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Research Paper

Fairy shrimp *Branchinella kugenumaensis* displays sensitivity to microplastic exposure*

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Abstract The increasing global concern regarding plastic pollution has prompted the research into the consequences of microplastics (MPs) on aquatic ecosystems. Fairy shrimp *Branchinella kugenumaensis* are freshwater planktonic organisms that have existed for 250 million years. This study aimed to uncover the harmful effects of MPs, with a particular focus on their size variations (0.1, 1, and 5 μm), on the fairy shrimp. We focused on how MPs could significantly affect the survival and growth of fairy shrimp. Notably, larger MPs, especially those measuring 5 μm, caused higher mortality rates and hindered the growth compared to smaller ones. The impact of MPs continued even subsequent to depuration in clean water. The accumulation of MPs within the intestines of fairy shrimp resulted in intestinal blockages, disrupted excretory functions, and harmed intestinal epithelial cells. Examinations at the histological, cellular, and molecular levels showed that exposure to MPs triggered necroptosis in intestinal cells, accompanied by alterations in pathways related to transcription, translation, digestion, energy metabolism, and neurological functions. Furthermore, the effects of MPs on gene expression and pathways varied based on particle size, with larger MPs having a more significant effect and causing a strong response in xenobiotic biodegradation and metabolism pathways. We suggest that the increasing severity of MPs pollution could pose a significant threat to the survival of fairy shrimp. This study provided vital insights into the complex relationship between microplastics and aquatic organisms, and highlighted the urgent need to address the potential devastating impact of plastic pollution on freshwater ecosystems. Additionally, due to their rapid growth, strong reproductive capacity, sensitivity, and ease of cultivation, fairy shrimp hold the potential candidate to serve as a model organism for studying the effects of MPs and other pollutants on freshwater ecosystems.

Keyword: microplastic; freshwater ecosystem; toxic effects; fairy shrimp; model organism

1 INTRODUCTION

Plastics, synthetic organic polymers primarily made from petroleum or natural gas, have gained wide usage due to their outstanding performance and cost-effectiveness. Notable among examples include polyethylene (PE), polypropylene (PP), and polystyrene (PS) (Kannankai et al., 2022). However, the widespread use of plastic products along with insufficient recycling systems has led to a growing crisis of plastic pollution (Su et al., 2022). Plastic particles with a diameter smaller than 5 mm are defined as microplastics (MPs), and MP pollution has become an urgent global environmental issue, attracting increasing attention (Borriello, 2023). Microplastics can be classified into primary and secondary sources. Primary MPs come from cleaning products, cosmetics, personal hygiene items, paints, and detergents, while secondary microplastics result from the breakdown of larger plastic objects like bottles, fishing nets, and bags due to processes such as exposure to sunlight, weathering, and the action of waves (Kumar et al., 2021). The distribution of MPs affects various environments worldwide, making it a significant pollutant in aquatic ecosystems, from small bodies

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of freshwater like ponds, rivers, and lakes to the deep oceans, extending even to polar regions (Huang et al., 2021a; Kumar et al., 2021; Citterich et al., 2023; Nirmala et al., 2023).

China exhibits an extensive production and use of plastic products, leading to a significant pollution of water sources with MPs. Investigations have revealed the profound extent of this issue: in the surface waters of the Changjiang (Yangtze) River, the concentration of MPs with a diameter ≥48 μm ranges from 1 597 to 12 611 particles/m^3 (Di and Wang, 2018). The surface water of the Huanghe (Yellow) River, the concentration of MPs with a diameter of ≥50 μm falls within the range of 380 000–1 392 000 particles/m^3 (Han et al., 2020). Within the surface waters of Zhanjiang River, MPs with a diameter of ≥5 mm are found at concentrations ranging from 50 to 725 particles/m^3 (Pan et al., 2020). The surface water of the Zhujiang (Pearl) River exhibits an exceptionally high concentration of MPs with a diameter of ≥45 μm, reaching levels as remarkable as 19 860 particles/m^3 (Yan et al., 2019). In the surface waters of the Suzhou River, the concentration of MPs with a diameter of ≥20 μm falls within the range of 1 800–2 400 particles/m^3 (Luo et al., 2019b). Furthermore, the surface waters of the Wei River contain MPs with a diameter of ≥75 μm, with concentrations fluctuating between 3 670 and 10 700 particles/m^3 (Ding et al., 2019). Notably, in the surface waters of rivers in Wuhan, MPs with a diameter of ≤20 cm are present at concentrations ranging from 1 660 to 8 925 particles/m^3, as reported by Wang et al. (2017).

Microplastics exert significant impacts on aquatic organisms, with fish being a primary focus of study. MPs tend to accumulate in various fish tissues, influencing functions like survival, growth, reproduction, behavior, and immunity (Hossain and Olden, 2022). Research on the effects of MPs on crustaceans, especially shrimp, has also expanded. Field studies have found the accumulation of microplastics in the digestive systems of both freshwater and marine shrimp species (Nan et al., 2020; Wu et al., 2020; Valencia-Castañeda et al., 2022). Experimental evidence has shown that exposure to MPs can disrupt feeding behavior, cause oxidative stress, change the distribution of gut microorganisms, interfere with immune responses and reproduction, and even result in shrimp mortality (Timilsina et al., 2023). Planktonic organisms, critical components of aquatic ecosystems, have also drawn attention due to their potential to ingest MPs (Rodrigues et al., 2021). However, a thorough understanding of the effects of microplastics on planktonic organisms requires further experimental investigation.

To address the breadth of planktonic species, ongoing research primarily employs brine shrimp (*Artemia salina*) and *Daphnia magna* as model organisms. Existing studies have demonstrated that MPs can induce oxidative stress, immune stress, intestinal damage, growth inhibition, and an increase in mortality in brine shrimp (Han et al., 2021; Kim et al., 2021b; Jeyavani et al., 2022). Furthermore, MPs have been shown to cause intestinal blockage, growth inhibition, increased mortality, as well as morphological and life history changes in *D. magna* (Eltemsah and Bøhn, 2019; Schrank et al., 2019; Chen et al., 2022). Brine shrimp have a short life cycle and prolific egg production but are limited to saline environments. In contrast, *D. magna*, a short-lived, rapidly reproducing freshwater organism, faces challenges in extracting RNA and proteins and making tissue sections because of its small size and hard outer shell (Lee et al., 2019; Ntungwe et al., 2020; Kim et al., 2021a; Tkaczyk et al., 2021). Consequently, the search for additional freshwater planktonic organisms becomes extremely important in investigating the toxic effects of MPs and other pollutants.

