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Original Article

Chronic exposure to polystyrene microplastics disrupts reproductive functions and hormonal balance in Zebrafish, *Danio rerio*: A dose- and time-dependent study

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Abstract: Microplastic pollution is a growing global concern with potential adverse effects on aquatic ecosystems and organisms. Although many studies have documented the presence of microplastics in aquatic environments, information on their long-term impacts on aquatic life remains limited. The current study aimed to investigate the effects of polystyrene microplastics on reproductive, hormonal, and molecular indices in zebrafish (*Danio rerio*) over 21- and 60-day periods. Zebrafish were exposed to various concentrations of polystyrene microplastics (0 (control), 20, 200, and 2000 µg/L). Several parameters were evaluated, including absolute and relative fecundity, gonadosomatic index, oocyte diameter, sex hormone levels (T and E2), cyp19a gene expression, and fertilization, hatching, and larval survival rates. The results indicated that as concentration and exposure duration increased, microplastic accumulation in fish bodies also increased. During the 60-day period, significant decreases were observed in relative fertility, gonadosomatic index, and oocyte diameter. Levels of sex hormones and cyp19a gene expression decreased significantly, indicating a disruption of the reproductive axis. In addition, fertilization, hatching, and larval survival rates were significantly reduced in high-concentration treatments, especially in the long term. Polystyrene microplastics can significantly impair zebrafish reproductive performance by disrupting physiological, hormonal, and genetic processes. These findings emphasize the need to investigate and manage plastic pollutants in aquatic ecosystems to prevent their long-term consequences on aquatic populations.

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Introduction

Aquatic ecosystems are often exposed to pollutants from multiple sources, including industrial waste, agricultural effluents, and urban sewage. Among these, plastics have emerged as one of the most concerning pollutants, attracting increasing attention over the past century (Derraik, 2002; Andrady, 2011). Plastics have become a global environmental concern because their increasing production and improper disposal contribute to widespread ecological damage (Thompson, 2006). These materials degrade into smaller particles, known as microplastics, through exposure to UV radiation, mechanical erosion, and biodegradation (Andrady, 2011; Peters and Bratton, 2016). The term microplastic was first used in 2004 to describe plastic particles smaller than 50 µm in the water column and sediments (Thompson et al., 2004). When these plastic particles are released into aquatic ecosystems, due to the slowness of the degradation process, they remain in aquatic environments for long periods, thereby posing a serious threat to environmental health and ecological security (Moore, 2008; Hopewell et al., 2009), and due to their size, similar to the size of sediments and several planktonic species, they are available to a wide range of aquatic organisms, including fish (Baldwin et al., 2016; Dai et al., 2018).

Aquatic organisms can be exposed to microplastics through ingestion, inhalation, and dermal absorption, resulting in various physical and chemical effects (Garrido et al., 2019; Prokić et al., 2019). Numerous studies show that aquatic organisms, including Copepods, Nematodes, Shrimps, Crabs, Oysters, and Fish, ingest plastic particles, which accumulate in vital

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tissues such as the liver, muscle, and brain. These particles can suppress the immune system and lead to numerous side effects, such as death, reduced feeding activity, inhibited growth and development, endocrine disorders, energy disorders, oxidative stress, immunodeficiency, and even genetic disorders (Besseling et al., 2015; Galloway, 2015; Peters and Bratton, 2016; Ding et al., 2018; Foley et al., 2018). The accumulation of these particles in aquatic organisms' tissues, combined with the widespread consumption of seafood, has raised concerns that humans may be exposed to these substances directly or indirectly through aquatic animals (Jovanovic, 2017).

Microplastics can disrupt the hypothalamic-pituitary-gonadal (HPG) axis by reducing the transcription of gonadotropin-releasing hormone (GnRH), thereby interfering with steroidogenesis and gametogenesis. This disruption alters the expression of reproductive genes and steroid hormones, ultimately affecting reproduction in different ways (Rochman et al., 2014; Sun et al., 2015). Previous studies have reported that microplastics reduce the expression of genes involved in reproduction, thereby decreasing steroid hormone levels (Wang et al., 2019; Hanachi et al., 2021). In addition, microplastic exposure can induce oxidative stress, leading to adverse effects on fish growth and reproduction, as well as significant impacts on fish embryo development (Prokić et al., 2019). Embryonic development stage is a critical phase in the life cycle of fish. Even minor alterations during early embryogenesis can lead to significant changes across various tissues and organs, potentially causing developmental disorders and reducing hatching success and survival rates (Scholz et al., 2008; Luís et al., 2015).

