Article:
Wu, D., Carter, L., Kay, P. et al. (3 more authors) (2025) Female zebrafish are more affected than males under polystyrene microplastics exposure. Journal of Hazardous Materials, 482. 136616. ISSN 0304-3894

https://doi.org/10.1016/j.jhazmat.2024.136616

Female zebrafish are more affected than males under polystyrene microplastics exposure

Di Wu[1, 2], Laura Carter[2], Paul Kay[2], Joseph Holden[2], Ying Yin[1]*, Hongyan Guo[1]

[1] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, 210023, China

[2] water@leeds, School of Geography, University of Leeds, Leeds, LS2 9JT, UK

*Corresponding author. Tel.: +86-25-89680263; Fax: +86-25-89680263.

E-mail address: yinying@nju.edu.cn (Yin Y).

Abstract

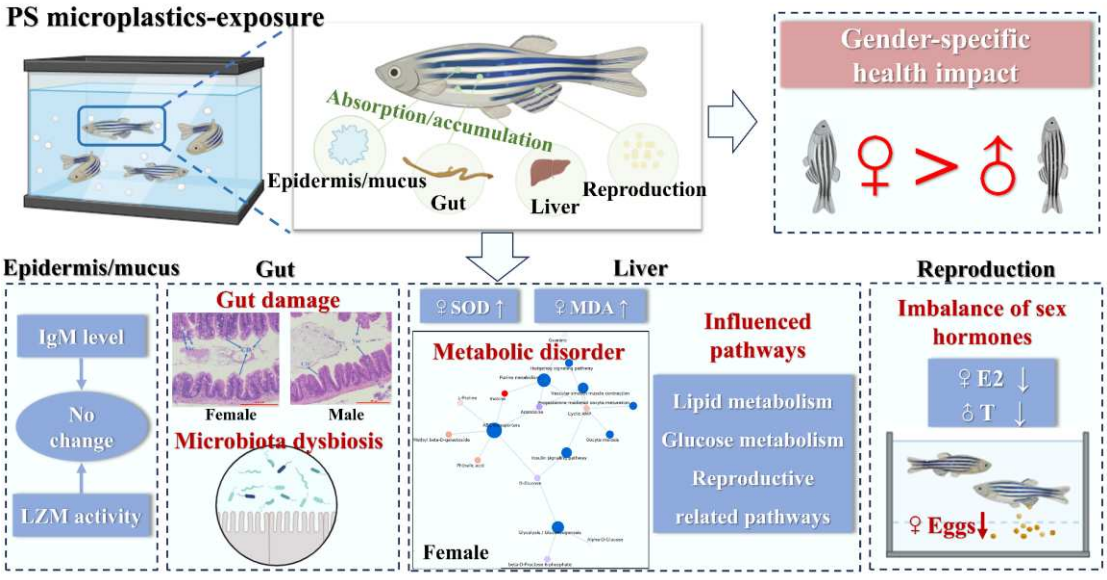
Microplastics are ubiquitous in freshwater and can be absorbed into fish skin and gills, accumulate in the gut, and be transported to other tissues, thus posing a risk to fish health. Further studies are needed, however, to investigate effects such as endocrine disruption and multi-tissue toxicity. In this study, zebrafish were exposed to polystyrene (PS) microplastics and health-related indicators were measured, including skin mucus, gut damage, oxidative stress, stable isotope composition and reproduction as well as an assessment of changes to metabolites using a metabolomics approach. Results showed that concentrations of PS microplastics were higher in gills than those in the gut. Minimal impact to immunoglobulin M level and lysozyme activity in mucus indicated, however, that microplastic toxicity primarily stemmed from ingestion rather than disruption of skin mucus immunity. Female zebrafish were more affected by PS microplastics. Gut microbiota dysbiosis was induced, especially in females. Significant alterations in pathways associated with lipid and energy metabolism were observed in the liver of female fish. PS microplastics also induced sex steroid hormone disorder and reduced female egg production, possibly linked to the alteration of gut microbiota and hepatic metabolism. Combined, these results highlight the gender-specific toxicity of PS microplastics to zebrafish health, potentially harming their population.

Keywords: polystyrene microplastics, zebrafish, gender-specific toxicity, metabolism, gut microbiota

Synopsis: Little research exists on gender-specific effects of microplastics on fish health. This study shows that polystyrene microplastics have a greater impact on the

health of female zebrafish, potentially posing a threat to their population stability.

Graphic abstract



1. Introduction

Microplastics, as novel pollutants, are widely detected in water environments, posing a threat to organisms and ecosystems [1]. Detected microplastic concentrations vary across different water environments, and can even reach ~70 mg/L in wastewater treatment plants and ~ 0.10 g/L in surface water in Malaysia and Brazil [2-4]. Concerns about microplastics in freshwater ecosystems have become higher profile [5, 6]. Within freshwater ecosystems, fish communities are important receptors and bioindicators of microplastic pollution because of their localized habitats and diverse traits [7]. Much research in recent years has focused on the toxicity of microplastics to freshwater fish [8-10]. When fish are exposed to microplastics in freshwater, the main absorption pathways include oral, gill and skin routes [11]. Microplastics may adhere to the skin surface due to sticky mucus found on fish and this skin mucus acts as the main non-specific defense mechanism [12-14]. Yang et al. [15] found that co-exposure of polystyrene (PS) microplastics and 6:2 chlorinated polyfluoroalkyl ether sulfonate reduced lysozyme activity, providing evidence of inflammation.

Apart from absorption on the skin and gills, ingested microplastics mainly accumulate in the gut and may be transported to other tissues, including the liver and gonads [16]. The gut is a target tissue for microplastic accumulation, leading to adverse effects on fish health [17-19]. Qiao et al. [20] found microplastics induced gut microbiota dysbiosis, mucosal damage and inflammation in zebrafish gut. Dysbiosis of gut microbes is linked to gut inflammation and can indicate host metabolic disorders [21, 22]. The liver plays a vital role in maintaining biological energy homeostasis and

health [23, 24]. Studies have shown that PS microplastics can disturb metabolism in fish [18, 25], and stable isotope analysis has recently been applied to investigate changes in metabolic pathways after stress exposure [26]. Toxic exposure could influence $\delta^{15}N$ and $\delta^{13}C$ of organisms by affecting growth, metabolism, and element turnover [26-28]. However, current knowledge of the isotopic effects of microplastics on fish remains limited.

In addition to research on the effects of microplastics on gut microbiota and metabolism, studies on reproductive toxicity are emerging, indicating microplastics induced alteration in tissue formation of zebrafish gonads, female zebrafish fecundity rate and hormonal homeostasis [29, 30]. Gut dysfunction may influence energy and material supply for reproduction [31]. Sun et al. [32] demonstrated that PS nanoplastics disturbed the endocrine regulation pathways of the brain-pituitary-gonadal axis, inducing sex-specific reproductive toxicity to zebrafish.

While many studies have demonstrated the adverse effects of microplastics on fish health [33], gender is usually a neglected factor when assessing their impacts, despite the fact that microplastics have gender-specific effects in other organisms [34, 35]. Yang et al. [36] found that aged PS microplastics induced gender-specific effects on mice liver lipid metabolism. Shen et al. [37] also demonstrated that the response of gut microbiota and fecal metabolites of mice to the co-exposure of PS microplastics and lead was sex-specific: more fecal metabolites were influenced in female mice than in male mice. Differential responses of male and female organisms can cause variations in health impacts, affecting offspring and population stability [38, 39]. However, a

comprehensive understanding of the gender-specific impacts of microplastics on fish, encompassing absorption, ingestion, and multi-tissue responses, is still lacking.

