Acta Oceanol. Sin., 2024, Vol. 43, No. 8, P. 104–110
https://doi.org/10.1007/s13131-024-2289-8
http://www.aosocean.com
E-mail: ocean2@hyxb.org.cn

Dietary exposure to sulfamethazine, nanoplastics and their binary mixture disrupts the spermatogenesis of marine medaka (*Oryzias melastigma*)

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Received 29 August 2023; accepted 8 November 2023

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Abstract

In the coastal environment, the co-occurrence of antibiotic and nanoplastic pollution is common. Investigating their individual and combined toxicity to marine organisms is of great necessity. In the present study, the reproductive toxicity of sulfamethazine (SMZ) and nanoplastics (polystyrene, PS) via the dietary route on the spermatogenesis of marine medaka (*Oryzias melastigma*) was examined. After 30 d of dietary exposure, SMZ alone decreased the gonadosomatic index (GSI) value (~35%) and the proportion of undifferentiated type A spermatogonia (A_{und}) (~40%), probably by disrupting the testicular sex hormone production, the spermatogenesis-related growth factor network and the balance of apoptosis. Individual exposure to PS did not affect the GSI value or the proportions of germ cells at different developmental stages, but dysregulated the expression of several spermatogenesis-related genes. Interestingly, the presence of PS alleviated the decreased GSI value caused by SMZ. This alleviation effect was achieved by enhancing the spermatogonia differentiation instead of reversing the suppressed self-renewal of A_{und}, suggesting that the mixture of PS and SMZ could cause reproductive effects in a different way. These findings expand our knowledge of threats of ubiquitous antibiotic and nanoplastic pollution to fish reproduction and population.

Key words: nanoplastics, antibiotics, spermatogenesis, combined toxicity, *Oryzias melastigma*

Citation: Zhang Yuting, Chen Ruanni, Chen Zhiqiang, Fu Xiaoyu, Wu Ziyi, Chen Jinwan, Xie Lingtian, Zong Humin, Mu Jingli. 2024. Dietary exposure to sulfamethazine, nanoplastics and their binary mixture disrupts the spermatogenesis of marine medaka (*Oryzias melastigma*). Acta Oceanologica Sinica, 43(8): 104–110, doi: 10.1007/s13131-024-2289-8

1 Introduction

The co-occurrence of two or more pollutants is common in aquatic environments, especially in areas with high human activity. Certain environmental pollutants can easily interact, causing unexpected combined toxicity to aquatic organisms. For example, nanoplastics decrease the developmental toxicity caused by polycyclic aromatic hydrocarbons (PAHs) (Trevisan et al., 2019), but enhance the cardiac toxicity induced by DDT in zebrafish larvae (Varshney et al., 2023). Therefore, to fully assess the ecological risk of an environmental pollutant, it is crucial not only to clarify the individual effects, but also to understand its interaction and combined effects with concomitant pollutants.

Plastics have been used intensively by human society for more than 50 years, and the consequent environmental problems caused by plastics have received much attention in recent years. Marine ecosystems are severely suffering from plastic pollution, as about 10% of annual plastic production ends up in the oceans (Avio et al., 2015). Once entering the oceans, plastic debris keeps releasing microplastics (smaller than 5 mm) and nanoplastics (smaller than 1 μm) under environmental weathering (Andrady, 2011). The reported environmental concentrations of microplastics and nanoplastics are up to 10 000 mg/L in marine surface waters (Allen et al., 2022). A recent study shows that the average concentration of nanoplastics for Antarctic sea ice is 52.3 ng/ml (Materić et al., 2022), indicating a high prevalence of nanoplastics in the oceans. Aquatic organisms can ingest microplastics and nanoplastics passively and actively (Li et al., 2021). The ingested microplastics and nanoplastics can be transferred

Foundation item: The National Natural Science Foundation of China under contract No. 42106119; the Department of Science and Technology of Fujian Province under contract Nos 2022J02052, 2020J05175 and 2020J05178; the Fujian Provincial Department of Ocean and Fisheries under contract No. FJHJF-L-2022-12; the Yancheng Fishery High Quality Development Project under contract No. YCSCYJ2021023.