Polystyrene microplastics were utilized in this study as they are among the most commonly produced plastic polymers and serve as model particles in biological studies of microorganisms. They are widely used in aquaculture and fishing activities and, due to their widespread presence in aquatic ecosystems and potential adverse effects on aquatic organisms, have become an important environmental concern (Andrady and Neal, 2009; Besseling et al., 2015). Polystyrene particles are found in high concentrations in marine and river environments. Because their density is similar to that of water, they disperse evenly throughout the water column, enhancing interactions between microplastics and aquatic organisms (Sadri and Thompson, 2014).

Understanding the effects of polystyrene microplastics on zebrafish (*Danio rerio*) at different life stages is crucial for assessing their implications for aquatic biodiversity and ecosystem health. This fish is used because of its short reproductive cycle, high adult fertility, the ability of both males and females to produce large numbers of gametes, and the transparency of its embryos and larvae. Additionally, this species has been proposed as a model for rapid analysis of gene function and the biological activities of organic molecules, and its genome has been fully sequenced (Zon and Peterson, 2005; Howe et al., 2013). Most studies have focused on the short-term effects of microplastics on fish, with limited information on the long-term impacts of exposure to these particles. The interaction between microplastics and biological systems, particularly in fish, has raised concerns regarding the long-term effects of these particles on aquatic life cycles.

Therefore, the aim of this study is to investigate the short-term and long-term (21- and 60-day periods) effects of exposure to polystyrene microplastics on reproductive, hormonal, and molecular indices in zebrafish. This study examines various aspects of polystyrene microplastics' impact on zebrafish and clarifies their consequences. Our objective is to fill the knowledge gap regarding the potential impacts of microplastics to better understand their ecological consequences in aquatic environments.

Materials and Methods

Preparation of microplastics: For this study, polystyrene pellets supplied by Tabriz Petrochemical Company were used. To prepare microplastics, the polystyrene pellets were frozen in liquid nitrogen and then ground into powder using an industrial grinder.

