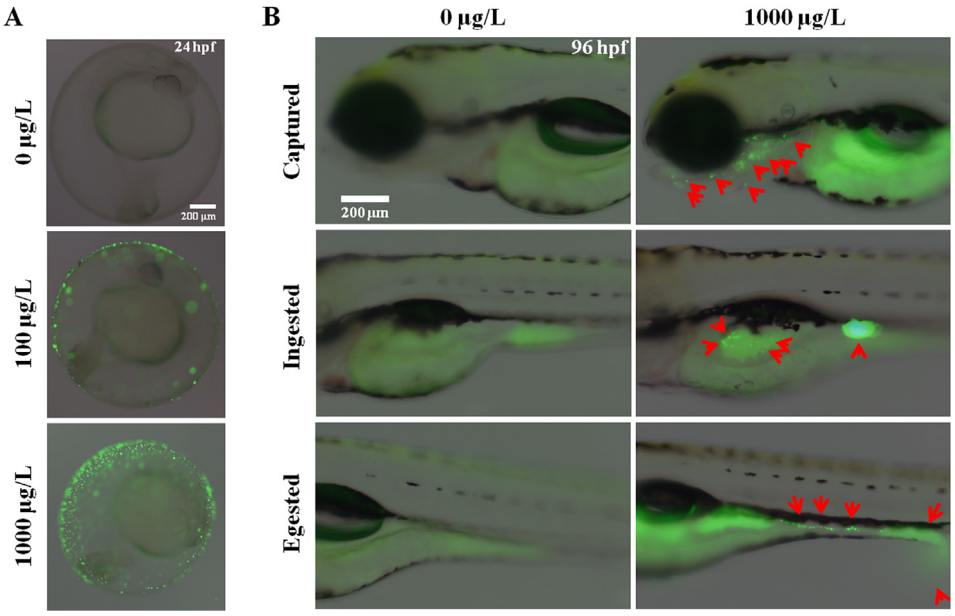

Fig. 2. (A) Representative images of microplastic distribution in 0, 100 and 1000 μg/L exposed zebrafish embryos at 24 hpf. Magnification × 40. n = 15 embryos from each concentration; (B) Representative images of microplastic distribution in 0 μg/L and 1000 μg/L exposed zebrafish larvae at 96 hpf. n = 15 larvae from each concentration. Red arrows indicate the existence of microplastics in zebrafish larvae. Magnification × 100. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

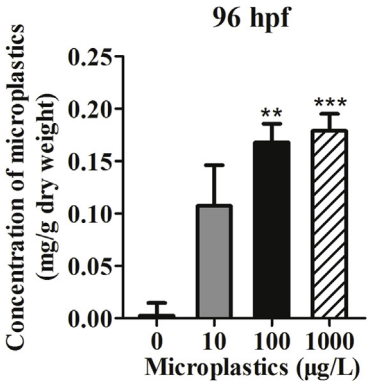

Fig. 3. Uptake of microplastics in zebrafish larvae exposed to 0, 10, 100 and 1000 μg/L of microplastics from 4 to 96 hpf. Three wells were set up at each concentration; each well with 30 embryos; all 30 embryos from each well were pooled as a sample for analysis (n = 3 wells/pools). Error bars represent the mean ± standard error. Asterisks represent the significant difference to the control (One-way ANOVA followed by a Dunnett post hoc test, $^{**}P < 0.01$, $^{***}P < 0.001$).

compared to the control group (0.002 ± 0.012 mg/g dry weight) (p = 0.007 and p = 0.0004).

3.3. Impacts on the early development of zebrafish

To investigate the impacts of microplastics on the early development of zebrafish, three developmental parameters (hatching rate, body length and yolk sac area) were examined. As shown in Fig. 4, the hatching rates (%) of zebrafish embryos at 48, 52 and 56 hpf were slightly reduced upon exposure to microplastics, especially at the concentration of 1000 μg/L at 52 and 56 hpf. However, these were not statistically significant (p = 0.18 and 0.65, Fig. 4A). The development speed of zebrafish larvae was not significantly affected by exposure to microplastics from 4 to 72 hpf (Fig. 4B and C) in terms of body length and yolk sac area. At 72 hpf, larvae exposed to the highest concentration of microplastics (1000 μg/L) had smaller body length compared to the control (0 μg/L), but this trend was insignificant (p = 0.38, Fig. 4B). Similarly, average yolk sac area showed increase in treatments containing microplastics (100 and 1000 μg/L) when compared to the control (0 μg/L), especially in the treatment of 1000 μg/L, but once again this difference was insignificant (p = 0.61, Fig. 4C). In addition, the larvae treated with microplastics were not observed to have any obvious malformations.