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to and accumulated in many organs, leading to various adverse effects such as shortened lifespan, aging acceleration, metabolic disorders, neural dysfunctions, etc (Xiang et al., 2022; Abdolahpur Monikh et al., 2023; Xiao et al., 2023). Additionally, microplastics and nanoplastics with large surface/volume ratios and hydrophobicity have a high adsorption capacity for other pollutants, resulting in combined toxicity (Wang et al., 2016).

As another widespread anthropogenic pollutant, antibiotics have been frequently detected in the water ranging from ng/L to μg/L. Sulfamethazine (SMZ) is one of the most commonly used sulfonamide antibiotics. The environmental concentration of SMZ can be over 1 000 ng/L in some aquatic environments, higher than many other sulfonamide antibiotics (Zhao et al., 2016; Bu et al., 2013; Ji et al., 2012). For instance, the maximum concentration of SMZ in the Huangpu River is 623.27 ng/L, much higher than that of sulfapyridine (57.39 ng/L), sulfadiazine (40.55 ng/L), sulfamethoxazole (55.24 ng/L), sulfachlororyridazine (58.29 ng/L), oxytetracycline (37.17 ng/L), chlortetracycline (16.80 ng/L), florfenicol (46.63 ng/L), etc (Jiang et al., 2011). Exposure to SMZ inhibits microalgae growth, hampers crustacean reproduction, and causes developmental malformation in fish embryos (De Liguoro et al., 2009; Yan et al., 2018). Furthermore, SMZ is easily adsorbed on different types of microplastics and nanoplastics (including PS, PE, PET, PP, etc.) with partition coefficient K_d values ranging from 15.1 L/kg to 38.7 L/kg (Guo et al., 2019), higher than that of other antibiotics, such as sulfadiazine-microplastics (ranging from 6.61 L/kg to 7.85 L/kg) and trimethoprim-microplastics (ranging from 8.38 L/kg to 17.1 L/kg) (Li et al., 2018), implying that environmental SMZ and nanoplastics are likely to interact and cause potential combined toxicity. In aquatic environments, pollutants can reach and affect aquatic animals through either a waterborne or dietary exposure route, and differences in toxicity have been documented after exposure to the same pollutant (Geens et al., 2012; Wang, 2013; Xie et al., 2010). Nanoplastics and SMZ are reported to be highly bioaccumulated in aquatic animals (Rist et al., 2017; Zhao et al., 2016), suggesting that their toxicity via the dietary exposure route (trophic transfer) could be dominant. Given that the co-occurrence of nanoplastics and SMZ is common in coastal environments, clarifying their combined toxicity, especially via the dietary exposure route, in marine animals is of great importance.

Reproductive success ensures the continuity of fish species. Changes in spermatogenesis or oogenesis can be the main drivers of alterations in fish population growth (Segner, 2011). In male fish, spermatogenesis, in which spermatogonia proliferate and differentiate to form mature spermatozoa, is a highly coordinated and organized process (Schulz et al., 2010). First, undifferentiated type A spermatogonia (A_{und}), which have the potential for self-renewal and differentiation, differentiate into differentiated type A spermatogonia (A_{diff}) with reduced potential for self-renewal. Then A_{diff} irreversibly divide into type B spermatogonia. After the final mitosis, type B spermatogonia give rise to spermatocytes which then enter meiosis and differentiate into spermatids. At last, spermatids undergo a final differentiation period and become functional spermatozoa. Disorders in spermatogenesis would lead to low fertility in males and consequently affect fish populations. Therefore, exploring the toxicity of nanoplastics and SMZ in fish spermatogenesis is of necessity when evaluating their potential threat to ecological structures and functions.