Fairy shrimp *B. kugenumaensis*, a small crustacean belonging to the Branchiopoda class, is ubiquitous in seasonal lakes. Its life cycle is closely synchronized with seasonal water fluctuations: eggs hatch, grow, and reproduce when water levels increase, while they go into a dormant state during water shortages, waiting for favorable conditions to hatch again (Velu and Munuswamy, 2006). Our laboratory has successfully cultivated fairy shrimp *B. kugenumaensis*, which exhibits qualities like fast growth, strong reproductive abilities, and the ability to adapt to various diets. These characteristics make it an ideal subject for experiments. Therefore, this study aims to employ fairy shrimp as a model organism to elucidate the toxic effects and underlying mechanisms of MPs with varying sizes and concentrations. This will help improve our understanding of the threat MPs pose to aquatic ecosystems and explore the potential use of fairy shrimp in toxicology studies.

2 MATERIAL AND METHOD

2.1 Experimental animal and MP

The experimental fairy shrimp used in this study were obtained by hatching dormant fairy shrimp

eggs preserved in the laboratory. They were reared in a light incubator under a 13-h:11-h light:dark cycle with daily aeration using tap water. The water temperature was maintained at 28±0.5 ℃. After 24 h of hatching, the fairy shrimp were fed with fresh *Chlorella proteinucleus* twice daily. The 0.1-, 1-, and 5-μm green fluorescent polystyrene plastic microspheres (PS-MPs) used in the experiments were purchased from Tianjin Base Line Chrom Tech Research Centre (Tianjin, China) and stored as dispersions (2.5% w/v, 10 mL). We observed the ultrafine morphological characteristics of MPs using field emission scanning electron microscopy (FE-SEM) (Zeiss GeminiSEM 300, Germany) and fluorescence microscopy (Olympus BX53, Japan). The ultrafine morphological and fluorescence images of the MPs are shown in Fig.1a & b, respectively.

2.2 MPs exposure

Three days post hatching (dph), healthy and uniform-sized fairy shrimps with a length of 4.13± 0.13 mm were selected and exposed to MPs of three sizes (0.1, 1, and 5 μm) at concentrations of 10 μg/L and 1 mg/L, respectively. The exposure concentration was chosen based on the previous studies (Yang et al., 2020; Yu et al., 2020; Xie et al., 2021; Zhang et al., 2021). The experiments were conducted in 2 000-mL beakers with three replicates for each treatment, with an initial placement of 150 fairy shrimp in each beaker. Furthermore, we also established a control (Con) group without MPs, with the same rearing conditions as the MPs-exposed groups. All the fairy shrimp were feed with microalgae twice a day. The preliminary experiments revealed

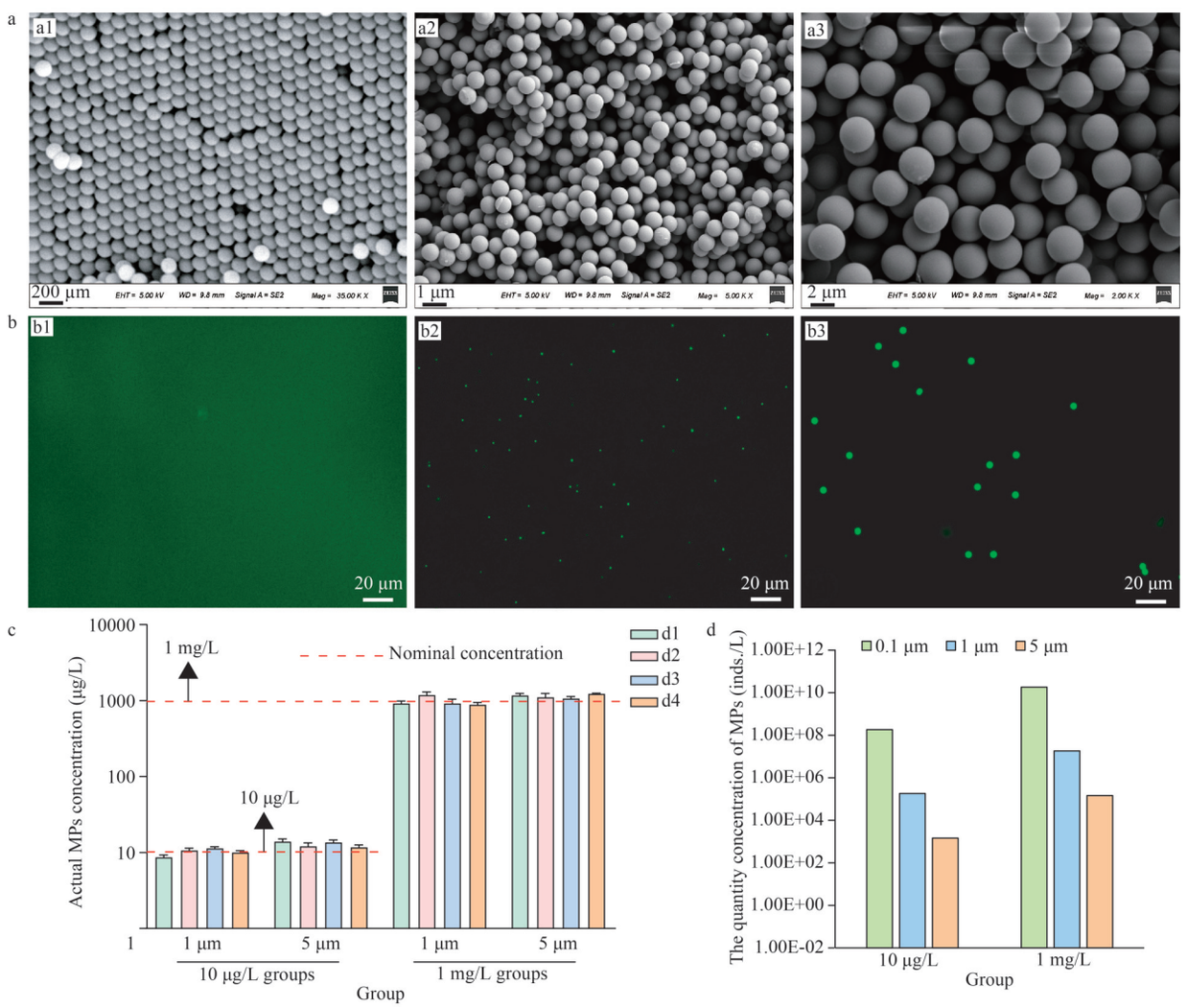

Fig.1 Characteristics and actual exposure concentration analysis of MPs

a. scanning electron microscopy images of MPs; b. fluorescence images of MPs; c. actual exposure concentrations of MPs; d. the nominal quantity concentration of MPs in each exposure group.

that fairy shrimps are highly sensitive to MPs exposure, with a high mortality rate. Therefore, we conducted a short-term exposure experiment lasting 4 d. The exposure solution was replaced every 24 h to maintain a constant concentration of MPs. After 4 d of exposure, the fairy shrimps were transferred to clean water and the exposure solution was replaced with fresh aerated tap water every 24 h to observe whether MPs could be excreted from their bodies. A certain amount of exposure water samples was collected to count the number of 1- and 5-μm MPs under a fluorescence microscope with excitation light at 488 nm (Olympus BX53, Japan) (0.1-μm MPs could not be counted under the microscope). Based on the density and diameter information of the MPs, the actual mass concentration of MPs was calculated. The results show that the actual exposure concentration of larger MPs was close to the nominal concentration (Fig.1c). Based on the diameter and density of MPs, we calculated the nominal quantity concentration of MPs in each exposure group, as shown in Fig.1d. On the 1st and 4th days after exposure and the 3rd day after being transferred to clean water, the length and mortality of the fairy shrimp were recorded. Prior to sample collection, all fairy shrimp were anesthetized with a final concentration of 1-mg/L MS-222 (Sigma, USA).