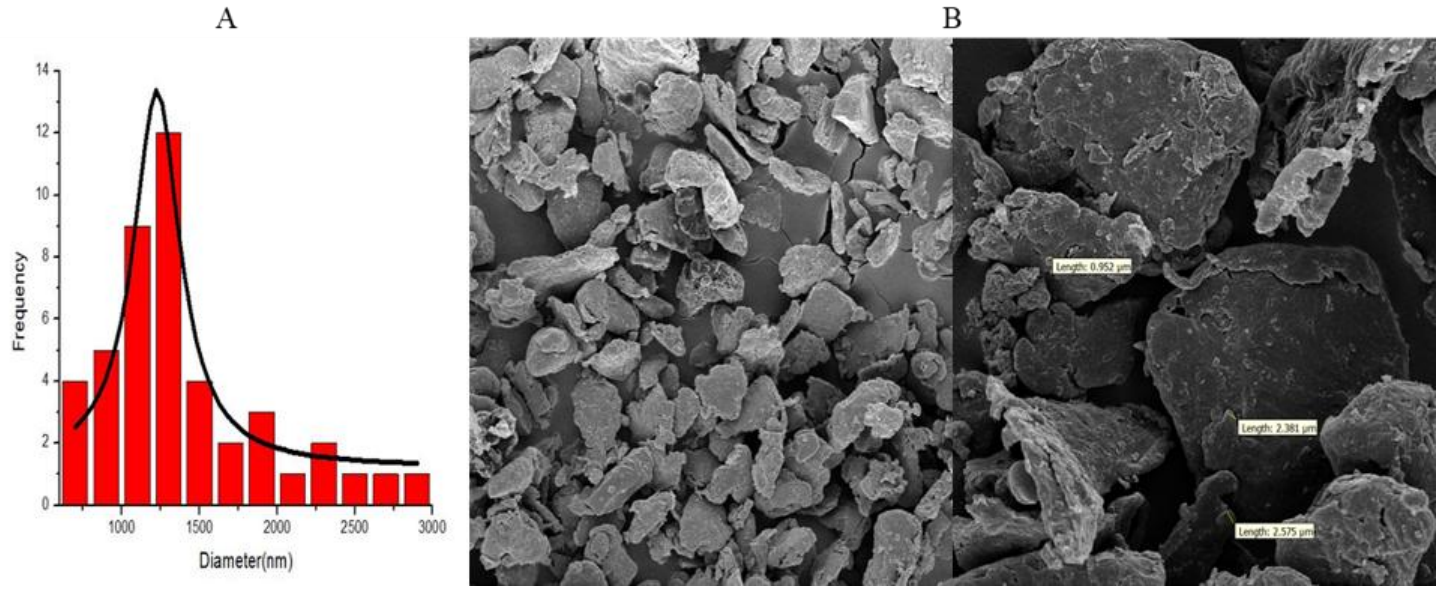

Figure 1. (A) The size distribution histogram of the particles, and (B) scanning electron microscope (SEM) image of polystyrene microplastics used in this study.

The resulting powder was sieved to obtain particles smaller than 10 µm (Fig. 1). Finally, the microplastic suspension was prepared in distilled water and sonicated in an ultrasonic water bath (60 W/L, 30 kHz) for 60 minutes before use.

Experiment design: A total of 480 adult zebrafish (0.38±0.018 g; 3:2 ratio of females and males) were obtained from an ornamental fish distribution center in Karaj, Iran. They were then transferred to the Faculty of Natural Resources at the University of Tehran in Karaj, Iran, and adapted to laboratory conditions for two weeks. Following the adaptation period, the fish were divided into two groups based on the duration of exposure. Each group was further divided into four treatment groups, with three replicates per treatment (n = 60 per treatment). The first group was exposed for 21 days, and the second group for 60 days, to different concentrations of polystyrene microplastics: 0 µg/L (control), 20 µg/L (T1), 200 µg/L (T2), and 2000 µg/L (T3) (Wang et al., 2019; Qiang and Cheng, 2021). During the exposure periods, the fish were maintained in 10-liter aquaria with continuous aeration provided by an air compressor. They were fed twice daily with commercial pelleted fish food at approximately 3% of their biomass. To maintain water quality, 100% water renewal was performed every 48 hours, and microplastics were replenished accordingly. Fecal matter was removed daily by siphoning, and any dead fish were recorded and removed from the tanks. The experiment was conducted under controlled physicochemical conditions: water temperature of 26±1°C, pH of 6.8–7.0, dissolved oxygen at 6.5 mg/L, and a 12:12 h light/dark photoperiod.

Sampling: At the end of each period, 5 fish (female = 3, male = 2, per replicate) were transferred to spawning aquariums, and the rest of the fish were anesthetized in an ice water bath to measure the amount of microplastic accumulation in the body, absolute fecundity, relative fecundity, gonadosomatic index (GSI), oocyte diameter, sex hormones and examination of cyp19a gene expression.

Microplastics concentration in water and fish bodies: On days 21 and 60, water samples (100 mL) were collected from each aquarium 12 hours after water renewal and the addition of microplastics. The collected water was filtered through a 0.45 µm membrane filter (Millipore), and retained particles were rinsed with deionized water. The number of microplastics was then quantified under a light microscope. Additionally, 100 mL of water was sampled from aquaria containing microplastics but without fish to determine the initial microplastic concentration.

To quantify microplastic concentrations in fish (n = 6 per treatment), individuals were anesthetized and dried in an incubator at 70°C. Once dried, the samples were powdered and transferred into 10 mL glass centrifuge tubes. Approximately 4 mL of H_2O_2 was added to each tube, and the mixture was incubated at 65°C for 24 hours to digest the organic matter (Li et al., 2015). Subsequently, 5 mL of saturated NaI solution (1.6 g/cm³) was added to facilitate the formation of a microplastic suspension. The supernatant was filtered through 5 µm filter paper, and the retained particles were rinsed with deionized water. The number of microplastics was then counted under a light microscope.