3.4. Exposure to microplastics decreased larvae free swimming competence

At 120 hpf, zebrafish can live freely and catch moving prey (Bai et al., 2016), thus the effects of microplastic exposure on swimming competence of zebrafish embryos were assessed at 120 hpf. Using a video-track system, the distance and duration of swimming for each larvae examined was recorded. The data were divided into 2 min intervals and represented by the sum of distance and duration every 2 min. Average speed was calculated by dividing the distance travelled by the duration. At 120 hpf, the exposure study showed that microplastics caused a significant decrease in activity in the 20 min free swimming test under the dark condition after a 10 min dark adaptation. As shown in Fig. 5, the swimming speed of the 1000 μg/L exposure group (1.47 ± 0.31 mm/s) was 3.5% lower (p = 0.027) than that of the control (1.52 ± 0.29 mm/s) (Fig. 5B). Furthermore, the total distance of swimming of the 1000 μg/L exposure group (15.28 ± 2.81 cm) was reduced by 3.2% (p = 0.021) compared to the control group (15.78 ± 2.68 cm) (Fig. 5A). The total distance moved and swimming speed of zebrafish larvae decreased at 120 hpf in the treatment with 1000 μg/L of microplastics (Fig. 5A&B) compared to the control group, thus microplastics significantly inhibited the swimming performance of larvae.

3.5. Exposure to microplastics decreased larvae swimming competence under alternating light-to-dark photoperiod stimulation

Swimming competence of zebrafish larvae at 120 hpf was further assessed by a 60-min alternating light-to-dark photoperiod stimulation test. After a 10 min dark adaption, larvae were then stimulated with three cycles of 10 min light and 10 min dark alternating photoperiods. The travelled distances and total durations of movements in each 10 min light/dark period were collected once every 2 min. Larval swimming distance and speed were determined by calculating the average distance and speed during three 10 min light/dark periods. In the experiments assessing activity response to alternating light-to-dark photoperiod stimulation, no significant difference in the swimming distance and speed was found between the control group and microplastics treated groups in the light period (Fig. 6A&C). In contrast, a

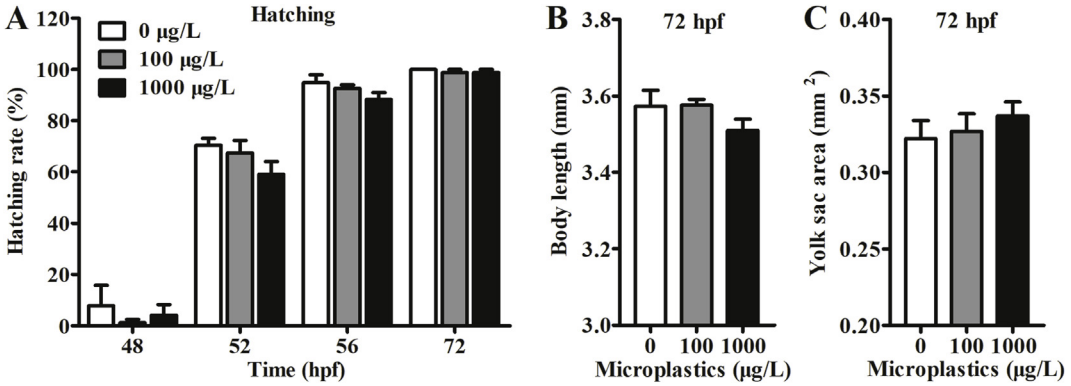

Fig. 4. Hatching rates (%) and development of zebrafish that were exposed to microplastics from 4 to 72 hpf. Error bars represent the mean ± standard error. (One-way ANOVA followed by a Dunnett post hoc test, no significant differences in the larval hatching rate (four wells were set up at each concentration; each well with 20 embryos; $n = 4$ wells) (A), body length (three wells were set up at each concentration; each well with 20 embryos; 10 larvae from each well were randomly selected for observation and imaging; a total of 30 larvae for each concentration were used for analysis; $n = 30$ larvae) (B) and yolk sac area (three wells were set up at each concentration; each well with 20 embryos; 10 larvae from each well were randomly selected for observation and imaging; a total of 30 larvae for each concentration were used for analysis; $n = 30$ larvae) (C) were observed following microplastic exposure).