2.3 MPs enrichment analysis

On the 1st and 4th days after exposure, randomly selected surviving fairy shrimp from each group were observed under a fluorescence microscope (Olympus BX53, Japan) to assess the accumulation and excretion of MPs in the fairy shrimp. As fairy shrimp are prone to movement and difficult to immobilize, they were anesthetized with MS-222 (100 mg/L; Sigma, USA) in advance for photography and collection. In addition, dead fairy shrimp from each group were also observed under the fluorescence microscope to examine the presence of MPs in their bodies. Similarly, on the 3rd day after transferring to clean water, MPs excretion was observed under the fluorescence microscope (Olympus BX53, Japan).

2.4 Lysosome and apoptosis detection

The lysosome red fluorescent probe Lyso-Tracker Red (LTR) (Beyotime, China) can penetrate cell membranes and emits intense red fluorescence upon binding to the acidic environment within lysosomes. This allows accurate observation and localization of lysosome distribution and quantity. After 4 d of MPs exposure, three replicates were set for each group, with six fairy shrimp selected for each replicate. LTR solution was diluted in 1∶20 000 ratio to create LTR working solution. After incubating the fairy shrimp at 37 °C in the dark for 60 min, the excess probe was washed off using phosphate buffer solution (PBS). The fairy shrimp were then anesthetized and immobilized on glass slides for observation and photography under a fluorescence microscope. The fluorescence was analyzed using Image J. Acridine orange (AO) (Beyotime, China) is a nucleic acid-selective vital dye that emits bright green fluorescence upon binding to fragmented DNA fragments, making it suitable for indicating cell apoptosis. As the MPs microspheres used in the experiment also emit bright green fluorescence, fairy shrimp that had excreted MPs for 3 d in clean water were selected for the AO staining. Three replicates were set for each group, with six fairy shrimp randomly selected for each replicate. After washing the fairy shrimp with PBS, AO dye with a final concentration of 2 mg/L was added for staining at room temperature in the dark for 10 min. The fairy shrimp were then washed with PBS. Before inspection, the fairy shrimp were fixed on glass slides using MS-222 and identified under a fluorescence microscope (Olympus BX53, Japan), with apoptotic cells exhibiting bright green fluorescence being counted and analyzed.

2.5 Ultrastructure observation

The ultrastructural alterations of fairy shrimp in 1-mg/L MPs treated groups were meticulously examined using transmission electron microscopy (TEM) after 4 d of MPs exposure. Each group consisted of three replicates, with each replicate containing one shrimp. The shrimps were fixed overnight in 2.5% glutaraldehyde at 4 °C for 48 h and thoroughly rinsed with 0.1-mol/L PBS. Subsequently, they were fixed in 1% osmium tetroxide at 4 °C for 2 h and underwent an additional round of PBS rinsing. To further process the samples, the shrimps were dehydrated using a graded ethanol series, followed by embedding in epoxy resin and careful drying at 55 °C for 48 h. Afterward, precise semi-thin sections (1 μm) were generated to accurately identify the regions of interest, which were then further refined into ultra-thin sections (80 nm). The samples were then meticulously examined and documented using a TEM (Hitachi HT7700, Japan).

2.6 Transcriptome sequencing and data analysis

After 4 d of exposure to MPs, 30 shrimps were

collected from Con and 1-mg/L MPs (0.1, 1, and 5 μm) treated groups in triplicate, and total RNA was extracted using the Trizol (Vazyme, China) one-step method. The ultramicrospec-trophotometer (Thermo NanoDrop 2000, USA) was used for RNA quantification, and samples with an optical density 260/280 ($OD_{260/280}$) ratio of 1.8–2.0 were further processed. The library preparation and sequencing were conducted by Lianchuan Biotechnology Co., Ltd. (Hangzhou, China). Differentially expressed genes (DEGs) were analyzed, and pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG). Bioinformatic analysis was conducted using the OmicStudio tools available at https://www.omicstudio.cn.

Six differentially expressed genes (DEGs), including two up-regulated genes, two down-regulated genes, and two genes with unchanged expression, were randomly selected from each treatment group for real-time quantitative PCR (RT-qPCR) validation. Eukaryotic translation elongation factor 1 alpha (*ef1a*) served as reference gene. The specific primers used for qRT-PCR are listed in Supplementary Table S1. The amplification efficiency (E) ranged from 90% to 110%. The RNA samples used for qRT-PCR analysis were the same as those utilized in the RNA-seq experiment. HiScript II 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, China) was used to reverse the selected total RNA samples with the required quality to produce cDNA. RT-qPCR results were quantified with QuantStudio3 (Thermo Fisher Scientific, USA). Relative transcript changes were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7 Statistical analysis

All data were presented as mean±standard deviation (SD). Data were tested for normality of distribution (Kolmogorov-Smirnoff test) and homogeneity of variance (Levene's test) prior to analysis. Data that did not meet normality and homoscedasticity were transformed and then analyzed by two-way Analysis of Variance (ANOVA), and post-hoc multiple comparisons were carried out using Tukey's multiple-comparison tests. SPSS 17.0 software was used for statistical analysis.

3 RESULT

3.1 Impact of MPs on fairy shrimp survival and growth

Following 1-d exposure to MPs, the mortality rate of fairy shrimp exhibited a significant increase across all treatment groups. The mortality rate increased as the size of MPs' particles grew, with a weaker connection to the exposure concentration. Extending the exposure duration to 4 d continued to increase the mortality rate in all treatment groups. Importantly, the mortality caused by 0.1- and 5-μm MPs exceeded that caused by 1-μm MPs. After a 3-d purification period in clean water, the previously elevated mortality trended to decrease, although the mortality rate induced by 0.1- and 5-μm MPs remained higher compared to 1-μm MPs (Fig.2a). One day post-exposure, both 1- and 5-μm MPs caused a significant reduction in the body length of fairy shrimp. After 4 d of exposure, low-concentration 1- and 5-μm MPs, as well as high-concentration 0.1- and 5-μm MPs, significantly hindered the growth of the fairy shrimp. After 3 d of depuration in clean water, the body length of fairy shrimp in all MPs exposure groups remained significantly lower than that of the Con group (Fig.2b).