This study aimed to investigate the gender-specific effects of PS microplastics on the health of zebrafish and explore correlation between multi tissue toxic effects. Adult zebrafish were exposed to 30 mg/L PS microplastics (1 μm) and the distribution of PS microplastics in different zebrafish tissues (guts and gills) was determined. We then explored the effects of PS microplastics on skin mucus, gut damage, hepatic metabolism, stable isotope composition and reproduction to provide novel insights into the toxicity of PS microplastics to freshwater fish populations, helping to clarify their potential effects on freshwater ecosystem stability and health.

2. Materials and methods

2.1 Microplastics

As one of the most abundant polymers in the environment, PS microplastics were chosen [40]. PS microplastics with a diameter of 1 μm were purchased from BaseLine ChromTech Research Centre (Tianjin, China). Fluorescent microplastics (excitation wavelengths, 488 nm; emission wavelengths, 518 nm) were used in the uptake and accumulation test whereas virgin microplastics were used for the toxicity test.

2.2 Zebrafish maintenance and experimental setup

Healthy wild-type adult zebrafish (AB strain), aged 5 months, were purchased from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The zebrafish were kept in ultraviolet-sterilized and aerated water at 24 ± 1 °C under a 14 h light/10 h dark cycle and were fed brine shrimp twice daily. For the accumulation test,

acclimated zebrafish of both genders were randomly placed into 16 glass tanks, assigned to control and fluorescent PS microplastics (30 mg/L) treatment groups. Each treatment had four replicates containing 10 zebrafish, with female and male fish exposed separately. After 21 days of exposure, fish were rinsed with ultra-pure water, euthanized using a 100 mg/L MS-222 (tricaine) solution, and the gut and liver tissues dissected for concentration analysis.

For the toxicity test, acclimated female and male zebrafish were exposed to 0 (control) and 30 mg/L PS microplastics separately for 21 days. Each group contained four replicate glass tanks: approximately 30 zebrafish were included in each tank randomly, and female and male fish were exposed separately. After exposure, the fish underwent a 24-hour fasting period before sampling and rinsing with ultra-pure water. Fish were euthanized using a 100 mg/L MS-222 (Tricaine) solution and the gut, liver and gonads dissected for further analysis.

2.3 Uptake and accumulation of PS microplastics in fish tissues

According to methods outlined by previous studies [18, 41, 42], the zebrafish tissues (the liver and gut) were homogenized and then digested in KOH (100g/L) at 60 ℃ for 24h. The fluorescence intensity of fish samples was assessed using a microplate reader (Tecan Inifinite M200). The content of microplastics in zebrafish tissues was calculated using a standard curve. The standard curve was obtained using serial concentration of fluorescent PS microplastic solutions.

2.4 Mucus determination

The skin mucus indicators were analyzed according to Wang et al. [43]. Briefly, 20

zebrafish from each treatment were anaesthetized, washed and transferred to a sterile polyethylene bag of 5 mL NaCl (100 mM). Fish were then gently rubbed back and forth on the bag for 2-3 minutes to collect the mucus. The collected samples were centrifuged (12000 rpm, 30 min, 4 ℃) and the supernatant was filtered using a 0.22 μm filter for further analysis. Immunoglobulin M (IgM) and lysozyme (LZM) of the mucus were measured using the Immunoglobulin M Assay Kit and lysozyme assay kit (Jiancheng, Nanjing, China) based on turbidity assays.

2.5 Histopathological analysis of gut

The entire gut tissues from three zebrafish in each treatment were selected and immersed in 4% paraformaldehyde at 4 ℃ for one day for fixation [20, 44]. Gut samples were dehydrated in ethanol of gradient concentrations before embedment in paraffin wax. Subsequently, samples were cut to 4 μm thick using Leica microtomes (RM2016) and stained with hematoxylin and eosin (H&E) and observed under microscopy.

2.6 Oxidative damage of zebrafish

To evaluate the degree of oxidative stress in zebrafish liver, superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were measured. Liver tissue homogenates (10%) were prepared with sterilized PBS at 4 ℃ and then centrifuged (12000 rpm, 10 min, 4 ℃) [45]. Commercial kits (superoxide dismutase assay kit and malondialdehyde assay kit) (Jiancheng, Nanjing, China) were used to measure the SOD activity and MDA content in collected supernatants.

2.7 16S rRNA gene sequencing analysis of gut microbiota

The gut samples were collected and kept at -80 ℃ before further analysis. The

details of extraction of genome DNA, amplicon generation, PCR products quantification and qualification, library preparation and sequencing, and data analysis are listed in SI. The sequencing data were deposited in the NCBI database (BioProject accession: PRJNA1116557).

2.8 Liver metabolomics

Liver samples were collected and kept at -80 ℃ before further analysis. For metabolomics analysis, samples were extracted and detected by LC-MS. Metabolite identification was conducted by MS-MS analysis under the same conditions of MS analysis. The metabolites were identified by accuracy mass (< 30 ppm) and MS/MS data which were matched with HMDB, massbank, LipidMaps, mzcloud and KEGG. P values $<$ 0.05 and variable importance projection (VIP) values $>$ 1 were considered statistically significant for metabolites. Differential metabolites were subjected to pathway analysis by MetaboAnalyst. The identified metabolites in metabolomics were then mapped to the KEGG pathway for biological interpretation of higher-level systemic functions. Details on sample extraction, LC-MS conditions and data analysis are provided in SI.

2.9 Stable isotope analysis

After freeze-drying (BIOSAFER-18A), zebrafish samples were milled for 1 minute (Retsch MM301) to obtain powder (< 100 μm). The stable isotope composition of samples was analyzed using isotope ratio mass spectrometry (IRMS, Delta V Advantage, USA). The abundance of ^{13}C and ^{15}N was calculated using Eq. (1):

$$\delta X\ (‰) = ((R_{sample} - R_{reference})/R_{reference} \times 1000‰ \quad (1)$$

where R_{sample} is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of the sample, and $R_{reference}$ is the $^{13}C/^{12}C$

or $^{15}N/^{14}N$ ratio of the reference.

2.10 Impact of PS microplastics on sex hormone levels and reproduction of zebrafish

Concentrations of 17β-estradiol (E2) and testosterone (T) were analyzed by using an estradiol 2 assay kit and a testosterone assay kit (Jiancheng, Nanjing, China), following the manufacturer's instructions. In brief, zebrafish blood was collected and centrifuged (3000 rpm, 5 min, 4 °C) and the supernatants used for hormone concentration analysis [46]. For reproduction, the spawning groups are ♀ck × ♂ck, ♀ck × ♂1μm, ♀1μm × ♂ck, ♀1μm × ♂1μm. The control check group is represented by ck. After removing spawning zebrafish, the eggs spawned in each group were collected using a Pasteur pipette and counted manually.

2.11 Statistical analysis

The data are presented as mean ± standard deviation. Mean differences between treated groups and controls were analyzed by one-way analysis of variance (ANOVA) using IBM SPSS Statistics 22, with a p-value of < 0.05 considered statistically significant.

3. Results and discussion

3.1 Effect of PS microplastics on the physiochemical properties of zebrafish

3.1.1 Accumulation of PS microplastics in zebrafish tissues and mucus analysis

PS microplastics accumulated in the gills and guts of fish (Fig. S1A). The concentration of PS microplastics in gills was higher than that in the gut for both genders. No significant difference was observed in IgM level and LZM activity of zebrafish mucus in both genders compared to controls (Fig. S1B and C).