Marine medaka (*Oryzias melastigma*) is a useful marine model fish in toxicology studies, as its generation time is short (2–3 months), the breeding and culturing technique is well developed, and the whole genome information is available (Gao et al., 2018; Kim et al., 2018; Zheng et al., 2024). In this study, we exposed marine medaka (*O. melastigma*) to diet-borne SMZ and nanoplastics, individually and in combination, for 30 d. The effects on spermatogenesis (including the gonadosomatic index, proportions of germ cells at different developmental stages, and expression of spermatogenesis-related genes) were investigated. The results demonstrated that dietary exposure to SMZ reduced the GSI value and the self-renewal of A_{und} in the male *O. melastigma*. The presence of PS alleviated the SMZ-induced low GSI value by enhancing the spermatogonia differentiation instead of reversing the suppressed self-renewal of A_{und}. Our findings would help better understand the toxicity of antibiotics and nanoplastics in fish and assess their potential ecological risk in marine ecosystems.

2 Materials and methods

2.1 *Chemicals and test organisms*

Sulfamethazine (SMZ) and square polystyrene (PS) fragments with a side length of 100 nm were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Xi'an Ruixi Biological Technology Co. Ltd. (Shanxi, China), respectively.

Oryzias melastigma was cultured in glass aquaria with a recirculatory system. Automatic photoperiod controllers were used to keep the fish in 16 h light: 8 h darkness. The pH, salinity, and temperature of seawater were set at (8.0 ± 0.1)‰, (30 ± 1)‰ and (24 ± 2)℃, respectively. The fish were fed *Artemia salina* twice a day.

2.2 *Experimental design*

The preparation of SMZ and PS-enriched diet was described in our previous study (Zhang et al., 2021b). First, the working solutions of SMZ and PS were mixed with dry commercial feed pellets. Then the wet mixture was freeze dried in a drier for 2 d for a long storage life. For the control feed, double-distilled water (the solvent for nanoplastics and SMZ) was used to mix with dry commercial feed pellets. The exposure experiment consisted of 5 groups: Control (fed with the control feed); SMZ at a low concentration (0.28 mg/g dry feed, L-SMZ); SMZ at a high concentration (4.62 mg/g dry feed, H-SMZ); PS (3.45 mg/g dry feed) and a mixture group (4.62 mg/g SMZ and 3.45 mg/g PS, SMZ + PS). Measurement of SMZ and PS in the diet was previously described (Zhang et al., 2021b). Briefly, the measurement of SMZ was performed on Agilent 1290 ultra-high performance liquid chromatography coupled with Agilent 6400 Series triple quadrupole mass spectrometer (Agilent Technologies, California, USA) using sulfamerazine as internal standard. Fluorescent PS was adopted to measure the realistic concentration of PS. The fluorescent intensity in the diet was determined using the Nanodrop 3300. The choice of SMZ concentration was selected according to the legal doses in aquaculture (1–10 mg/g in feed) in China, the United States and Italy (Lalumera et al., 2004; Limbu et al., 2018) and recent toxicological studies using the dietary exposure route (Zhou et al., 2018; Ming et al., 2020). Nanoplastics can be highly bioaccumulated in lower trophic animals (up to ~10 mg/g body weight in *Daphnia magna*) (Rist et al., 2017). The concentration of nanoplastics in the diet was set at 3.45 mg/g. During exposure, adult fish (with a male-to-female ratio of 1:1) were fed twice a day. The feces were removed, and the medium in tanks was renewed every 2 d. The whole exposure experiment lasted for 30 d. After exposure, the fish were anesthetized on ice and then dissected for further analysis. The gonadosomatic index (GSI) was calculated as GSI (%) = [gonad weight (g)/total body weight (g)] × 100%.

2.3 *Histological analysis*

The testes were dissected and fixed in 4% paraformaldehyde for 12 h and then transferred to 70% ethanol. After being dehydrated in ethanol (70%–100%), the testes were embedded in the paraplast. The embedded testes were sectioned into 5 μm- thick slides using a retracting microtome. After deparaffinization and rehydration, the sections were stained with hematoxylin.