Primary reproductive indicators: To evaluate absolute fecundity, relative fecundity, and Gonadosomatic Index (GSI), the fish (n = 9 per treatment) were randomly selected and weighed after anesthesia. Then their gonads were carefully dissected and weighed. After weighing the gonads to facilitate counting, the eggs were placed in 1 mL of Phosphate-Buffered Saline, counted under the stereomicroscope, and calculated using the following formulas (Biswas, 1993).

GSI = (gonad weight (g) / body weight (g) × 100

Absolute fecundity = number of eggs

Relative fecundity = number of eggs/body weight (g)

To measure oocyte diameter, 30 oocytes were randomly selected from each female fish and placed on a graduated slide. Using a light microscope, the diameter of each oocyte was measured.

Steroid hormones (Testosterone and 17β-Estradiol): For the measurement of steroid hormones, including testosterone (T) and 17β-estradiol (E2), 6 fish were randomly selected from each treatment group (2 per replicate). Following anesthesia, the fish were immediately frozen and stored at −80°C until the experiment. For sample preparation, whole-body homogenates were prepared by homogenizing the fish in ice-cold phosphate buffer (0.1 M; pH 7.4) at a 1:9 (w/v) ratio. The homogenates were centrifuged at 12,000 rpm for 15 minutes at 4°C, and the resulting supernatants were collected for hormone analysis (Bartoskova et al., 2013; Blahová et al., 2013). Hormone concentrations of T and E2 were determined using commercially available ELISA kits (PARS Azmoon, Iran) following the manufacturer's instructions. All measurements were performed in triplicate.

Gene expression (cyp19a): For the measurement of cyp19a gene expression, 6 fish (2 per replicate) were randomly selected from each treatment group. Total RNA was extracted from homogenized samples using the DENAzist Column RNA Isolation Kit (DENAzist, Iran), following the manufacturer's instructions. The quality and quantity of the extracted RNA were assessed using 1% agarose gel electrophoresis and a NanoDrop 2000C, respectively. To remove potential genomic DNA contamination, DNase treatment was performed according to the protocol provided by SinaClon (SinaClon, Iran). cDNA was synthesized from purified RNA using the Eacy™ cDNA Synthesis Kit (Pars Tous, Iran) according to the manufacturer's instructions. The primers used in this study were adopted from the research of Paknejad et al. (2019). The specificity of the primers was verified using the NCBI BLAST database. To determine the optimal annealing temperature for each primer pair, a temperature gradient PCR was performed, and the best amplification conditions were selected accordingly. For normalization of gene expression data, β-actin was used as the housekeeping gene. The primer sequences used in the study are listed in Table 1. Real-time PCR was performed using the Step One Real-Time PCR System (Applied Biosystems, USA). The Real-time Q plus 2n dye kit was used to evaluate gene expression. The polymerase chain reaction conditions were set as follows: first, a preheating step was performed at 95°C for 15 min. Then, 45 reaction cycles were performed at 95°C for 15 s (denaturation), 60°C for 30 s (primer annealing), and 72°C for 40 s (amplification). Gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method, with normalization to the reference gene β-actin. All reactions were performed in triplicate to ensure reproducibility and accuracy.

Reproductive performance indices: To evaluate reproductive performance parameters, including fertilization rate, hatching rate, and larval survival,

Table 1. Gene-specific primers for cyp19a and β-actin as a reference gene of zebrafish.

Target gene	Primer sequence (5′~3′)	Amplicon size (bp)	GenBank Number
Cyp19a	F: TCTGCTTCAGAAGATTCATAA ATACTTT R: CCTGCAACTCCTGAGCATCTC	121	AF226620.1
β-actin	F: AGGTCATCACCATCGGCAAT R: GATGTCCACGTCGCACTTCAT	140	NM_131031

spawning was induced in the experimental fish. For this purpose, 15 fish from each treatment group (5 per replicate, with a female-to-male ratio of 3:2) were transferred into separate spawning aquaria filled with clean, microplastic-free water. After spawning, a total of 300 eggs from each treatment (100 eggs per replicate) were examined under a light microscope to assess fertilization rate. Subsequently, 300 healthy fertilized eggs (100 per replicate) were carefully collected using wide-mouth pipettes and transferred to hatching containers maintained at 26°C. Hatching rate was recorded from 48 hours post-spawning, and larval survival rate was evaluated on day 14 post-hatching.