193 Previous studies have shown that the gills and guts are important tissues for the
194 uptake and accumulation of microplastics, as they have a large surface area and are
195 involved in nutrient absorption and immune defense [47]. The accumulation
196 concentration in different tissues depends on various factors, such as exposure
197 concentration, time, and particle size of microplastics [48]. Once PS microplastics enter
198 the gut, there is the potential for them to result in adverse effects as well as translocation
199 to other tissues via the circulatory system [49]. As with our study, Yang et al. [15] also
200 found that PS microplastics did not influence LZM activity and IgM level in larval
201 zebrafish. This suggests no humoral immunotoxicity after exposure to PS microplastics
202 and that the toxicity of PS microplastics was related to ingestion into the gut instead of
203 interaction with skin mucus.
204 3.1.2 Hepatic oxidative stress and gut histopathology
205 The female fish treatment showed significantly increased SOD activity and MDA
206 content in the liver compared to the controls, while there was no such difference for
207 male zebrafish (Fig. S1D and E). There was no histological change in the gut of control
208 fish although the guts of zebrafish exposed to PS microplastics displayed obvious
209 damage, including vacuolization and cilia defects (Fig. S2).
210 The PS microplastic-induced oxidative stress in female zebrafish that we observed
211 is similar to that found in earlier studies [20, 50]. The increased SOD activity could
212 reflect severe membrane damage in the liver, while as the indicator of cell membrane
213 lipid peroxidation damage [51], increased MDA content might lead to ROS generation
214 and apoptosis of hepatocytes. Apart from oxidative stress, gut damage was also

observed. It has been previously noted that PS microplastic beads (15 μm) can result in vacuolization in the zebrafish gut [20] and Lei et al. [52] revealed that gut damage was related to physical uptake and accumulation rather than the chemical composition of microplastics.

3.2 Effects of PS microplastics on zebrafish gut microbiota composition and function

3.2.1 Gut microbiota composition

The Shannon and Simpson indices of gut microbiota increased significantly after exposure to PS microplastics in both genders (Table S2). There was also a large number of unique OTUs in female and male zebrafish when exposed to PS microplastics (Fig. 1A and B). The PCoA assessment (Fig. 1C and D) showed that the female group deviated from the control group significantly, whereas this was not the case in the male group. Results indicated that PS microplastics increased the alpha diversity and caused changes in the beta-diversity of gut fauna in both genders. The increased Shannon and Simpson diversity indices indicated that PS microplastics enhanced the evenness of the gut microbial community [53], indicating that exposed zebrafish were more vulnerable to the invasion and infection of opportunistic pathogens and the niche of dominant bacteria was also suppressed [54].

A significant change in the gut microbial composition was found at the phylum level. In female zebrafish, as shown in Fig. 1E and S3A, Fusobacteria were dominant (over 70%) in the control while Proteobacteria were most abundant (over 50%) in the 1μm PS microplastics treatment. The relative abundance of Proteobacteria, Firmicutes and Bacteroidota increased in the 1μm PS microplastics treatment compared to the

control group whereas the abundance of Fusobacteria decreased. In male zebrafish, the most abundant phylum was Proteobacteria (Fig. 1E and S3B) but the exposure to PS microplastics led to a lower relative abundance of Fusobacteria and Proteobacteria while the abundance of Actinobacteria and Firmicutes was greater. At genus level, results showed that, in female fish, PS microplastics decreased the content of *Cetobacterium* significantly, whereas the relative abundance of *Pseudomonas*, *Aeromonas* and *Vibrio* increased (Fig. S3C). Meanwhile, in the male fish gut, PS microplastics decreased the abundance of *Cetobacterium*, *Plesiomonas*, *Aeromonas* and *Vibrio* (Fig. S3D). Apart from phylum and genus level, in the female microplastics exposure group, the abundance of the classes Gammaproteobacteria and Clostridia, the order Pseudomonadales, Lachnospirales and Bacteroidales, and the families Pseudomonadaceae and Lachnospiraceae were higher compared to the control (Fig. S4). In the male microplastics exposure group, the abundance of the class Bacteroidia and the unidentified Actinobacteria, the orders Pseudomonadales, Bacteroidales and Burkholderiales, and the family Pseudomonadaceae were greater than in the male control group (Fig. S5).

Several studies have demonstrated that microplastics accumulation can alter gut microbiota composition in fish [20, 53, 55]. This is important because the dysbiosis of fish gut microbiota composition can lead to the development of many diseases in hosts, including obesity and diabetes [56]. At phylum level, previous research found that gut inflammation was related to microbiota disorder and inflammatory-induced oxidation could promote the growth of Proteobacteria, which are able to cope with this adverse

host environment [57]. In our study, gut inflammation, as observed by histopathological results, was accompanied by increased abundance of Proteobacteria in the female gut. As Bacteroidetes and Firmicutes are related to lean body weight and fat absorption [55, 58, 59], the variation of Bacteroidetes and Firmicutes abundance that we identified might influence the energy and lipid metabolism of zebrafish. At genus level, the decreased abundance of *Cetobacterium* (as a beneficial bacterium that can protect the host from pathogens [60]) and the enriched pathogens (including *Aeromonas* and *Pseudomonas* [61-63]) in female PS microplastic treatments suggested that female zebrafish gut function and health was influenced by PS microplastics.

3.2.2 Predicted function of gut microbiota

Changes in the gut microbiota of zebrafish were accompanied by significant changes in the predicted functions of the microbiota, particularly in females (Fig. 2 and S6-S8). At level 1, predicted pathways of gut microbiota including metabolism, genetic information processing and organismal systems showed under-representation. Conversely, pathways such as environmental information processing, cellular processes and human diseases showed significant enrichment in females in the treatments compared to the control. In the male group, only metabolism and environmental information processing were significantly affected by PS microplastics.

According to the level 2 and level 3 functional prediction map of the metabolic pathway (Fig. S6-S8), more metabolic pathways in female fish were significantly affected than those in male fish after PS microplastics exposure. In the female zebrafish group, the pathways related to nutrient transport and metabolism, as well as energy

production and conversion (including amino acid, carbohydrate, nucleotide, cofactors and vitamins, and energy metabolism) were significantly influenced by PS microplastics, indicating impacts on the basic metabolism of female zebrafish.

Huang et al. [57] revealed that energy imbalance might lead to reduced replication, recombination and repair pathways in the guppy gut when exposed to PS microplastics. The impact of PS microplastics on energy metabolism, along with reduced energy reserves caused by gut inflammation might lead to under-representation of replication and repair pathways in our study, and an imbalance of energy metabolism can affect growth and reproduction [64]. It has also been reported that gut microbiota can regulate sex hormone levels by interactions among their metabolites, the immune system, chronic inflammation, and the gut-brain-gonad axis [65]. Some microorganisms are involved in steroid production and estrogen metabolism [66]. In our study, the endocrine system pathways (glucagon signaling pathway, PPAR signaling pathway, insulin resistance, estrogen signaling pathway, adipocytokine signaling pathway, progesterone-mediated oocyte maturation) were under-represented in females, which might be related to sex steroid hormone disorder.

In the male zebrafish group, relatively few functions were influenced by PS microplastics. Enriched pathways included folding, sorting and degradation, and biosynthesis of other secondary metabolites, while under-represented pathways included membrane transport and cellular community-prokaryotes.