3.2 MPs accumulation in fairy shrimp intestine

Accumulation of MPs was observed in the intestines of both surviving and dying fairy shrimp following MPs exposure. The Con group exhibited no MPs accumulation in their intestines. Surviving fairy shrimp in all treatment groups displayed MPs accumulation within their intestines. These shrimps maintained normal excretion function, indicated by the presence of excretion products and MPs at the excretion pore (Fig.3a). In the 1- and 5-μm MPs treatment groups, intestines of dying fairy shrimp displayed obvious MPs accumulation, leading to evident intestinal blockages and impaired excretion function. Notably, the obstruction caused by 5-μm MPs was more severe than that induced by 1-μm MPs. Dying fairy shrimp subjected to 0.1-μm MPs did not show noticeable blockages (Fig.3b). After 3 d of purification treatment, the MPs have been effectively eliminated from the fairy shrimp's bodies (Supplementary Fig.S1).

3.3 Impact of MPs on fairy shrimp intestinal lysosomes and apoptosis

The number of lysosomes in fairy shrimp intestines experienced a significant increase across all MPs exposure groups, with the 5-μm MPs treatment group showing the most pronounced elevation in lysosome count among the low-concentration MPs treatment groups. In the high-concentration MPs treatment group, all three sizes

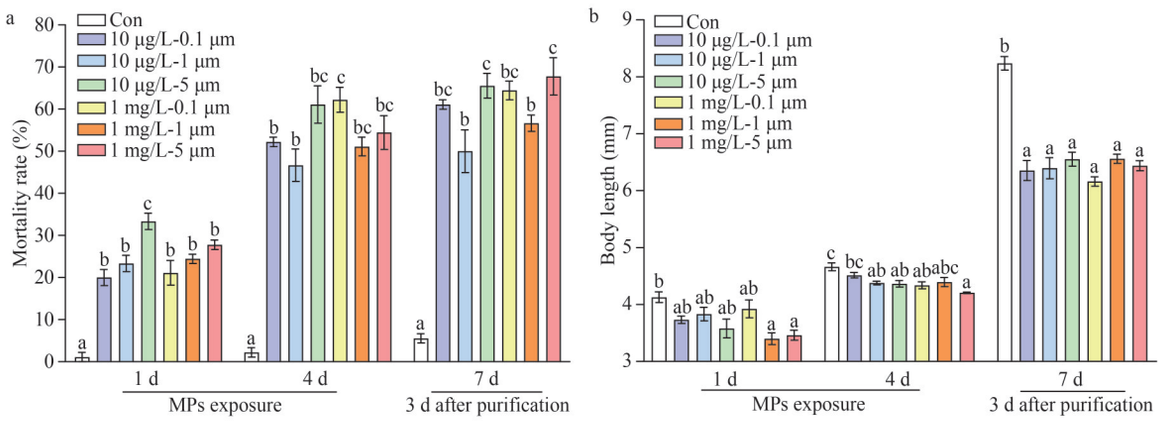

Fig.2 Impact of MPs on fairy shrimp growth and survival

a. mortality rate; b. body length. Data is shown as mean±SD, significant differences between two data points within the same group are indicated by different letters (P<0.05).

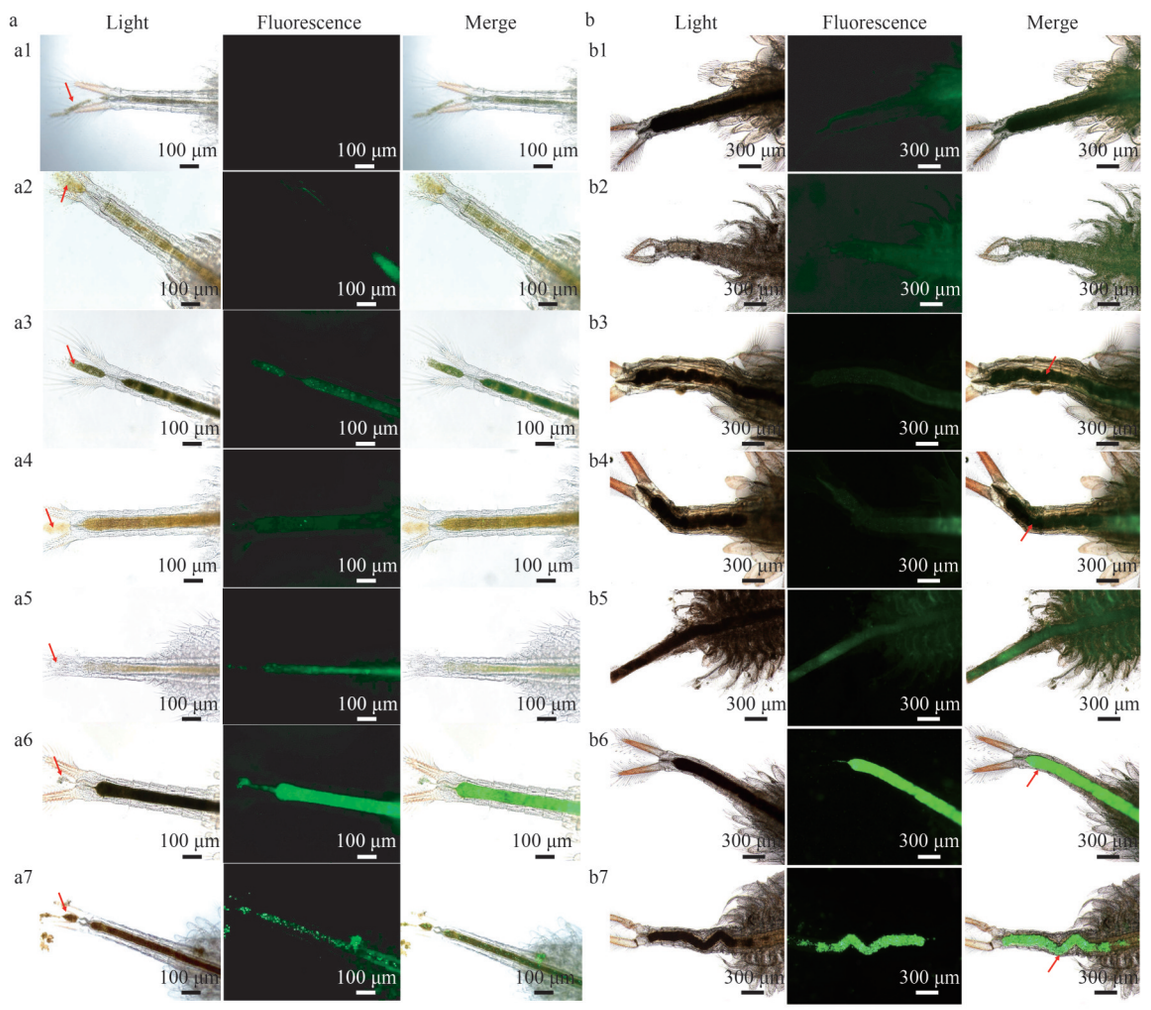

Fig.3 Accumulation of MPs in fairy shrimp intestines

a. accumulation of MPs in intestines of live fairy shrimp. a1: Con group; a2: 10 μg/L-0.1 μm group; a3: 10 μg/L-1 μm group; a4: 10 μg/L-5 μm group; a5: 1 mg/L-0.1 μm group; a6: 1 mg/L-1 μm group; a7: 1 mg/L-5 μm group; b. accumulation of MPs in intestines of moribund fairy shrimp. b1: Con group; b2: 10 μg/L-0.1 μm group; b3: 10 μg/L-1 μm group; b4: 10 μg/L-5 μm group; b5: 1 mg/L-0.1 μm group; b6: 1 mg/L-1 μm group; b7: 1 mg/L-5 μm group. Bright green color represents MPs in the images. Accumulation of MPs in live fairy shrimp varies with particle size and exposure concentration, and MPs can be excreted normally through feces, indicated by arrows in (a). Moribund fairy shrimp treated with 1- and 5-μm MPs showed obvious intestinal obstructions, unable to excrete, indicated by arrows in (b).