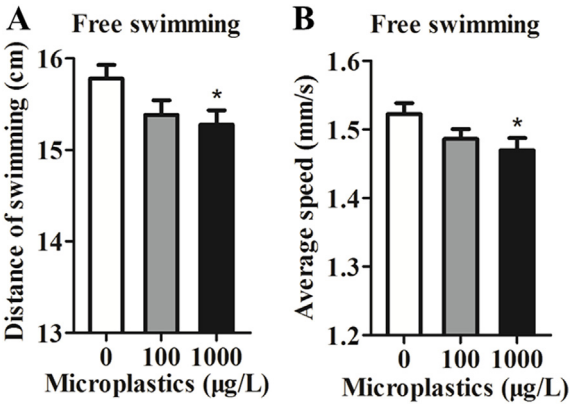

Fig. 5. Zebrafish larvae free swimming under dark condition, exposed to 0, 100 and 1000 μg/L of microplastic from 4 to 120 hpf. Error bars represent the mean ± standard error. Asterisks represent the significant difference to the control (One-way ANOVA followed by a Dunnett post hoc test, $^*P < 0.05$). Four wells were set up at each concentration; each well with 20 embryos; eight larvae from each well were randomly selected for these assays; a total of 32 larvae for each concentration were used for analysis; $n = 32$ larvae.

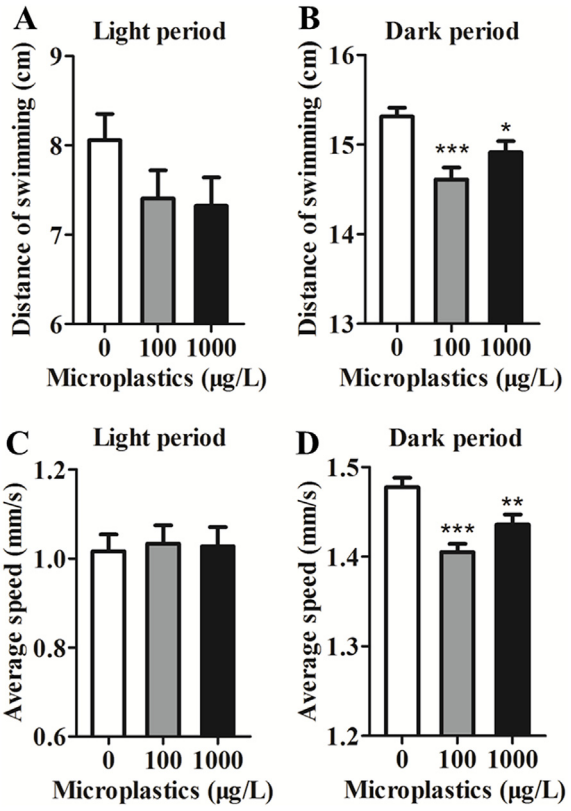

Fig. 6. (A & B) The distance of swimming of zebrafish larvae during dark and light cycles, exposed to 0, 100 and 1000 μg/L of microplastic from 4 to 120 hpf. (C & D) The average swimming speed of zebrafish larvae during dark and light cycles, exposed to 0, 100 and 1000 μg/L microplastic from 4 to 120 hpf. Error bars represent the mean ± standard error. Asterisks represent the significant difference to the control (One-way ANOVA followed by a Dunnett post hoc test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Four wells were set up at each concentration; each well with 20 embryos; eight larvae from each well were randomly selected for these assays; a total of 32 larvae for each concentration were used for analysis; $n = 32$ larvae.

significant reduction in larvae swimming competence was observed in the dark period. As shown in Fig. 6, the total swimming distance of the 100 μg/L exposure group (14.61 ± 2.86 cm) was reduced by 4.6% ($p = 0.00002$) compared to the control group (15.31 ± 2.15 cm) (Fig. 6B). Similarly, the total swimming distance of the 1000 μg/L exposure group (14.91 ± 2.73 cm) was also reduced compared to the control (15.31 ± 2.15 cm) by 2.6% ($p = 0.012$) (Fig. 6B).

The swimming speed of the microplastic exposure groups also showed a significant decrease under the dark condition of the alternating light-to-dark photoperiod stimulation test (Fig. 6D). At 120 hpf, the swimming speed of the 100 μg/L exposure group (1.41 ± 0.21 mm/s) was lower than that of the control (1.48 ± 0.23 mm/s) by 4.9% ($p = 0.0000003$) (Fig. 6D). The swimming speed of the 1000 μg/L exposure group (1.44 ± 0.24 mm/s) was also lower than that of the control (1.48 ± 0.23 mm/s) by 2.8% ($p = 0.0065$) (Fig. 6D).