3.3 Metabolic alterations induced by PS microplastics

3.3.1 Liver metabolomics

In the female PS microplastics treatment, there were a total of 53 metabolites that were significantly different to those in the control. Among them, 8 were up-regulated and 45 were down-regulated (Fig. S9 and 3A). For the male treatment group, by contrast, fewer metabolites were influenced by PS microplastics; 11 metabolites were up-regulated and 1 metabolite was down-regulated (Fig.S9 and 3B).

KEGG metabolic pathway analysis was conducted to better understand the functions of these differential metabolites. As shown in Fig. 4, in the female groups of PS microplastics compared to the control, the main metabolic pathways of differential metabolites included the insulin signaling pathway, retinol metabolism, glycolysis/gluconeogenesis, progesterone-mediated oocyte maturation, oocyte meiosis, FoxO signaling pathway, and steroid hormone biosynthesis. In the male groups, the main metabolic pathways impacted by PS microplastics included D-arginine and D-ornithine metabolism, valine, leucine and isoleucine biosynthesis, glycine, serine and threonine metabolism, and steroid hormone biosynthesis.

Small molecule metabolites, including glucose, lipids, amino acids, peptides, and nucleic acids, are crucial indicators that reflect the physiological state and alterations in biosystems [67]. In this study, the metabolites belonging to carbohydrates (D-glucose, alpha-D-glucose and beta-D-fructose-6-phosphate) in the female PS microplastics treatment showed significant alteration. The altered metabolites were involved in the glycolysis/gluconeogenesis pathway, which is the primary pathway related to energy metabolism [9]. Meanwhile, according to KEGG pathway analysis, D-glucose is also involved in the FoxO signaling pathway and insulin signaling pathway, the latter of

which is associated with glycolipid metabolism and adipogenesis [68]. Several studies have found similar results to ours indicating that microplastics could affect glucose metabolism, glycolysis, lipid metabolism, and amino acid metabolism in fish [25, 69, 70].

Alterations in amounts of several lipid metabolites, such as prenol lipids (retinol, all-trans-retinoic acid, and 9-cis-retinoic acid), steroids (17a-estradiol, dihydrocortisol, 21-hydroxy-5b-pregnane-3,11,20-trione, and 11-dehydrocorticosterone), and fatty acyls (9,10-epoxyoctadecenoic acid), were also observed in the female PS microplastics treatment compared to the control. These metabolites are involved in lipid-related metabolic pathways, such as retinol metabolism and steroid hormone biosynthesis, suggesting the disordering of lipid metabolism. The affected lipid and retinol metabolic pathways could interfere with the energy supply for ovarian steroidogenesis, thereby affecting the growth and maturation of zebrafish oocytes [71]. Meanwhile, KEGG pathway analysis revealed that steroid differences between the PS microplastics treatments and the control involved those associated with hormone biosynthesis, and several pathways related to regulation of reproduction, including progesterone-mediated oocyte maturation and oocyte meiosis. Medrano et al. [72] found that polyester fiber could alter the regulation of pivotal sex hormones such as estrogen and androgen, which might be regulated by gut microbiota. In our study, the endocrine system pathway of gut microbiota was under-represented and pathways of liver metabolism related to reproduction were influenced in females, suggesting the connection between gut microbiota and liver metabolism leads to a decreased E2 level

and decreased egg production in female fish.

For male zebrafish treatments, the metabolites affected by PS microplastics, compared to those in the control, were mainly amino acids (such as L-leucine, D-ornithine, and L-cystathionine), suggesting PS microplastics had significant effects on amino acid metabolism, which is linked to basic metabolism, such as lipid and carbohydrate metabolism [73, 74]. Rehman et al. [75] also found that PS nanoplastics influenced amino acid metabolism of male zebrafish, indicating they affected the energy metabolism pathway.

3.3.2 Stable isotope analysis

Both female and male fish showed a significant decrease of $\delta^{13}C$ and $\delta^{15}N$ following PS microplastic exposure (Fig. 5A and B). Ouyang et al. [76] also found that PS microplastics reduced $\delta^{13}C$ of koi carp, which resulted from the alteration of nutrient absorption. $\delta^{13}C$ has been found to be related to energy metabolic or nutrient stress while $\delta^{15}N$ could be influenced by metabolic changes involving amino acids metabolism, protein synthesis and carbon turnover [26]. A few studies have shown an interrelationship between gut microbiota and metabolism in zebrafish after exposure to pollutants [77-79]. The change in gut microbiota after PS microplastic exposure might be related to altered metabolites in liver (mainly carbohydrates, amino acids, nucleic acid), which could contribute to the changes of $\delta^{13}C$ and $\delta^{15}N$.

3.4 Sex hormone level and egg production

As shown in Fig. 5C-E, for female zebrafish, the concentration of E2 and the E2/T ratio was significantly lower in the treatments compared to the controls, despite no

obvious difference in T concentration. Male zebrafish exhibited a significant decrease in T concentration under exposure to PS microplastics and an increased E2/T ratio. Egg production was lower in ♀1μm×♂ck treatments and ♀1μm×♂1μm treatments, whereas a slight but not significant decrease was found in ♀ck×♂1μm (Fig. 5F).

E2 and T, as gonadal steroids, can regulate the sexual functions of fish, including gametogenesis, gamete maturation and reproduction [80]. Decreased E2 levels in females could contribute to the reduction of vitellogenesis in fish ovaries [81] and inhibit VTG expression, which would have an adverse effect on reproductive processes, leading to decreased egg production [81, 82], which could be one of the reasons for the decreased egg production in females in our study. Similarly, Gupta et al. [83] demonstrated that PS microplastics decreased plasma E2 level in female zebrafish, contributing to delayed ovarian development. We found that PS microplastics hindered reproduction of female zebrafish by causing oxidative stress, apoptosis, and hormone level imbalance. Teng et al. [38] found that exposure of male and female zebrafish to charged PS microplastics disrupted the reproductive system, sex hormone balance and steroidogenic pathway. Mature female zebrafish should possess higher food consumption and metabolic costs when compared to males, thus any potential disruption of energy or metabolism is expected to result in more serious response in females than in males [57, 84].

3.5 Potential toxicity mechanisms of PS microplastics on metabolism and reproductive process in females

In zebrafish, various axes govern health regulation, such as the gut-liver axis,

391 hypothalamus-pituitary-gonadal-liver axis (HPGL axis), and gut-brain-gonad axis. The
392 gut-liver axis refers to the bidirectional relationship between the gut, gut microbiota,
393 and liver [85]. Dysbiosis of gut microbiota increases intestinal permeability, facilitating
394 the translocation of microbial-derived substances to the liver through the gut-liver axis,
395 leading to glycolipid metabolism disorder and liver damage [86]. Feng et al. [53]
396 reported that PS microplastics perturbed gut microbiota composition in medaka, along
397 with disrupted glycolipid and energy metabolism, leading to gut-liver axis disruption.
398 In our study, PS microplastics induced dysbiosis in gut microbiota, potentially further
399 disturbing lipid metabolism and glucose metabolism in the liver via the gut-liver axis
400 (Fig. 6). Evidence of oxidative stress in the liver further suggests liver damage.

401 The HPGL axis is important in the regulation of the reproductive system, regulating
402 the synthesis of steroid hormones [87]. Meanwhile, the gut-brain axis can exert
403 influence on the gonads via the endocrine pathway [88]. The gut microbiota can
404 modulate endocrine control of reproduction by impacting hormone levels, thereby
405 altering their bioavailability and efficacy [66]. In our study, disturbance of gut
406 microbiota induced underrepresentation of pathways associated with the endocrine
407 system. Significant alterations were found in the reproductive-related metabolic
408 pathways within the liver. Consequently, these results led to disruptions in sex hormone
409 levels and compromised egg production in the ovaries.