of MPs induced a notable lysosome induction effect, displaying insignificant differences between particle sizes (Fig.4a & c). Apoptosis within intestinal epithelial cells exhibited a significant increase across all treatment groups. In the low-concentration treatment groups, the 5-μm MPs treatment group displayed a significantly higher count of apoptotic cells compared to the other two groups. In high-concentration treatment groups, the number of apoptotic cells in intestines was generally elevated and correlated with MPs' size (Fig.4b & d).

3.4 Impact of MPs on fairy shrimp intestinal epithelial cell ultrastructure

Within the Con group, intestinal epithelial microvilli remained intact, mitochondria exhibited typical morphology, and tight junction structures remained unaltered (Fig.5a). The 0.1-μm MPs treatment induced increased lysosomes within fairy shrimp intestinal epithelial cells, abnormal mitochondria with augmented quantity, disrupted tight junctions featuring vacuolar structures, and cell rupture (Fig.5b). In the 1-μm MPs treatment group, fairy shrimp intestinal epithelial cells displayed increased lysosomes and mitochondria, alongside endoplasmic reticulum expansion and disrupted tight junctions containing vacuolar structures (Fig.5c). The 5-μm MPs treatment prompted augmented lysosomes and abnormal mitochondria with heightened quantity within fairy shrimp intestinal epithelial cells. Additionally, it led to significant disruption of tight junctions, partial cell breakage, and cytoplasmic leakage (Fig.5d).

3.5 Impact of different size MPs on fairy shrimp transcriptome

The number of genes exhibiting differential expression increased as the size of MPs grew. Specifically, within the 5-μm MPs treatment group, 3 845 genes showed significant upregulation, while 2 633 displayed downregulation. Similarly, within the 1-μm MPs treatment group, 580 genes experienced significant upregulation, and 420 displayed downregulation. In the 0.1-μm MPs treatment group, 360 genes demonstrated significant upregulation, while 249 exhibited downregulation (Fig.6a). Principal Component Analysis (PCA) highlighted that 5- μm MPs exerted the most significant impact on fairy shrimp at the gene expression level, trailed by 1-μm MPs, whereas 0.1-μm MPs exerted the least influence (Fig.6b). Six genes from the RNA-Seq results were selected for RT-qPCR to validate the RNA-Seq data. As shown in Fig.6c, for both up- and down-regulated genes, the results of RT-qPCR matched well with those of RNA-seq. The top 10 KEGG

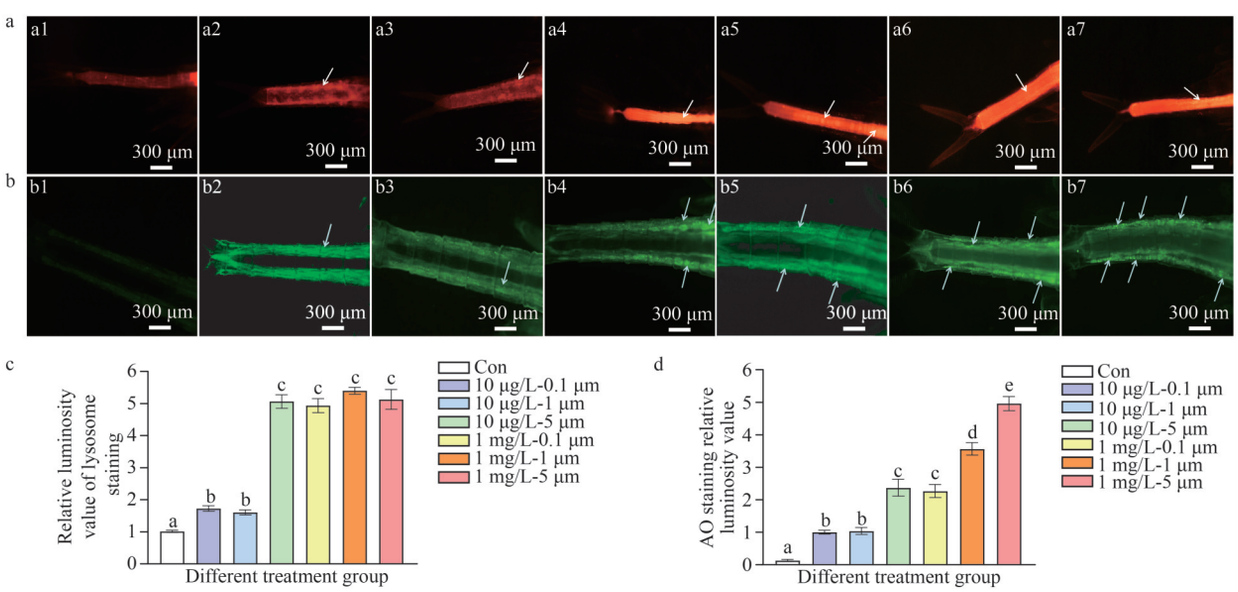

Fig.4 Effect of MPs on fairy shrimp intestinal lysosomes and cell apoptosis

a. lysosomal staining. a1: Con group; a2: 10 μg/L-0.1 μm group; a3: 10 μg/L-1 μm group; a4: 10 μg/L-5 μm group; a5: 1 mg/L-0.1 μm group; a6: 1 mg/L-1 μm group; a7: 1 mg/L-5 μm group. Red fluorescence intensity correlates with lysosome count; b. intestinal cell apoptosis staining. b1: Con group; b2: 10 μg/L-0.1 μm group; b3: 10 μg/L-1 μm group; b4: 10 μg/L-5 μm group; b5: 1 mg/L-0.1 μm group; b6: 1 mg/L-1 μm group; b7: 1 mg/L-5 μm group. Bright green fluorescence dots indicate apoptotic cells; c. fluorescence abundance statistics of lysosomal staining; d. statistics of intestinal apoptotic cell count. Data are presented as mean±SD, significant differences between two data points within the same group are denoted by different letters (P<0.05). The arrows in (a) indicates lysosomes. The arrows in (b) indicates apoptotic intestinal epithelial cells.