3.6. Exposure to microplastics upregulated the expression of inflammation and oxidative stress genes

After exposure to microplastics, the *il1b* expression was significantly upregulated to 165% ($p = 0.021$) in the 1000 μg/L exposure group at 96 hpf (Fig. 7A). The *cat* expression was significantly upregulated to 121% ($p = 0.044$) in the 1000 μg/L exposure groups at 96 hpf (Fig. 7B). No significant changes in *sod* expression were observed in the exposure groups at 96 hpf ($p = 0.095$) (Fig. 7C).

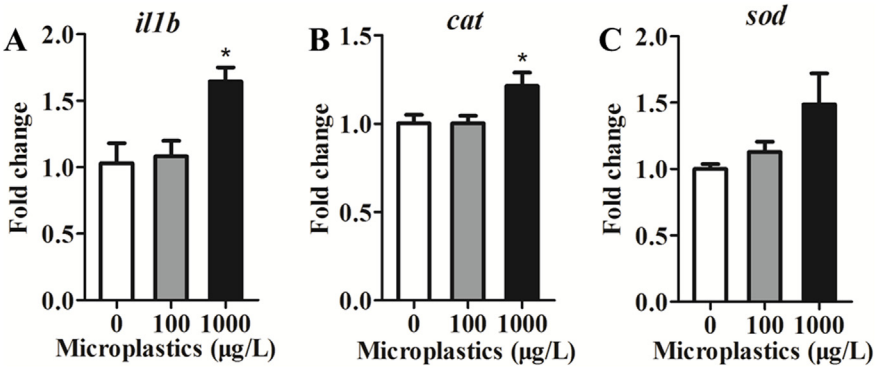

Fig. 7. Inflammation and oxidative stress genes (*il1b*, *sod* and *cat*) of zebrafish larvae exposed to 0, 100 and 1000 μg/L of microplastic from 4 to 96 hpf. Error bars represent the mean ± standard error. Asterisks represent the significant difference to the control (One-way ANOVA followed by a Dunnett post hoc test, $*P < 0.05$). Four wells were set up at each concentration; each well with 20 embryos; all 20 embryos from each well were pooled as a sample for analysis ($n = 4$ wells/pools).

4. Discussion

4.1. Uptake of microplastics in zebrafish larvae

The embryo chorion has pores with a diameter of approximately 0.5–0.7 μm (Rawson et al., 2000) which are necessary for oxygen and nutrient transportation from the outer aquatic environment to the embryo and for the elimination of wastes (Cheng et al., 2007). The measurement result of hydrodynamic diameter of microplastics showed that the size of polystyrene microplastics used in this study was larger than 0.9 μm in embryo culture medium. Microplastics could only adhere to the outer layer of the chorion of the zebrafish embryos and were too large to enter the chorion under the exposure conditions.

After hatching, the uptake of microplastics by zebrafish larvae is a more complicated issue. The particles might be taken up as food items by mistake or brought inside the bodies by the water flow. In this study, microplastic content in larvae significantly increased when the concentrations of microplastics were equal to or greater than 100 μg/L. The microplastics were detected in the stomach of zebrafish larvae after exposure to microplastics for 92 h, and larvae could intake the plastic particles through ingestion and egest microplastics from their intestinal tract. These results are in line with the study of Jovanović et al. (2018), which reported that for gilt-head seabream (*Sparus aurata*), after 45 days of exposure at 0.1 g/kg/bodyweight to six common types of microplastics, the microplastics were present in the fish gastrointestinal tract and effectively eliminated from the body of the fish. Similarly, Ding et al. (2018) reported that after 14 days of exposure to microplastics for the freshwater fish red tilapia (*Oreochromis niloticus*), the fish gut seemed to accumulate the most microplastics compared to other tissues. Chen et al. (2017) reported that after exposure to 47 ± 0.2 nm microplastics (1 mg/L) from 128-cell stage to 120 hpf, the content of microplastics in zebrafish larvae was 115 ± 28 mg/g wet weight. Compared to the Chen et al. (2017) study, this study detected the concentrations of microplastics in the exposed larvae to be 0.179 ± 0.016 mg/g dry weight in the 1000 μg/L treatments. There is a difference between the microplastic uptake in zebrafish larvae in these two studies although the zebrafish were exposed to the same concentration of microplastics. Disparity of detected concentrations of microplastics in exposed larvae between Chen et al. (2017) study and this study may be mainly due to differences in size of microplastics as Chen et al. (2017) study used 47 ± 0.2 nm and this study used 1 μm. Jovanović et al. (2018) evaluated that particle size of microplastics plays a major factor in determining their ingestion by organisms. And in the study of Lu et al. (2016), smaller particles (5 μm) can more easily be accumulated in zebrafish gills, liver, and gut after seven days of exposure than larger-sized 20 μm diameter microplastics. In addition, it was found from the study of Lu et al. (2016) that microplastics with a smaller size (5 μm) mainly accumulated in internal tissues (liver and gut) rather than in external tissues (gills). Besides, the difference of larvae weight used in microplastic uptake analysis between the study of Chen et al. (2017) and this study may also be one of the reasons for the differences of microplastic concentration in zebrafish larvae. In the study of Chen et al. (2017), wet weight was used, however; this study used dry weight. There are differences between these two weights of zebrafish larvae. This difference, however, may be attributed to the concentration of microplastics in zebrafish larvae.