Statistical analysis: Statistical analyses were performed using SPSS software (version 26.0). The normality of the data was assessed using the Kolmogorov–Smirnov test. One-way analysis of variance (ANOVA), followed by Duncan's multiple range test, was used to determine significant differences among treatment groups at the 5% significance level ($P<0.05$). All results are expressed as mean ± standard deviation (SD).

Results

Microplastic concentration in water and fish body: The number of microplastic particles in water without fish at three concentrations of 20, 200, and 2000 μg/L was 1569.5±293.8, 14209.2±421.3, and 120360.7±495.9 particles, respectively. While in the presence of fish, the number of particles on day 21 was 1304.3±77.1, 12201.4±339.4, and 112050.3±438.9, respectively, and on day 60 was 967.7±64.7, 9693.3±397.8, and 101885.4±383.5. The results showed a clear dose-dependent increase in microplastic particle concentration in the absence of fish. However, in Aquariums containing fish, particle numbers were reduced at all concentrations and both time periods (21 and 60 days), indicating uptake or ingestion of microplastics by the fish. This reduction was particularly evident on day 60, especially in the higher concentration groups.

On the other hand, the assessment of microplastic accumulation in fish bodies revealed a significant increase in particle numbers as exposure concentrations increased. On day 21, the mean number of microplastic particles accumulated in the bodies of fish in the three exposure groups was 39.3±4.1, 95.6±5.7, and 176.7±7.4 particles, respectively. By day 60, these values increased to 101.3±3.8, 179.7±4.5, and 273.7±5.7 particles, respectively. Overall, a clear time-dependent accumulation pattern was observed, with significantly higher particle counts in fish tissues on day 60 than on day 21. This suggests a progressive accumulation of polystyrene microplastics within the fish over time, especially at higher exposure levels.

Primary reproductive indicators: On day 21, there were no significant differences ($P>0.05$) in absolute fecundity, relative fecundity, gonadosomatic index (GSI), and oocyte diameter between the control and treatment groups. However, on day 60, although no significant difference in absolute fecundity was observed among the treatments, significant changes were detected in other reproductive parameters. In particular, relative fecundity and oocyte diameter were significantly reduced in T3 (exposed to the highest microplastic concentration) compared with the control ($P<0.05$). Furthermore, the gonadosomatic index (GSI) showed a significant reduction in both T2 and T3 groups compared to the control group ($P<0.05$). Although the extent of increase was considerably lower in the microplastic-exposed groups, particularly in T3, than in the control group. Overall, exposure to polystyrene microplastics,

Table 2. Reproductive indices: absolute fecundity, relative fecundity, GSI, and OD in zebrafish following 21- and 60-day exposure to polystyrene microplastic. Lowercase letters indicate significant differences among treatments (P<0.05; ANOVA, Duncan).

Duration of exposure (days)	Treatment	Absolute fecundity	Relative fecundity	Gonadosomatic index (%)	Oocyte diameter (mm)
21	control	229.8±19^{a}	609.6±23.8^{a}	15.51±0.61^{a}	0.55±0.01^{a}
	T1	225.3±19.3^{a}	608.7±6.7^{a}	15.38±0.36^{a}	0.55±0.02^{a}
	T2	221.8±18.9^{a}	588.3±31.8^{a}	14.93±0.74^{a}	0.55±0.01^{a}
	T3	218.8±19.4^{a}	580.2±22.6^{a}	14.84±0.61^{a}	0.54±0.01^{a}
60	control	361.5±13.1^{a}	711.2±4.7^{a}	17.11±0.14^{a}	0.56±0.01^{a}
	T1	349.5±26.5^{a}	694.2±19.3^{a}	16.73±0.43^{ab}	0.55±0.01^{a}
	T2	341.7±19.7^{a}	690.1±15^{a}	16.15±0.53^{b}	0.55±0.01^{a}
	T3	335.8±26.6^{a}	664.4±23.2^{b}	14.96±0.73^{c}	0.53±0.02^{b}