410 Microplastics could cause adverse impacts on fish health, including gut microbiota
411 dysbiosis, liver metabolism disorder and sex steroid hormone imbalance. However, the
412 effects observed in this study combined with the potential for long-term interaction of

413 microplastics with fish from natural environments, suggest that the gender-specific risk
414 of microplastics should not be overlooked. We found that female zebrafish health was
415 more affected than males through gut microbiota, metabolism and reproduction after
416 PS microplastics exposure. When females experience higher toxicity levels, it will lead
417 to adverse effects and impact the survival and reproductive processes of the organism.
418 Importantly, any impairment of reproductive function can have cascading effects on
419 population dynamics and the overall community structure [38, 39, 89]. Thus, our results
420 provide evidence that PS microplastics can induce gender-specific toxicity on
421 freshwater fish populations. Further research should pay increased attention to the
422 gender-specific toxicity of microplastics to organisms and communities in freshwater
423 environment.

424 3.6 Limitations and future work

425 Several limitations should be acknowledged with our study. First, in order to
426 investigate the toxicity mechanism of PS microplastics on zebrafish health, the
427 concentration of PS microplastics in our study is higher than typical environmental
428 concentrations in freshwater (though not higher than for total microplastic
429 concentrations [4]). However, we used high concentrations specifically to provide us
430 with insights into mechanistic responses. Future work should examine impacts at a
431 broader range of environmentally-relevant concentrations. Second, our findings suggest
432 that gut microbiota alterations of zebrafish possibly further influence liver metabolism
433 and reproductive processes. However, to derive more definitive and comprehensive
434 conclusions, further investigations into the intricacies of these axes are needed. Third,

while our results showed decreased sex hormone level and egg production of mother zebrafish, further studies should consider the cascading effects on offspring survival and development.

4. Conclusion

In summary, our results showed that the toxicity of PS microplastics was related to the ingestion of microplastics rather than the effects of non-specific skin defense. Female zebrafish were more affected than male zebrafish by PS microplastics. PS microplastics significantly influenced zebrafish metabolism, specifically lipid metabolism, carbohydrate metabolism, energy metabolism and regulation of reproduction. PS microplastics also destroyed the balance of sex hormones and disturbed the reproductive process in zebrafish, especially in females. The adverse impacts on reproduction are related to gut microbiota dysbiosis and metabolic disorder. The overall effects of PS microplastics on zebrafish metabolism and reproduction might influence individual fish health and whole population stability, which would further degrade freshwater ecosystems. The more severe impact of PS microplastics on females, compared to males, may lead to severe effects on zebrafish populations.

Acknowledgements

This study was supported by The Science and Technology Innovation Program of Jiangsu Province (BK20220036) and Primary Research& Development Plan of Jiangsu Province (BE2021706) and a joint University of Leeds – Nanjing University PhD studentship awarded to Di Wu.

References

[1] Y. Wang, B. Zhou, H. Chen, R. Yuan, F. Wang, Distribution, biological effects and biofilms of microplastics in freshwater systems - A review, Chemosphere, 299 (2022) 134370.

[2] S. Zhang, L. Zhang, P. Chen, N.R. Maddela, S. Li, Insights into the membrane fouling aggravation under polyethylene terephthalate microplastics contamination: From a biochemical point of view, J Clean Prod, 424 (2023).

[3] J. Xu, X. Wang, Z. Zhang, Z. Yan, Y. Zhang, Effects of chronic exposure to different sizes and polymers of microplastics on the characteristics of activated sludge, Sci Total Environ, 783 (2021) 146954.

[4] C. Li, R. Busquets, L.C. Campos, Assessment of microplastics in freshwater systems: A review, Sci Total Environ, 707 (2020) 135578.

[5] A.K. Mishra, J. Singh, P.P. Mishra, Microplastics in freshwater ecosystem: A serious threat for freshwater environment, Int J Environ Sci Te, 20 (2022) 9189-9204.

[6] K. Bexeitova, A. Baimenov, E.A. Varol, K. Kudaibergenov, U. Zhantikeyev, Y. Sailaukhanuly, K. Toshtay, Z. Tauanov, S. Azat, R. Berndtsson, Microplastics in freshwater systems: A review of classification, sources, and environmental impacts, Adv Chem Eng, 20 (2024).

[7] B. Parker, D. Andreou, I.D. Green, J.R. Britton, Microplastics in freshwater fishes: Occurrence, impacts and future perspectives, Fish Fish (Oxf), 22 (2021) 467-488.

[8] J. Chen, C. Rao, R. Yuan, D. Sun, S. Guo, L. Li, S. Yang, D. Qian, R. Lu, X. Cao, Long-term exposure to polyethylene microplastics and glyphosate interferes with the behavior, intestinal microbial homeostasis, and metabolites of the common carp

(*Cyprinus carpio* L.), Sci Total Environ, 814 (2022) 152681.

[9] C. Wang, M. Hou, K. Shang, H. Wang, J. Wang, Microplastics (polystyrene) exposure induces metabolic changes in the liver of rare minnow (*Gobiocypris rarus*), Molecules, 27 (2022).

[10] C.W. Mak, K. Ching-Fong Yeung, K.M. Chan, Acute toxic effects of polyethylene microplastic on adult zebrafish, Ecotoxicol Environ Saf, 182 (2019) 109442.

[11] J. Bhagat, L. Zang, N. Nishimura, Y. Shimada, Zebrafish: An emerging model to study microplastic and nanoplastic toxicity, Sci Total Environ, 728 (2020) 138707.

[12] J.N. Huang, Y. Zhang, L. Xu, K.X. He, B. Wen, P.W. Yang, J.Y. Ding, J.Z. Li, H.C. Ma, J.Z. Gao, Z.Z. Chen, Microplastics: A tissue-specific threat to microbial community and biomarkers of discus fish (*Symphysodon aequifasciatus*), J Hazard Mater, 424 (2022) 127751.

[13] S. Karbalaei, P. Hanachi, G. Rafiee, P. Seifori, T.R. Walker, Toxicity of polystyrene microplastics on juvenile *Oncorhynchus mykiss* (rainbow trout) after individual and combined exposure with chlorpyrifos, J Hazard Mater, 403 (2021) 123980.

[14] D. Montenegro, C. Astudillo-Garcia, T. Hickey, G. Lear, A non-invasive method to monitor marine pollution from bacterial DNA present in fish skin mucus, Environ Pollut, 263 (2020) 114438.

[15] H. Yang, H. Lai, J. Huang, L. Sun, J.A. Mennigen, Q. Wang, Y. Liu, Y. Jin, W. Tu, Polystyrene microplastics decrease F-53B bioaccumulation but induce inflammatory stress in larval zebrafish, Chemosphere, 255 (2020) 127040.

[16] J.H. Kim, Y.B. Yu, J.H. Choi, Toxic effects on bioaccumulation, hematological

parameters, oxidative stress, immune responses and neurotoxicity in fish exposed to microplastics: A review, J Hazard Mater, 413 (2021) 125423.

[17] M.S. Bhuyan, Effects of microplastics on fish and in human health, Front Environ Sci, 10 (2022).

[18] Y. Lu, Y. Zhang, Y. Deng, W. Jiang, Y. Zhao, J. Geng, L. Ding, H. Ren, Uptake and accumulation of polystyrene microplastics in zebrafish (*Danio rerio*) and toxic effects in liver, Environ Sci Technol, 50 (2016) 4054-4060.