4.2. Decreased swimming competence of zebrafish larvae upon exposure to microplastics

Behavioral assessment of developing zebrafish is becoming popular. Behavior represents the unique interface between internal and external forces that determine an organism's fit and survival (MacPhail et al., 2009). Behavior anomalies can happen without any obvious morphological deformations or subsequent decrease in survival rates. Behavior changes have proven to be more sensitive and act as the important endpoints for toxicological study (Little and Finger, 1990). In contrast, the development speed of zebrafish larvae was not significantly affected at such microplastic concentrations. Little data suggest that exposure to microplastics would result in acute lethal toxicity in wild fish populations. Previous study has shown that larval zebrafish become free-living to hunt for food and escape from predators since 120 hpf, suggesting that simple yet functional circuitries for processing reward and aversion are already in place (Bai et al., 2016). Eye development and photoreceptor maintenance of zebrafish is dependent on the retinal pigment epithelium (Easter and Nicola, 1996). At 120 hpf, the pigment cells of eyes have differentiated completely (Westerfield, 2000), and Clark (1981) described that from 96 hpf, zebrafish can begin to catch moving prey and distinguish light and dark across time. Free-swimming, zebrafish larvae can use eyes and lateral line to identify the interference of foreign materials. Chen et al. (2017) found that exposure to microplastics (1 mg/L) with a diameter of 41, 011 ± 420 nm significantly changed visual system related gene expression of *zfrho* (rhodopsin). Microplastics may affect the vision and weaken the recognition of larval fish in darkness.

The study of MacPhail et al. (2009) on ethanol toxicity on zebrafish larvae indicated that this alternating stimulus (light-to-dark transition test) is a useful tool for behavior assessment. In their study, after a period of darkness adaptation, locomotion in darkness increased initially, then decreased steadily to a low level by 14–50 min. Locomotion during light gradually increased to a stable level by 28–50 min. In contrast, when 10 min periods of light and dark were alternated, activity was low in light and high in dark, and activity during alternating dark periods was markedly higher than originally obtained during either extended dark (10–50 min) or light (10–50 min). From their results, the changes in locomotion in larval zebrafish can be better reflected in the alternating light-dark cycles than in extended dark or light periods. From previous studies, 10-min or 20-min periods and three cycles of light-dark photoperiod were often used in the light-to-dark transition test (Huang et al., 2010; Wang et al., 2013; Chen et al., 2017). In this study, based on the optimal combination, 10-min periods and three cycles of light-dark photoperiod were set up. Exposure to

microplastics caused a significant decrease in both swimming distance and speed under dark conditions in the light-to-dark alternation experiments, suggesting that the effect of microplastics mainly occurred during the light to dark transition.

The fish responses in the light-dark cycles are more severe at 100 μg/L than at 1000 μg/L microplastic concentrations. Similarly, in the study of Wang et al. (2013) on Bisphenol A toxicity on zebrafish larvae behavior, zebrafish larvae responses in the light-dark cycles are more severe at 1 μM and 5 μM than at 15 μM Bisphenol A concentrations. Another recent report demonstrated that exposure to low level Bisphenol A (0.01 and 0.1 μM) resulted in early life-stage hyperactivity in zebrafish larvae by 5 days post fertilization (dpf); however, exposure to higher Bisphenol A concentrations (1 and 10 μM) did not affect larval activity (Saili et al., 2012). Saili et al. (2012) explained that this phenomenon might be an adaptation to a toxic response; e.g., the reduction or lack of response at higher concentrations is the result of adaptive detoxification mechanisms. In this study, we also observed that the fish responses in the light-dark cycles are more severe at 100 μg/L than at 1000 μg/L microplastic concentrations. Fish swimming performance might be a good index to assess the toxicity of chemicals at low concentrations.