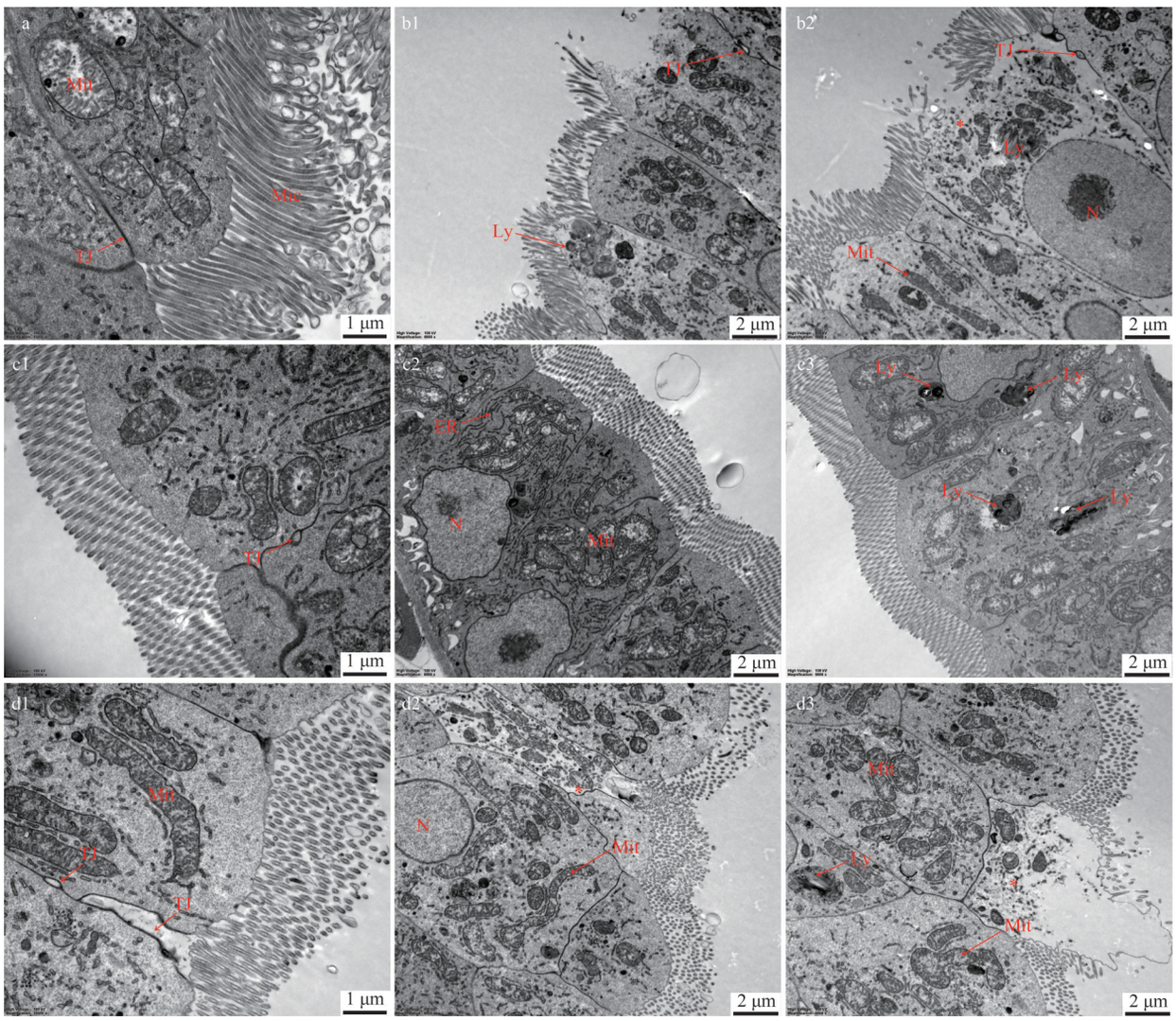

Fig.5 Impact of MPs on fairy shrimp intestinal epithelial cell ultrastructure

a. Con group; b. 1 mg/L-0.1 μm group; c. 1 mg/L-1 μm group. d. 1 mg/L-5 μm group. ER: endoplasmic reticulum; Mic: microvilli; Mit: mitochondria; N: nuclear; TJ: tight junction; Ly: lysosome. * indicates ruptured cells. Exposure to MPs led to an increase in lysosomes in intestinal epithelial cells, disruption of tight junctions, abnormal mitochondrial morphology, endoplasmic reticulum expansion, and even cell rupture.

pathways significantly altered in each MPs treatment group are presented in Fig.6d. Within the 0.1-μm MPs treatment group, the most affected pathways in fairy shrimp, including RNA polymerase, Ribosome, and Thermogenesis, chiefly involving Transcription, Translation, and Environmental adaptation (P<0.001). Additionally, pathways associated with neurological diseases like Parkinson's and Alzheimer's, nucleotide metabolism (Pyrimidine and Purine metabolism), as well as circulatory and immune systems (Cardiac muscle contraction and Platelet activation), exhibited marked impact (P<0.001). Similar significant impacts were evident in the 1-μm MPs treatment group, including RNA polymerase, Ribosome, Thermogenesis, neurological diseases, and metabolic pathways (P< 0.001). However, the 5-μm MPs treatment group delineated distinct pathways, primarily related to metabolism-xenobiotics biodegradation and metabolism, carbohydrate metabolism, glycolysis/gluconeogenesis, and amino acid metabolism. Notably, signaling molecules, interaction pathways, transport, and catabolism pathways were also affected, along with endocrine and metabolic disease pathways, including the AGE-RAGE signaling pathway in diabetic complications (P<0.001) (Fig.6d). We also conducted an analysis of the combined effects of MPs of different particle sizes on the fairy shrimp transcriptome. Classification analysis of KEGG pathways indicated a wide-reaching influence of different size MPs exposure on various aspects of fairy shrimp,