2.4 *Real-time PCR*

Real-time PCR was performed according to our previous method (Zhang et al., 2021a, 2021b). To obtain the relative expression level of interest genes, the delta-delta CT method was used (Schmittgen and Livak, 2008). The expression of genes involved in spermatogenesis (nanos homolog 2, *nanos2*; piwi-like protein 1, *piwil1*; deleted in azoospermia-like, *dazl*; synaptonemal complex protein 3, *sycp3*; outer dense fiber of sperm tails 3b, *odf3b*; septin 7, *sept7*; steroidogenic acute regulatory protein, *star* and *17β*-hydroxysteroid dehydrogenase, *17β-hsd*; cytochrome P450 family 19 subfamily A, *cyp19a*; androgen receptor α, *arα* and androgen receptor *β*, *arβ*; anti-Müllerian hormone, *amh*; gonadal soma derived factor, *gsdf*; insulin-like growth factor 3, *igf3*; insulin-like peptide 3, *insl3* and wnt family member 5A, *wnt5a*), apoptosis (caspase 3a, *cas3*; caspase 3b, *cas3b*; caspase 8, *cas8* and caspase 9, *cas9*) and vitellogenesis (vitellogenin-1, *vtg1*; vitellogenin-2, *vtg2*; choriogenin-H, *chgh* and choriogenin-L, *chgl*) were measured and normalized to the internal control gene *β-actin,* which is widely used in toxicological study of marine medaka (Zhang et al., 2021a, 2021b).

2.5 *Statistical analyses*

To evaluate the statistical differences, either Student's *t*-test (control vs. PS; H-SMZ vs. H-SMZ + PS) or one-way analysis of variance (ANOVA) analysis test followed by Fisher's least significant difference (LSD) post hoc test (control, L-SMZ and H-SMZ) was used (SPSS, IBM, Chicago, USA). Normality and homogeneity of variance of data were tested using Shapiro-Wilk and Bartlett's tests, prior to ANOVA analyses.

3 Results

In female, no significant differences were observed in the GSI value, fertilization rate, embryo hatching rate, or hepic *vtg1*, *vtg2*, *chgh* and *chgl* transcripts between all treatments after 30 d of exposure (Fig. S1).

3.1 *Gonadosomatic index*

A significant decrease in GSI was observed in the male fish from the L-SMZ and H-SMZ groups (32.3% and 36.5%, respectively) relative to the control males (Fig. 1). The GSI of the male fish from the H-SMZ + PS group was close to that of the control male fish and 42.6% higher (p = 0.007 4) than that of the H-SMZ group.

3.2 *Histopathological morphology*

Compared to the control, no clear histopathological changes were found in the testes from the L-SMZ, H-SMZ, PS or H-SMZ + PS groups (Fig. 2). Germ cells at different stages, i.e., undifferentiated type A spermatogonia (A_{und}), differentiated type A spermatogonia (A_{diff}), type B spermatogonia (B), spermatocytes (SC) and spermatids (ST), could be clearly identified and the structure of the cysts was intact and normal in all the five groups.

3.3 *Proportions of germ cells at different developmental stages*

Compared to the Control, the *nanos2* (a marker gene for A_{und}) transcripts in the H-SMZ group were significantly decreased by 39.4% (p = 0.048 1) (Fig. 3a). Relative to the H-SMZ group, a slight but insignificant (p = 0.119 6) increase of *nanos2* transcripts was found in the H-SMZ + PS group. The *dazl* (a marker gene for type B spermatogonia) and *sycp3* (a marker gene for SC) transcripts were respectively elevated by 52.1% (p = 0.000 5) and 22.3% (p = 0.046 9) in the male fish from the H-SMZ + PS group compared to the H-SMZ group (Figs 3b and c).