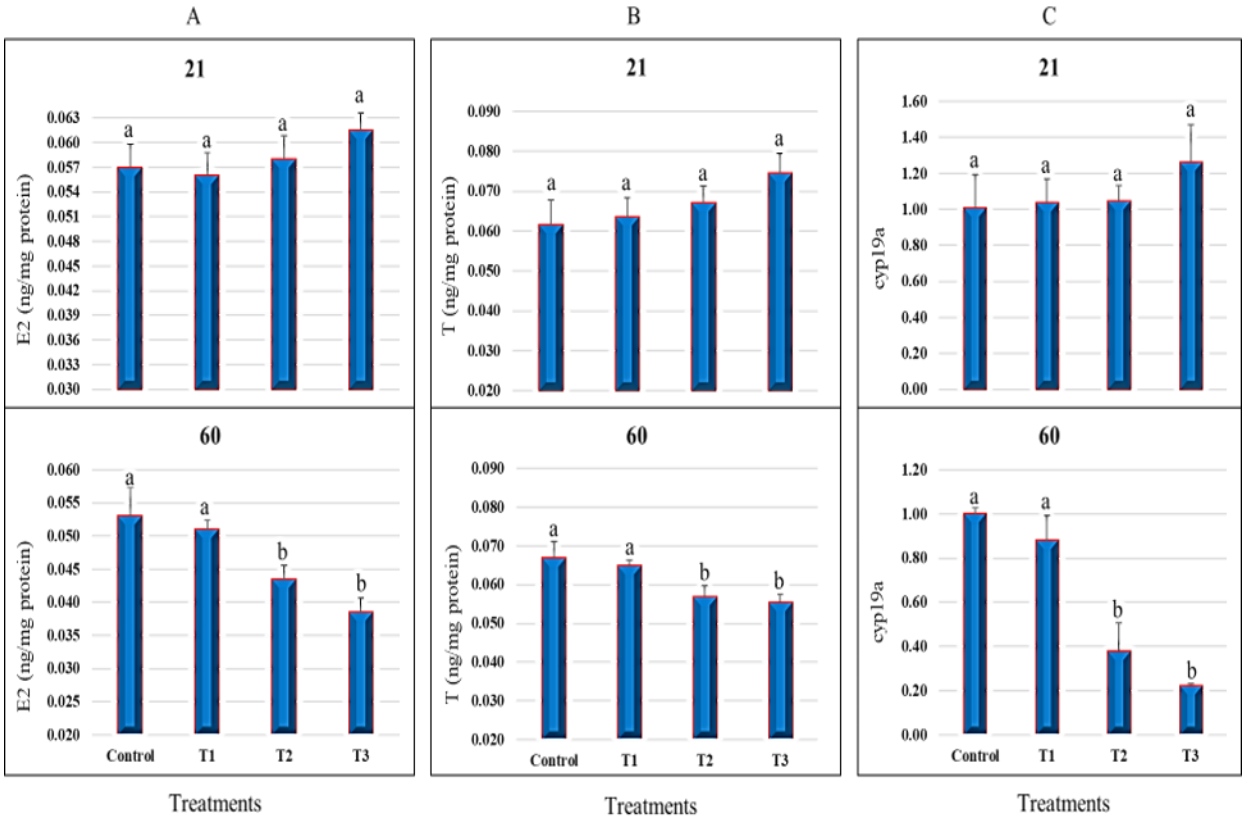

Figure 2. Levels of steroid hormones (A) E2, (B) T, and (C) transcription of the cyp19a gene in zebrafish following 21- and 60-day exposure to polystyrene microplastic. Lowercase letters indicate significant differences among treatments (P<0.05; ANOVA, Duncan).

particularly at higher concentrations, significantly reduced key reproductive parameters, indicating a dose-dependent negative impact on zebrafish reproduction (Table 2).