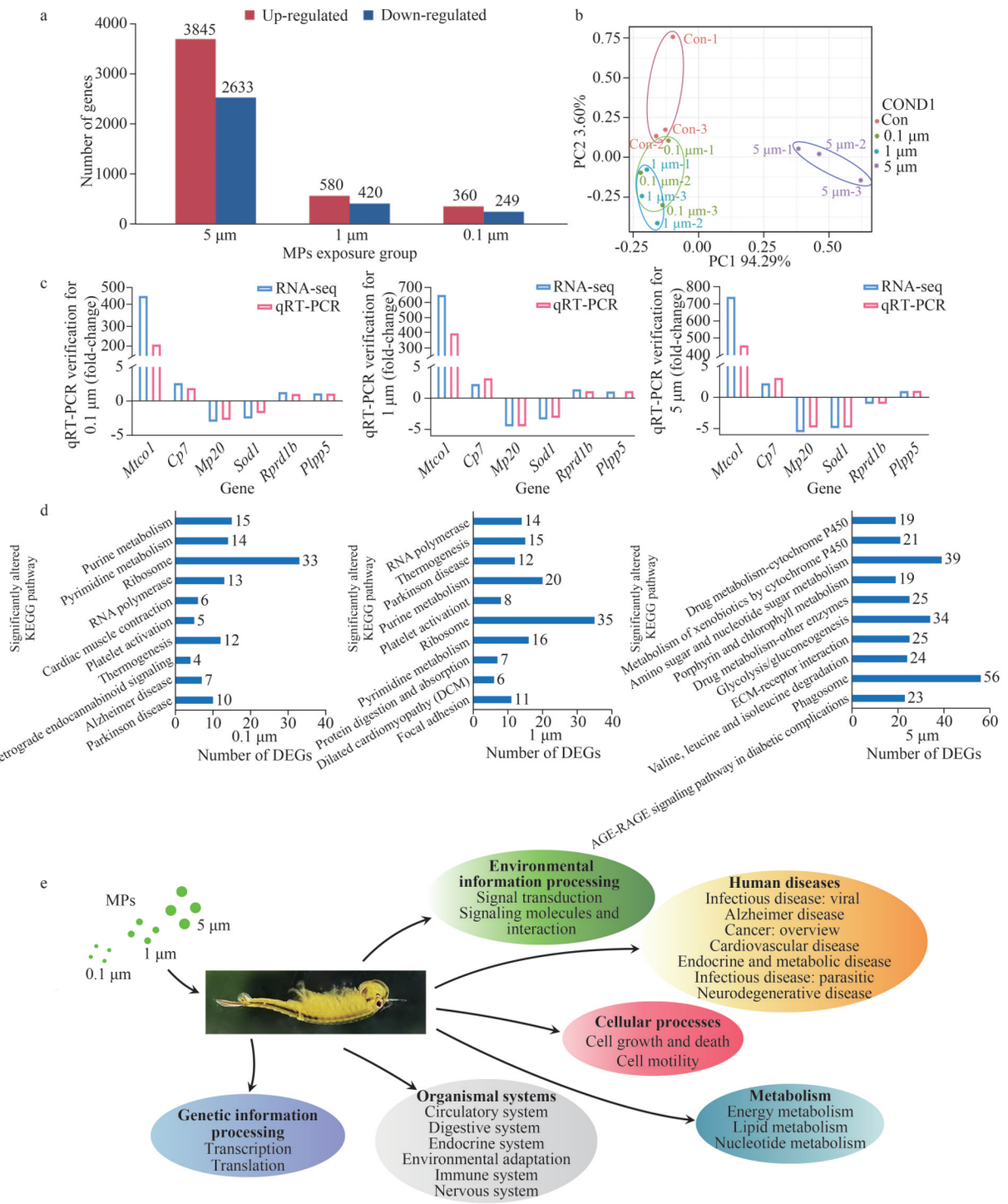

Fig.6 Impact of MPs on fairy shrimp transcriptome

a. number of significantly altered genes in each treatment group; b. PCA analysis of significantly altered genes; c. real-time quantitative PCR (RT-qPCR) validation of the RNA-Seq data; d. top 10 significantly altered KEGG pathways in each treatment group, numbers behind the bars indicate the count of DEGs; e. shared impact of different size MPs on fairy shrimp transcriptome. All significant changes are at the $P<0.05$ level.

including signal transduction, signaling molecules and interactions, transcription, translation, circulatory system, digestive system, endocrine system, environmental adaptation, immune system, nervous system, energy metabolism, lipid metabolism, nucleotide metabolism, cell growth and death, and

cell motility. Furthermore, MPs exposure induced notable changes in pathways related to human diseases, including infectious disease: viral, Alzheimer's disease, cancer: overview, cardiovascular disease, endocrine and metabolic disease, infectious disease: parasitic, and neurodegenerative disease. Notably, several significant changes in pathways within the cell growth and death category, including tight junction, necroptosis, and apoptosis, showed substantial modifications that align with the observed morphological alterations (Fig.6e).

4 DISCUSSION

Microplastic pollution has attracted global attention as a significant environmental concern, leading to a focused examination of its impact on aquatic ecosystems. This study assessing the effects of MPs on the ancient species of fairy shrimp, exploring their toxicity through survival, growth, histology, and transcriptomic analysis. The research highlighted significant negative impacts of MPs on fairy shrimp's survival and growth. Initial exposure to MPs resulted in elevated mortality rates across treatment groups on the first day, with larger MPs inducing more pronounced effects. The particle size emerged as a crucial factor, surpassing exposure concentration in influencing mortality. This size-dependent significance, particularly during early life stages, found support in the identification of severe intestinal blockages caused by larger MPs (1 μm and 5 μm). Comparable size-dependent outcomes of MPs have been observed; for instance, larger MPs (8 μm) caused more significant intestinal damage in goldfish compared to smaller particles (0.25 μm) (Abarghouei et al., 2021). As the exposure duration extended, a continuous increase in the mortality rate of fairy shrimp was noted, particularly concerning 0.1-μm and 5-μm MPs, which had a slightly greater impact than 1-μm MPs. This phenomenon could be attributed to the rapid growth rate of fairy shrimp (Dararat et al., 2011). With an increase in size, their excretion capacity for smaller MPs (1 μm) is enhanced. However, in the case of 0.1-μm MPs, their high permeability and larger quantity likely result in greater biological availability. Consequently, as the exposure time lengthens, the fatality rate for 0.1-μm MPs does not significantly decrease compared to larger-sized MPs. Similarity, exposure experiments using PS-MPs of different sizes (0.1, 1.0, 5.0, and 20.0 μm) on *Litopenaeus vannamei* demonstrated size-dependent ingestion and excretion of PS-MPs, with smaller particles causing more pronounced damage to gill and intestine tissues (Zhou et al., 2023). Experiments on larger Crucian carp also indicated that smaller PS-MPs (5.4–6.6 μm) led to a reduction in body length and fewer offspring compared to larger PS-MPs (18–22 μm) (Schwarzer et al., 2022). Considering the insights from this study and prior research, it becomes apparent that exposure to MPs does indeed result in a size-dependent toxic effect on organisms. However, whether this toxicity diminishes with increasing MPs' size or escalates is not an absolute rule. Instead, it is likely influenced by a combination of factors such as the specific particle size of MPs, organism size, and the organism's ability to ingest and excrete MPs. Just as in this study, different particle sizes of MPs all lead to a significant mortality of fairy shrimp. While it can be observed that the effects of MPs of different particle sizes exhibit some variations and patterns, in most cases, these differences do not reach a significant level. This is possibility attributed to the fact that the differences in particle size of MPs, relative to fairy shrimp, are not substantial enough.