Short, alternating light and dark periods cause swimming activity in unaffected zebrafish larvae to quickly increase (MacPhail et al., 2009; Wang et al., 2013). This could represent a stress response to the potential presence of a roving predator or a signal of lack of warmth. The transition from light to dark is undesirable for larval zebrafish and makes larval zebrafish behave differently (Bai et al., 2016). In previous studies, adult zebrafish have shown a marked preference for dark environments (Maximino et al., 2010), while larval zebrafish have been found to display dark avoidance (Lau et al., 2011). Many studies have demonstrated that dark avoidance in larval zebrafish is anxiety related (Steenbergen et al., 2011; Chen et al., 2015). Bai et al. (2016) ascertained that dark avoidance in larval zebrafish can be activated by different stressors, including heat, cold, UV, mechanical disturbance, and social isolation. The dissociation between the behavioral and physiological measures (relative cortisol level) of stress, provided that a number of anxiety related states are due to regulations by distinct neuronal circuits and activated by different stressors. Microplastics suspended in the exposure medium surrounding larval zebrafish could cause anxiety to the fish larvae.

These results demonstrated that exposure to 100 and 1000 μg/L of microplastics led to significantly decreased swimming competence of zebrafish larvae. Decreased swimming competence could decrease ecological fitness of the fish, such as decreased ability to avoid predation, which could ultimately lead to decreased competence and fitness in the wild environment (Pitt et al., 2018). The early social environment can persistently influence behavior and social competence later in vertebrate life, which has significant ecological consequences (Nyman et al., 2018). Swimming is an early social response; the alteration of swimming competence of zebrafish can be an indicator in monitoring early effects of pollutants on the fish community and the aquatic ecosystem (Strungaru et al., 2018). Early and larval stages of aquatic organisms are the key trophic link between primary producers and higher trophic level organisms, and any impacts on their population may have significant ecological consequences.

4.3. Increased defense response at molecular level of zebrafish larvae upon exposure to microplastics

In this study, microplastics significantly increased the mRNA expression of inflammation gene *il1b* and oxidative stress gene *cat*. Many studies have found that microplastics can induce the formation of reactive oxygen species (ROS) and oxidative stress, and alter the immune activity. For example, after exposure, microplastics caused cellular effects including changes to immunological responses, peroxisomal proliferation and antioxidant system (Avio et al., 2015). There were also increased expression of the glutathione S-transferase 4 enzyme in the intestine of *Caenorhabditis elegans*, these indicate that both intestinal damage and oxidative stress are major effects of microplastic exposure (Lei et al., 2018). Browne et al. (2013) found that exposure to microplastics can reduce the ability of coelomocytes of lugworms (*Arenicola marina*) to remove pathogenic bacteria and make them more susceptible to oxidative stress. In addition, Chen et al. (2017) found that exposure to 47 ± 0.2 nm microplastics can significantly decrease the total distance of swimming of zebrafish larvae in dark period at 120 hpf, and the oxidative damage (the altered CAT, Glutathione peroxidase (GPx) activities and glutathione (GSH) content in 120 hpf zebrafish larvae) was evaluated to be one of the main reasons for the behavior inhibition.

5. Conclusions

In this study, polystyrene microplastics were significantly taken up by zebrafish larvae at 96 hpf at the concentrations of 100 and 1000 μg/L. Exposure to microplastics significantly decreased swimming competence in zebrafish larvae not only in the free swimming test, but also in the light-to-dark alternation stimulation in dark period. The decreased swimming competence of zebrafish larvae during the light-to-dark alternation stimulation was possibly due to the inhibited competence of dark avoidance in zebrafish larvae. Additionally, exposure to microplastics led to increased defense response at the molecular level in treated zebrafish larvae, which may contribute to the decreased swimming performance. These results suggest that microplastics in the aquatic environment can affect the swimming performance of young fish. Decreased swimming competence could ultimately lead to decreased fish fitness (in terms of the ability to find food and avoid predation) in the wild environment, which may have significant impacts on its population fitness and further ecological consequences.

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