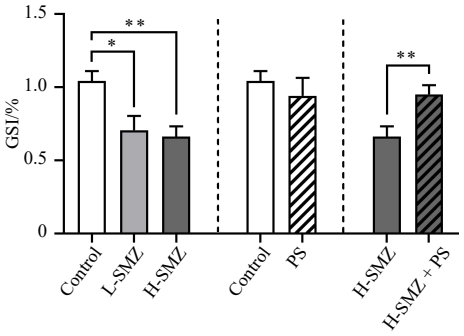

Fig. 1. Gonadosomatic indexes (GSI) in the sulfamethazine (SMZ) and nano polystyrene (PS) exposed male *O. melastigma*. Asterisks indicated significant differences (*0.01 < p < 0.05, ** $p \leqslant$ 0.01, $n \geqslant 5$).

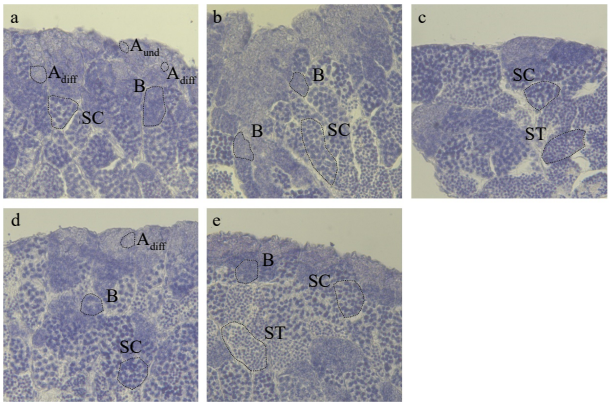

Fig. 2. Paraffin sections of testis from the Control (a), L-SMZ (b), H-SMZ (c), PS (d) and H-SMZ + PS (e) groups. No significant effects of PS, SMZ or their binary mixture on the morphological structure of testis were observed. A_{und}, undifferentiated type A spermatogonia; A_{diff}, differentiated type A spermatogonia; B, type B spermatogonia; SC, spermatocyte; ST, spermatids.

3.4 *Transcriptional expression of spermatogenesis related genes*

For the sex hormones production and biological functions related genes, their transcriptional expression was generally down-regulated by individual exposure to SMZ (Fig. 4). On the contrary, the expression of *star* was obviously upregulated in the PS alone group and the H-SMZ + PS group. In terms of the growth factors involved in spermatogenesis, the expression of *amh* and *gsdf* was down-regulated, while that of *igf3* was up-regulated in the four treatments. Individual exposure to H-SMZ dramatically down-regulated the expression of *insl3*. Interestingly, the expression of *insl3* was not dysregulated by PS alone, but the presence of PS slightly alleviated the inhibition caused by SMZ. For the apoptosis related genes, in general, exposure to SMZ and/or PS showed a promoting effect.