[19] Y. Li, G. Yang, J. Wang, L. Lu, X. Li, Y. Zheng, Z. Zhang, S. Ru, Microplastics increase the accumulation of phenanthrene in the ovaries of marine medaka (*Oryzias melastigma*) and its transgenerational toxicity, J Hazard Mater, 424 (2022) 127754.

[20] R. Qiao, Y. Deng, S. Zhang, M.B. Wolosker, Q. Zhu, H. Ren, Y. Zhang, Accumulation of different shapes of microplastics initiates intestinal injury and gut microbiota dysbiosis in the gut of zebrafish, Chemosphere, 236 (2019) 124334.

[21] M. Ganz, T. Csak, B. Nath, G. Szabo, Lipopolysaccharide induces and activates the Nalp3 inflammasome in the liver, World J Gastroenterol, 17 (2011) 4772-4778.

[22] C. Zhang, M. Zhang, S. Wang, R. Han, Y. Cao, W. Hua, Y. Mao, X. Zhang, X. Pang, C. Wei, G. Zhao, Y. Chen, L. Zhao, Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice, ISME J, 4 (2010) 232-241.

[23] T. Luo, C. Wang, Z. Pan, C. Jin, Z. Fu, Y. Jin, Maternal polystyrene microplastic exposure during gestation and lactation altered metabolic homeostasis in the dams and their F1 and F2 offspring, Environ Sci Technol, 53 (2019) 10978-10992.

[24] A. Castegna, R. Gissi, A. Menga, M. Montopoli, M. Favia, A. Viola, M. Canton, Pharmacological targets of metabolism in disease: Opportunities from macrophages, Pharmacol Ther, 210 (2020) 107521.

[25] G. Ye, X. Zhang, X. Liu, X. Liao, H. Zhang, C. Yan, Y. Lin, Q. Huang, Polystyrene microplastics induce metabolic disturbances in marine medaka (*Oryzias melastigmas*) liver, Sci Total Environ, 782 (2021).

[26] C. Ek, A.M. Karlson, S. Hansson, A. Garbaras, E. Gorokhova, Stable isotope composition in Daphnia is modulated by growth, temperature, and toxic exposure: implications for trophic magnification factor assessment, Environ Sci Technol, 49 (2015) 6934-6942.

[27] C. Ek, Z. Gerdes, A. Garbaras, M. Adolfsson-Erici, E. Gorokhova, Growth retardation and altered isotope composition as delayed effects of PCB exposure in *Daphnia magna*, Environ Sci Technol, 50 (2016) 8296-8304.

[28] A.M.L. Karlson, M. Reutgard, A. Garbaras, E. Gorokhova, Isotopic niche reflects stress-induced variability in physiological status, R Soc Open Sci, 5 (2018) 171398.

[29] L. Qiang, J. Cheng, Exposure to polystyrene microplastics impairs gonads of zebrafish (*Danio rerio*), Chemosphere, 263 (2021) 128161.

[30] P. Gupta, A. Mahapatra, A. Suman, S.S. Ray, G. Malafaia, R.K. Singh, Polystyrene microplastics disrupt female reproductive health and fertility via sirt1 modulation in zebrafish (*Danio rerio*), J Hazard Mater, 460 (2023) 132359.

[31] P. Xiao, X. Liu, H. Zhang, W. Li, Chronic toxic effects of isoflucypram on reproduction and intestinal energy metabolism in zebrafish (*Danio rerio*), Environ

Pollut, 315 (2022) 120479.

[32] Z. Sun, B. Wu, J. Yi, H. Yu, J. He, F. Teng, T. Xi, J. Zhao, J. Ruan, P. Xu, R. Tao, L. Jia, H. Ji, Impacts of environmental concentrations of nanoplastics on zebrafish neurobehavior and reproductive toxicity, Toxics, 12 (2024).

[33] B. Sulaiman, J.C. Woodward, H.A. Shiels, Riverine microplastics and their interaction with freshwater fish, Water Biology and Security, 2 (2023).

[34] S. El Kholy, Y. Al Naggar, Exposure to polystyrene microplastic beads causes sex-specific toxic effects in the model insect *Drosophila melanogaster*, Sci Rep, 13 (2023) 204.

[35] Y. Lee, D.S. Yoon, Y.H. Lee, J.I. Kwak, Y.J. An, J.S. Lee, J.C. Park, Combined exposure to microplastics and zinc produces sex-specific responses in the water flea *Daphnia magna*, J Hazard Mater, 420 (2021) 126652.

[36] X. Yang, J. Jiang, Q. Wang, J. Duan, N. Chen, D. Wu, Y. Xia, Gender difference in hepatic AMPK pathway activated lipid metabolism induced by aged polystyrene microplastics exposure, Ecotoxicol Environ Saf, 245 (2022) 114105.

[37] W. Shen, M. Zhao, W. Xu, X. Shi, F. Ren, P. Tu, N. Gao, J. Shan, B. Gao, Sex-specific effects of polystyrene microplastic and lead(II) co-exposure on the gut microbiome and fecal metabolome in C57BL/6 mice, Metabolites, 14 (2024).

[38] M. Teng, X. Zhao, L. Zhao, D. Shi, Y. Li, J. Sun, W. Zhao, L. Zhou, X. Wang, J.P. Giesy, F. Wu, Zebrafish (*Danio rerio*) reproduction is affected by life-cycle exposure to differently charged polystyrene nanoplastics with sex-specific responses, ACS ES&T Water, 2 (2022) 2558-2566.

[39] J. Wang, Y. Li, L. Lu, M. Zheng, X. Zhang, H. Tian, W. Wang, S. Ru, Polystyrene microplastics cause tissue damages, sex-specific reproductive disruption and transgenerational effects in marine medaka (*Oryzias melastigma*), Environ Pollut, 254 (2019) 113024.

[40] H. Wang, J. Zhu, Y. He, J. Wang, N. Zeng, X. Zhan, Photoaging process and mechanism of four commonly commercial microplastics, J Hazard Mater, 451 (2023) 131151.

[41] A. Dehaut, A.L. Cassone, L. Frere, L. Hermabessiere, C. Himber, E. Rinnert, G. Riviere, C. Lambert, P. Soudant, A. Huvet, G. Duflos, I. Paul-Pont, Microplastics in seafood: Benchmark protocol for their extraction and characterization, Environ Pollut, 215 (2016) 223-233.

[42] Y. Zou, Q. Xu, G. Zhang, F. Li, F. Li, Influence of six digestion methods on the determination of polystyrene microplastics in organisms using the fluorescence intensity, Huan jing ke xue, 40 (2019) 496-503.

[43] H. Wang, W. Tang, R. Zhang, S. Ding, Analysis of enzyme activity, antibacterial activity, antiparasitic activity and physico-chemical stability of skin mucus derived from *Amphiprion clarkii*, Fish Shellfish Immunol, 86 (2019) 653-661.

[44] Y. Zhao, R. Qiao, S. Zhang, G. Wang, Metabolomic profiling reveals the intestinal toxicity of different length of microplastic fibers on zebrafish (*Danio rerio*), J Hazard Mater, 403 (2021) 123663.