After 3 d of purification in clear water, almost all the MPs were essentially excreted, leading to a partial reduction in the mortality rate of fairy shrimp. However, there were still new instances of mortality, and growth inhibition remained significant, underscoring that the impact of MPs on fairy shrimp was not completely eliminated even after 3 d of purification. A similar perspective is drawn from Sun et al. (2022)' s synthesis of MPs-related research, emphasizing that MPs have a brief retention period within aquatic organisms and are mostly expelled within three days. Although the negative effects induced by MPs were notably alleviated during the depuration phase, the toxicity caused by MPs persisted, resulting in legacy effects. This suggests that the swift elimination of ingested MPs is a common strategy adopted by aquatic organisms to mitigate the adverse effects stemming from MPs' retention. However, incomplete recovery is often observed due to limited recovery capacity and/or insufficient depuration (Naidoo and Glassom, 2019; Xu et al., 2020; Huang et al., 2021b).

In this study, exposure to MPs of various sizes resulted in a range of detrimental effects on fairy shrimp intestinal epithelial cells, including cellular apoptosis, an increase in lysosomal counts, disruption of tight junctions, and abnormal mitochondrial morphology. Collectively, these observations indicate

that ingestion of MPs of different sizes results in significant stress and damage to fairy shrimp. Numerous studies have reported similar instances of MPs exposure leading to intestinal damage in aquatic organisms, such as abnormalities in intestinal epithelial cells in *Artemia* larvae, irregularly shaped and sized enlarged intestinal cells in earthworms, and inflammation and rupture of the goldfish intestine (Jabeen et al., 2018; Wang et al., 2019; Jiang et al., 2020). The accumulation of MPs in the fairy shrimp gut and the ensuing damage to intestinal epithelial cells impede feeding and digestion, ultimately resulting in the death or growth inhibition of fairy shrimp. Likewise, transcriptomic data from different treatment groups unveiled noteworthy alterations in pathways linked to the digestive system, energy metabolism, and other vital processes. Parallel to the impact on intestinal epithelial cells, the transcriptomic data also revealed substantial changes in pathways related to tight junctions, cell death (necroptosis and apoptosis), and mitochondrial function (oxidative phosphorylation) in various MPs treatment groups. MPs exposure has also been found to cause changes in the expression of genes linked to tight junctions and apoptosis in mouse intestines (Luo et al., 2019a; Jia et al., 2023), as well as changes in oxidative phosphorylation-related pathways in zebrafish intestines (Yu et al., 2023). Interestingly, AO staining results indicated that MPs induced apoptosis in intestinal epithelial cells, although the characteristic chromatin condensation typical of apoptosis was not observable in TEM images. This phenomenon in this study, deviating from common apoptosis, more closely resembled necroptosis. Necroptosis is a form of cell death demonstrating features of both apoptosis and necrosis, including potential DNA fragmentation but lacking chromatin condensation. When cells face specific stimuli like viral infections, cellular damage, or abnormal cytokine stimulation, the activation of apoptotic signaling pathways may falter, causing cells to undergo cell death via a pathway that blends apoptosis with necrosis (Pasparakis and Vandenabeele, 2015). A study by Xu et al. (2023) also found that exposure to MPs induced intestinal epithelial cell necroptosis in mice. Additionally, MPs have been documented to induce necroptosis in the liver of mice, the kidney of chickens, and the gill of fish (Meng et al., 2022; Xu et al., 2022; Chen et al., 2023a).

Furthermore, the transcriptomic data unveils a significant impact of MPs of different sizes on fairy shrimp, extending to transcription, translation, and pathways associated with neurological disorders. Upon toxin exposure, organisms exhibit a series of adaptive responses encompassing increased transcription of stress response genes, inflammatory reactions, cellular apoptosis, or necrosis. These changes typically influence the signal pathways governing gene transcription and translation, aimed at countering the harmful effects of toxins (Everds et al., 2013). The documented effects of MPs exposure on the biological nervous system are widespread, hypothesized to be linked to MPs' impact on the neurotransmitter AchE activity (Yin et al., 2021). Meanwhile, we also analyzed the distinct characteristics of the impact of MPs with different particle sizes on fairy shrimp. The fact is that, in the transcriptomic data of different size MPs treatment groups, the count of significantly upregulated and downregulated genes escalates with the size of MPs. Similarly, PCA analysis indicates that 5-μm MPs exert the most pronounced influence on fairy shrimp, followed by 1-μm MPs, while 0.1-μm MPs have the smallest impact. When compared to 0.1-μm and 1-μm MPs, the influence of 5-μm MPs on the xenobiotics biodegradation and metabolism pathway in fairy shrimp is particularly prominent. The transcriptome of grass carp liver exposed to MPs with diameters of 32–40 μm (100 μg/L and 1 mg/L) for 21 d, as well as the mouse liver exposed to 10-μm MPs (10 mg/L) for 28 d, exhibited similar findings (Liu et al., 2022; Chen et al., 2023b). It is possible that the shrimp are actively modulating pathways related to xenobiotic degradation and metabolism to mitigate the presence or transform these detrimental compounds. This adaptive response might result in an elevation in the expression of relevant genes, thus enhancing the organism's capacity to process these substances.

The data derived from this study, including survival, growth, histology, and gene expression of fairy shrimp after MPs exposure, clearly demonstrates the heightened sensitivity of fairy shrimp to toxic exposure. Moreover, our experiments have demonstrated the possibility of conducting research at tissue, cellular, and molecular levels utilizing techniques like TEM, lysosomal staining, AO staining, and transcriptomic sequencing. Additionally, fairy shrimp exhibit a remarkable ability to complete their growth and reproductive cycles within an exceedingly short period. Around ten days post-hatching, their body length can reach 1.5–2 cm, and they promptly commence continuous egg-laying. This accelerated growth and reproduction cycle facilitate the

observation of the effects of MPs exposure on their survival, growth, and reproduction under controlled laboratory conditions. Therefore, fairy shrimp hold the potential to serve as a model organism for the investigation of MPs' impacts and potentially other pollutants on freshwater ecosystems.

5 CONCLUSION

Findings of this study reveal the harmful impact of MPs of various sizes on fairy shrimp's intestinal tract, triggering changes in various metabolic pathways that ultimately affect the shrimp's growth and survival. Notably, larger-sized MPs exhibit a more pronounced adverse impact on fairy shrimp. As an ancient species with a history spanning 250 million years, fairy shrimp now face significant challenges due to modern industrial development and the increasing problem of MPs pollution. Thus, taking effective steps to prevent and control MPs pollution is essential. The sensitivity of fairy shrimp to MPs, their detectability at histological, cellular, and molecular levels, coupled with their attributes like ease of cultivation, rapid growth, and high reproductive capacity, position them as a promising model organism for studying the effects of MPs and potentially other pollutants on freshwater ecosystems. However, more extensive research is necessary to confirm and expand upon these findings, thus fully realizing the potential of fairy shrimp as a model organism.

6 DATA AVAILABILITY STATEMENT

All data generated and/or analyzed during this study are available from the corresponding author upon reasonable request.

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Electronic supplementary material

Supplementary material (Supplementary Table S1 and Fig.S1) is available in the online version of this article at https://doi.org/10.1007/s00343-023-3179-1.