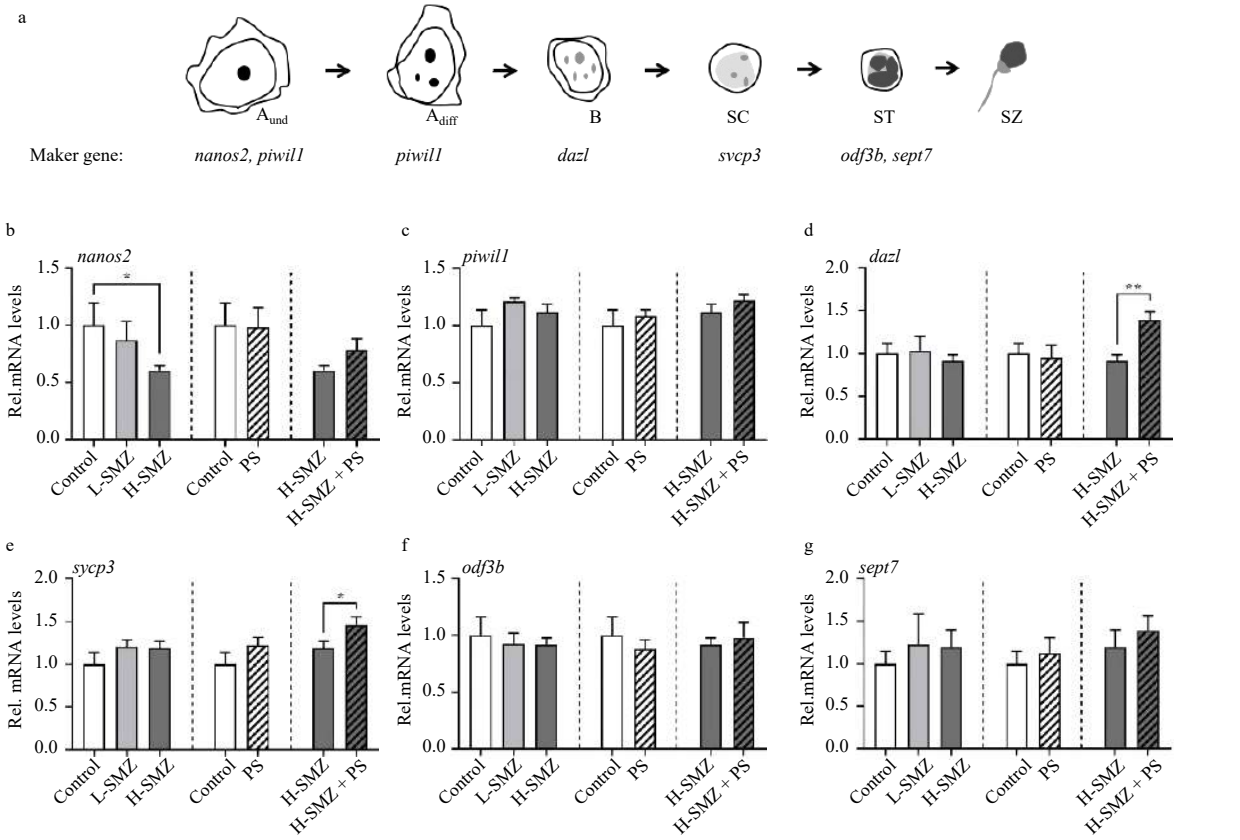

Fig. 3. Relative expression of germ cell marker genes in the sulfamethazine (SMZ) and nano polystyrene (PS) exposed male *O. melastigma*. Representation of spermatogenesis from type A undifferentiated spermatogonia to spermatozoa was modified from Schulz et al., (2010) (a). The transcriptional expression levels of *nanos2* (b), *piwil1* (c), *dazl* (d), *sycp3* (e), *odf3b* (f) and *sept7* (g) were measured. Asterisks indicated significant differences (*$0.01 < p < 0.05$, ** $p \leqslant 0.01$, $n \geqslant 5$). A_{und}, undifferentiated type A spermatogonia; A_{diff}, differentiated type A spermatogonia; B, type B spermatogonia; SC, spermatocyte; ST, spermatids; SZ, spermatozoa.

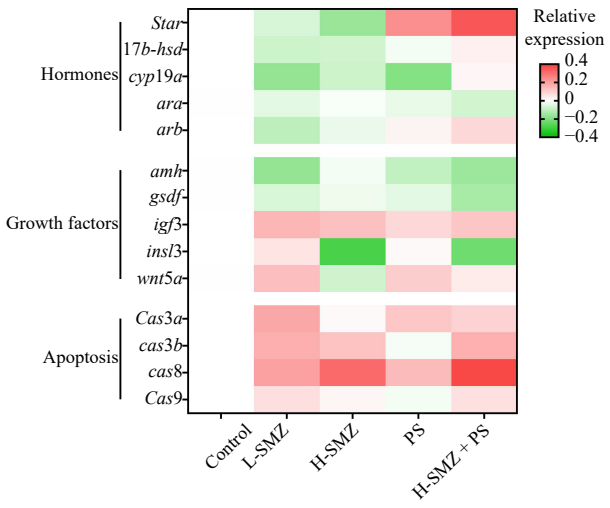

Fig. 4. Relative expression of spermatogenesis related genes in the sulfamethazine (SMZ) and nano polystyrene (PS) exposed male *O. melastigma*. The values of four treatment groups were normalized to the control which was set to 0. Color bar from red to green represented the fold change from increasing to decreasing ($n \geqslant 5$).