[45] D. Li, Z. Li, C. Qiu, B. Peng, Y. Zhang, H. Sun, S. Wang, 2-Amino-3-methylimidazo[4,5-f]quinoline induced oxidative stress and inflammation via

589 TLR4/MAPK and TLR4/NF-κB signaling pathway in zebrafish (*Danio rerio*) livers,
590 Food and Chem Toxicol, 157 (2021) 112583.
591 [46] G.L. Pedroso, T.O. Hammes, T.D. Escobar, L.B. Fracasso, L.F. Forgiarini, T.R. da
592 Silveira, Blood collection for biochemical analysis in adult zebrafish, J Vis Exp, (2012)
593 e3865.
594 [47] A. Batel, F. Borchert, H. Reinwald, L. Erdinger, T. Braunbeck, Microplastic
595 accumulation patterns and transfer of benzo[a]pyrene to adult zebrafish (*Danio rerio*)
596 gills and zebrafish embryos, Environ Pollut, 235 (2018) 918-930.
597 [48] J. Ding, S. Zhang, R.M. Razanajatovo, H. Zou, W. Zhu, Accumulation, tissue
598 distribution, and biochemical effects of polystyrene microplastics in the freshwater fish
599 red tilapia (*Oreochromis niloticus*), Environ Pollut, 238 (2018) 1-9.
600 [49] A.B. Mark, D. Awantha, S.G. Tamara, M.L. David, C.T. Richard, Ingested
601 microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis*
602 (L.), Environ Sci Technol, 42 (2008) 5026-5031.
603 [50] C.B. Jeong, H.M. Kang, M.C. Lee, D.H. Kim, J. Han, D.S. Hwang, S. Souissi, S.J.
604 Lee, K.H. Shin, H.G. Park, J.S. Lee, Adverse effects of microplastics and oxidative
605 stress-induced MAPK/Nrf2 pathway-mediated defense mechanisms in the marine
606 copepod *Paracyclopina nana*, Sci Rep, 7 (2017) 41323.
607 [51] M. Uchiyama, M. Mihara, Determination of malonaldehyde precursor in tissues
608 by thiobarbituric acid test, Anal Biochem, 86 (1978) 271-278.
609 [52] L. Lei, S. Wu, S. Lu, M. Liu, Y. Song, Z. Fu, H. Shi, K.M. Raley-Susman, D. He,
610 Microplastic particles cause intestinal damage and other adverse effects in zebrafish

611 *Danio rerio* and nematode *Caenorhabditis elegans*, Sci Total Environ, 619-620 (2018)
612 1-8.
613 [53] S. Feng, Y. Zeng, Z. Cai, J. Wu, L.L. Chan, J. Zhu, J. Zhou, Polystyrene
614 microplastics alter the intestinal microbiota function and the hepatic metabolism status
615 in marine medaka (*Oryzias melastigma*), Sci Total Environ, 759 (2021) 143558.
616 [54] C. Hu, Y. Bai, B. Sun, L. Tang, L. Chen, Significant impairment of intestinal health
617 in zebrafish after subchronic exposure to methylparaben, Sci Total Environ, 838 (2022)
618 156389.
619 [55] Z. Wan, C. Wang, J. Zhou, M. Shen, X. Wang, Z. Fu, Y. Jin, Effects of polystyrene
620 microplastics on the composition of the microbiome and metabolism in larval zebrafish,
621 Chemosphere, 217 (2019) 646-658.
622 [56] S.M. Snedeker, A.G. Hay, Do interactions between gut ecology and environmental
623 chemicals contribute to obesity and diabetes?, Environ Health Perspect, 120 (2012)
624 332-339.
625 [57] J.N. Huang, B. Wen, J.G. Zhu, Y.S. Zhang, J.Z. Gao, Z.Z. Chen, Exposure to
626 microplastics impairs digestive performance, stimulates immune response and induces
627 microbiota dysbiosis in the gut of juvenile guppy (*Poecilia reticulata*), Sci Total
628 Environ, 733 (2020) 138929.
629 [58] Y. Sheng, H. Ren, S.M. Limbu, Y. Sun, F. Qiao, W. Zhai, Z.Y. Du, M. Zhang, The
630 presence or absence of intestinal microbiota affects lipid deposition and related genes
631 expression in zebrafish (*Danio rerio*), Front Microbiol, 9 (2018) 1124.
632 [59] P.J. Turnbaugh, V.K. Ridaura, J.J. Faith, F.E. Rey, R. Knight, J.I. Gordon, The

633 effect of diet on the human gut microbiome: a metagenomic analysis in humanized
634 gnotobiotic mice, Sci Transl Med, 1 (2009) 6ra14.
635 [60] H. Gu, Y. Feng, Y. Zhang, D. Yin, Z. Yang, W. Tang, Differential study of the
636 *Parabramis pekinensis* intestinal microbiota according to different habitats and
637 different parts of the intestine, Ann Microbiol, 71 (2021).
638 [61] J.M. Janda, S.L. Abbott, The genus Aeromonas: taxonomy, pathogenicity, and
639 infection, Clin Microbiol Rev, 23 (2010) 35-73.
640 [62] H. Chandrarathna, C. Nikapitiya, S.H.S. Dananjaya, C.U.B. Wijerathne, S.
641 Wimalasena, H.J. Kwun, G.J. Heo, J. Lee, M. De Zoysa, Outcome of co-infection with
642 opportunistic and multidrug resistant Aeromonas hydrophila and A. veronii in zebrafish:
643 Identification, characterization, pathogenicity and immune responses, Fish Shellfish
644 Immunol, 80 (2018) 573-581.
645 [63] Oliver, A., High frequency of hypermutable *Pseudomonas aeruginosa* in cystic
646 fibrosis lung infection, Science, 288 (2000) 1251-1253.
647 [64] A.J. Watts, M.A. Urbina, S. Corr, C. Lewis, T.S. Galloway, ingestion of plastic
648 microfibers by the crab *Carcinus maenas* and its effect on food consumption and energy
649 balance, Environ Sci Technol, 49 (2015) 14597-14604.
650 [65] S. He, H. Li, Z. Yu, F. Zhang, S. Liang, H. Liu, H. Chen, M. Lu, The Gut
651 Microbiome and sex hormone-related diseases, Front Microbiol, 12 (2021) 711137.
652 [66] R. Haque, Das, II, P.B. Sawant, N.K. Chadha, L. Sahoo, R. Kumar, J.K. Sundaray,
653 Tenets in microbial rndocrinology: A new vista in teleost reproduction, Front Physiol,
654 13 (2022) 871045.

655 [67] W. Lin, K. Ouyang, Y. He, H. Yang, Y. Kuang, D. Li, L. Li, Combined effects of
656 microcystin-LR and rice straw-derived biochar on the hepatic antioxidant capacity of
657 zebrafish: Insights from LC-MS/MS-based metabolomics analysis, Sci Total Environ,
658 904 (2023) 166830.
659 [68] H. Guo, R. Khan, S.H. Abbas Raza, S.M. Suhail, H. Khan, S.B. Khan, A.H. Abd
660 El-Aziz, L. Zan, RNA-Seq reveals function of Bta-miR-149-5p in the regulation of
661 bovine adipocyte differentiation, Animals (Basel), 11 (2021).
662 [69] Y. Zhao, Z. Qin, Z. Huang, Z. Bao, T. Luo, Y. Jin, Effects of polyethylene
663 microplastics on the microbiome and metabolism in larval zebrafish, Environ Pollut,
664 282 (2021) 117039.
665 [70] B. Zhu, X. Chen, T. Zhang, Q. Zhang, K. Fu, J. Hua, M. Zhang, Q. Qi, B. Zhao,
666 M. Zhao, L. Yang, B. Zhou, Interactions between intestinal microbiota and metabolites
667 in zebrafish larvae exposed to polystyrene nanoplastics: Implications for intestinal
668 health and glycolipid metabolism, J Hazard Mater, 472 (2024) 134478.
669 [71] N. Fang, C. Zhang, H. Hu, Y. Li, X. Wang, X. Zhao, J. Jiang, Histology and
670 metabonomics reveal the toxic effects of kresoxim-methyl on adult zebrafish,
671 Chemosphere, 309 (2022) 136739.
672 [72] C.A. Medriano, S. Bae, Acute exposure to microplastics induces metabolic
673 disturbances and gut dysbiosis in adult zebrafish (*Danio rerio*), Ecotox Environ Safe,
674 245 (2022) 114125.
675 [73] X. Bai, J. Jia, Q. Kang, Y. Fu, Y. Zhou, Y. Zhong, C. Zhang, M. Li, Integrated
676 metabolomics and lipidomics analysis reveal remodeling of lipid metabolism and