4 Discussion

In this study, dietary exposure to SMZ caused obvious reproductive toxicity in the male *O. melastigma*, while no clear effect on egg production or hepatic vitellogenesis-related genes was observed in the female fish. It is reported that after exposure to 40 μg/L SMZ for 24 h, the bioconcentration factor (BCF) of SMZ in the testis (~6) is much higher than in the ovary (~27) (Zhao et al., 2016). Besides, exposure to SMZ causes different effects on the gut microbiota communities between the male and female *O. melastigma* (Zhang et al., 2021b; Zhao et al., 2016). Gut microbiota can interact with host's estrogen, androgens, insulin, and other hormones via microbial products and consequently affect host reproduction (Qi et al., 2021). For example, microbially secreted β-glucuronidase can metabolize host estrogens from their conjugate forms to their deconjugated forms (Plottel and Blaser, 2011). These results suggest that the gender-specific reproductive toxicity of SMZ in *O. melastigma* may be due to the different gonadal SMZ levels and the difference in reconstructed gut microbiota communities (as well as microbial products) between female and male fish. Similarly, a recent study shows that exposure to β-diketone antibiotics induces gender-specific reproductive toxicity in zebrafish. GSI values in male zebrafish are significantly decreased under 6.25 mg/L β-diketone antibiotics treatment, while GSI values in females are decreased only under higher concentration treatment (25 mg/L) (Wang et al., 2017). It seems that male fish are more vulnerable and are faced with a higher risk of SMZ pollution.

Exposure to SMZ downregulated the expression of the A_{und} maker gene *nanos2* in *O. melastigma*, suggesting that the self-re-

newal and proportion of A_{und} was suppressed. Since A_{und} is the primary source of subsequent differentiated germ cells (Schulz et al., 2010), the decreased amount of A_{und} could lead to a decrease in the number of all types of germ cells (including functional spermatozoa), which is in line with the observation of a decrease in GSI after SMZ exposure. The mechanism underlying the suppression of self-renewal and proportion of A_{und} by SMZ was investigated. A down-regulated expression of *star* (mediates the rate-limiting step in steroid biosynthesis) and *cyp19a* (converts androgens into estrogens) was observed after SMZ exposure, indicating a decrease in the 17β-estradiol (E2) level. In teleost, E2 plays an important role in spermatogonia renewal (Schulz et al., 2010). For instance, 10 pg/ml of E2 was sufficient to induce spermatogonial renewal divisions in cultured testicular tissue of Japanese eel (Miura et al., 1999), and a low dose of E2 promotes spermatogonial renewal in Japanese medaka (Song and Gutzeit, 2003). Therefore, the observed SMZ-induced suppression of spermatogonial renewal could be partially due to the declined E2 level (Manna et al., 2016). Besides of steroid hormones, growth factors participate the complex regulatory network to maintain the balance between self-renewal and differentiation of A_{und}. *Igf3* (produced by Sertoli cells) and *Insl3* (produced by Leydig cells) are reported to promote spermatogonia differentiation in teleost (Crespo et al., 2016; Nóbrega et al., 2015), while *Amh*, *Gsdf*, and *Wnt5a* are associated with spermatogonia self-renewal (Schulz et al., 2010; Crespo et al., 2020). In this study, L-SMZ downregulated *amh* and *gsdf* transcripts but upregulated *igf3*, *insl3*, and *wnt5a* transcripts, indicating that the balance of self-renewal/differentiation was shifted to differentiation. On the contrary, H-SMZ downregulated not only the self-renewal related genes (i.e., *amh*, *gsdf* and *wnt5a*) but also the differentiation promoting gene *insl3*, which implies a suppression in differentiation and less differentiated germ cells. This might account for the observed lower GSI in the H-SMZ group than that in the L-SMZ group. During spermatogenesis, there is a requirement of germ cell death by apoptosis to maintain normal germ cell development and to achieve a normal sperm output (Hikim and Swerdloff, 1999; Almeida et al., 2013). After SMZ exposure, increases in the expression of caspases in the testes were observed, suggesting that this balance was disrupted and abnormal cell apoptosis occurs. Taken together, the SMZ-induced decreases in the proportion of A_{und} and the GSI value in *O. melastigma* might be achieved via disrupting sex hormone production, growth factor network and the balance of apoptosis in the testes.

Different to SMZ, individual exposure to PS did not cause obvious effects on the GSI value or the proportions of germ cells at different developmental stages in the male *O. melastigma*. Interestingly, the presence of PS reversed the SMZ-induced decrease in GSI to the normal level. Recently, a growing body of evidence demonstrates that microplastics/nanoplastics could alleviate the toxicity of other environmental pollutants via interaction. For instance, polycyclic aromatic hydrocarbons (PAHs) are sorbing to the surface of the Nano-PS, decreasing the concentration, uptake, and developmental toxicity of free PAHs in the zebrafish embryos (Trevisan et al., 2019). Similarly, our previous study also shows that the mixture of SMZ and PS caused more modest effects on the gut microbiota and intestinal antioxidant physiology than the SMZ alone in *O. melastigma* (Zhang et al., 2021b). Importantly, in this study, the observed alleviation effect of PS was not achieved simply by reducing the free SMZ molecules. The decreased number of A_{und} by SMZ was unaltered in the H-SMZ + PS group. Instead, the levels of *star*, *17β-hsd*, *dazl* and *sycp3* transcripts were enhanced, suggesting that the production of sex hormones and the spermatogonial differentiation might be promoted, which compensates the decrease of GSI. Apparently, additional research is needed for a mechanistic understanding of this alleviation effect (e.g., the translocation of the PS and SMZ complex and the potential role of gut microbiota).

5 Conclusions

This research demonstrates that dietary exposure to SMZ reduces the GSI value and the self-renewal of A_{und} via disrupting the testicular sex hormone production, the growth factor network and the balance of apoptosis in the male *O. melastigma*. Individual exposure to PS does not affect the proportions of germ cells at different developmental stages or the GSI value, but dysregulates the expression of several spermatogenesis related genes. There is no simple antagonism between PS and SMZ regarding to the individual toxicity in the testes. Interestingly, the presence of PS alleviates the decreased GSI value by SMZ. The alleviation effect is achieved via enhancing spermatogonia differentiation instead of reversing the suppressed self-renewal of A_{und}, suggesting that the mixture of PS and SMZ could cause reproductive effects in a different way. Our findings expand our understanding of the ecological risk of antibiotics, nanoplastics, and their mixture to fish populations.

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Supplementary information:

Figure S1. Effects of PS, SMZ or their binary mixture on the reproduction of female marine medaka. The GSI value ($n \geqslant 6$) (A), egg production ($n = 3$) (B), fertilization rate ($n = 3$) (C), hatching rate ($n = 3$) (D), and transcriptional expression levels of *vtg1* (E), *vtg2* (F), *chgh* (G) and *chgl* in the liver ($n \geqslant 6$) (H) are measured. One-way ANOVA analysis followed by Fisher's least significant difference (LSD) post hoc test were conducted among the control, L-SMZ and H-SMZ groups. Two independent Student's *t*-test analyses were conducted (Control vs. PS; H-SMZ vs. H-SMZ+PS). No significant difference was observed.

The supplementary information is available online at https://doi.org/10.1007/s13131-024-2289-8 and http://www.aosocean.com/. The supplementary information is published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.