677 amino acid metabolism in glucagon receptor-deficient zebrafish, Front Cell Dev Biol,
678 8 (2020) 605979.
679 [74] L. Rui, Energy metabolism in the liver, Compr Physiol, 4 (2014) 177-197.
680 [75] A. Rehman, F. Huang, Z. Zhang, T. Habumugisha, C. Yan, U. Shaheen, X. Zhang,
681 Nanoplastic contamination: Impact on zebrafish liver metabolism and implications for
682 aquatic environmental health, Environ Int, 187 (2024) 108713.
683 [76] M.Y. Ouyang, J.H. Liu, B. Wen, J.N. Huang, X.S. Feng, J.Z. Gao, Z.Z. Chen,
684 Ecological stoichiometric and stable isotopic responses to microplastics are modified
685 by food conditions in koi carp, J Hazard Mater, 404 (2021) 124121.
686 [77] R. Qiao, C. Sheng, Y. Lu, Y. Zhang, H. Ren, B. Lemos, Microplastics induce
687 intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome
688 in zebrafish, Sci Total Environ, 662 (2019) 246-253.
689 [78] J. Jiang, L. Chen, S. Wu, L. Lv, X. Liu, Q. Wang, X. Zhao, Effects of
690 difenoconazole on hepatotoxicity, lipid metabolism and gut microbiota in zebrafish
691 (*Danio rerio*), Environ Pollut, 265 (2020) 114844.
692 [79] J. Gu, Y. Zhu, M. Guo, X. Yin, M. Liang, X. Lou, J. Chen, L. Zhou, D. Fan, L. Shi,
693 G. Hu, G. Ji, The potential mechanism of BPF-induced neurotoxicity in adult zebrafish:
694 Correlation between untargeted metabolomics and gut microbiota, Sci Total Environ,
695 839 (2022) 156221.
696 [80] K. Ji, X. Liu, S. Lee, S. Kang, Y. Kho, J.P. Giesy, K. Choi, Effects of non-steroidal
697 anti-inflammatory drugs on hormones and genes of the hypothalamic-pituitary-gonad
698 axis, and reproduction of zebrafish, J Hazard Mater, 254-255 (2013) 242-251.

699 [81] L. Qian, S. Qi, J. Zhang, M. Duan, D. Schlenk, J. Jiang, C. Wang, Exposure to
700 boscalid induces reproductive toxicity of zebrafish by gender-specific alterations in
701 steroidogenesis, Environ Sci Technol, 54 (2020) 14275-14287.
702 [82] B.J. Reading, C.V. Sullivan, The reproductive organs and processes | Vitellogenesis
703 in Fishes, in: Encyclopedia of Fish Physiology, 2011, pp. 635-646.
704 [83] P. Gupta, A. Mahapatra, A. Suman, S.S. Ray, G. Malafaia, R.K. Singh, Polystyrene
705 microplastics disrupt female reproductive health and fertility via sirt1 modulation in
706 zebrafish (*Danio rerio*), J Hazard Mater, 460 (2023) 132359.
707 [84] N. Kurchaba, B.J. Cassone, C. Northam, B.F. Ardelli, C.M.R. LeMoine, Effects of
708 MP polyethylene microparticles on microbiome and inflammatory response of larval
709 zebrafish, Toxics, 8 (2020).
710 [85] A. Albillos, A. de Gottardi, M. Rescigno, The gut-liver axis in liver disease:
711 Pathophysiological basis for therapy, J Hepatol, 72 (2020) 558-577.
712 [86] Q. Liu, B. Wang, S. Wang, H. Jing, S. Xu, Carbon black nanoparticles exposure
713 induces intestinal flora dysbiosis and consequent activation of gut-liver axis leading to
714 liver lipid accumulation in zebrafish, Research Square, (2021).
715 [87] M. Teng, X. Chen, C. Wang, M. Song, J. Zhang, S. Bi, C. Wang, Life cycle
716 exposure to propiconazole reduces fecundity by disrupting the steroidogenic pathway
717 and altering DNA methylation in zebrafish (*Danio rerio*), Environ Int, 135 (2020)
718 105384.
719 [88] H. Wang, X. Liu, C. Zhao, J. Yan, Z. Wang, R.A. Dahlgren, Q. Qian, X. Wang,
720 Interference of gut-brain-gonad axis originating from triclocarban exposure to parent

721 zebrafish induces offspring embryonic development abnormality by up-regulation of
722 maternal circSGOL1, Aquat Toxicol, 266 (2024) 106782.
723 [89] S. Vannuccini, V.L. Clifton, I.S. Fraser, H.S. Taylor, H. Critchley, L.C. Giudice, F.
724 Petraglia, Infertility and reproductive disorders: impact of hormonal and inflammatory
725 mechanisms on pregnancy outcome, Hum Reprod Update, 22 (2016) 104-115.
726

Figure captions

Figure 1. Assessment of difference or similarity of microbiota composition among different treatment groups after exposure to1μm PS microplastics for 21 days. Venn diagram of bacterial OTUs unique within the gut microbiota of zebrafish after exposed to PS microplastics. A, female groups; B, male groups. Principal coordinate analysis (PCoA) of the gut microbiota beta-diversity of the control and PS microplastics treated groups, C, female groups; D, male groups. E, the chord diagram of gut microbiota abundance at phylum level after PS microplastics exposure.

Figure 2. Welch's t-test analysis of predicted function (level 1) of zebrafish gut microbiota in female groups by Tax4Fun analysis after exposure to1μm PS microplastics for 21 days. A, female groups; B, male groups.

Figure 3. Heatmap of the differential metabolites in zebrafish liver after exposure to1μm PS microplastics for 21 days. A, female groups; B male groups.

Figure 4. KEGG pathway enrichment ($p < 0.05$) in zebrafish liver after exposure to 1μm PS microplastics for 21 days. A, female groups; B male groups.

Figure 5. Stable isotope analysis, sex hormone level and egg production of zebrafish after exposure to1μm PS microplastics for 21 days. Ratios of $\delta^{13}C$ (A) and $\delta^{15}N$ (B) of zebrafish (n=8). E2 (C), T (D), and E2/T ratio (E) of zebrafish (n=4). Egg production of paired zebrafish (F, n=4), ck represents control check group. Data represent mean ± SD. Different letters indicate significant differences between treatments ($p < 0.05$), the lowercase letters represent significant difference in female groups and uppercase letters represent those in male groups.

Figure 6. Potential toxicity mechanisms of PS microplastics on gut microbiota, liver metabolism, and reproductive processes in females after exposure to1μm PS microplastics for 21 days.