Steroid hormones: Analysis of testosterone (T) and estradiol (E2) levels showed no significant differences between the control and treatment groups on day 21 (P>0.05). However, on day 60, a significant alteration in the concentrations of both hormones was observed across treatments (P<0.05), suggesting that prolonged exposure to polystyrene microplastics affects endocrine function in zebrafish. Regarding E2 levels, fish exposed to higher concentrations of polystyrene microplastics (T2 and T3) exhibited a significant reduction compared to the control (P<0.05). This reduction was associated with both increasing microplastic concentration and exposure duration (Fig. 2A). Similarly, T levels were significantly reduced (P<0.05) in treatments T2 and T3 compared to the control. Finally, these results indicated a decreasing pattern in sex hormone levels as polystyrene microplastic concentrations increased

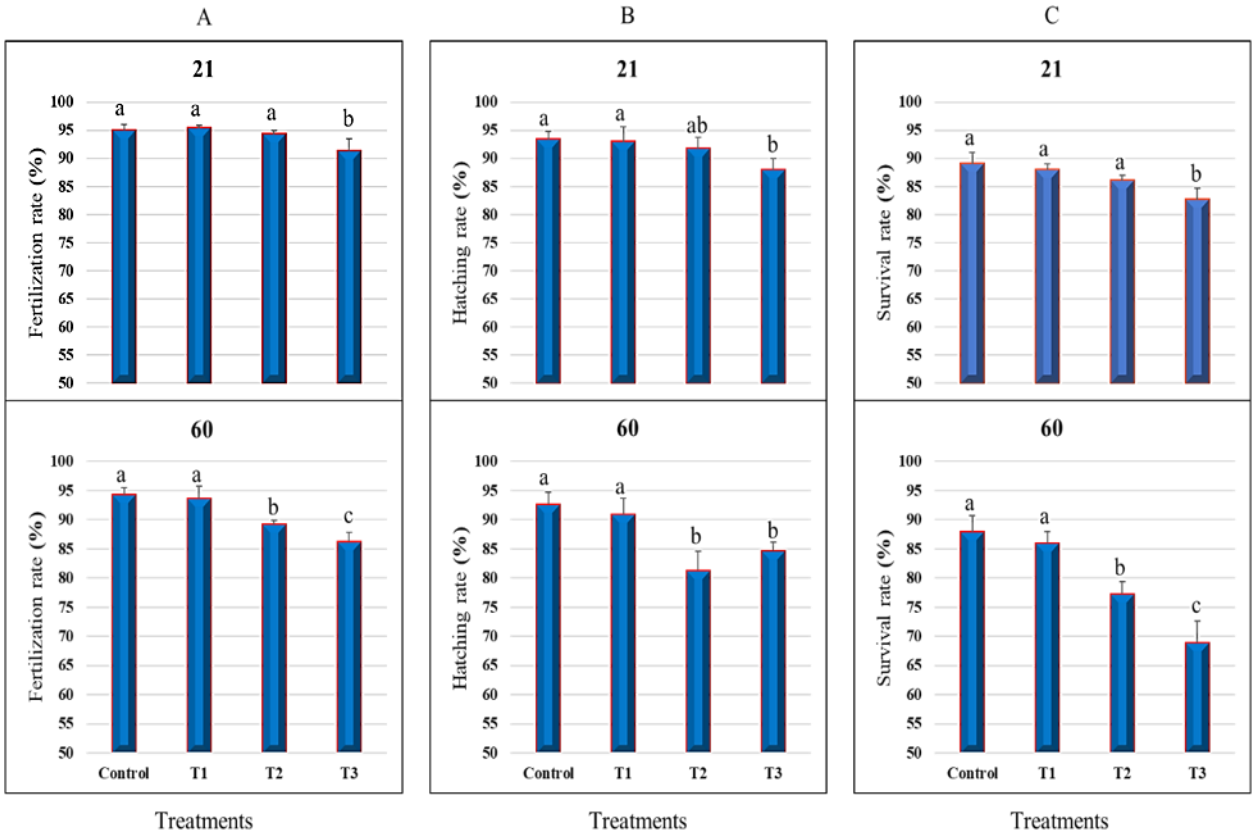

Figure 3. Reproductive performance Indices (A) fertilization rate, (B) hatching rate, and (C) larval survival rate in zebrafish following 21- and 60-day exposure to polystyrene microplastic. Lowercase letters indicate significant differences among treatments (P<0.05; ANOVA, Duncan).

over time, with the effect more pronounced in the higher-concentration treatments and on day 60 (Fig. 2B).

Gene expression: On day 21, cyp19a expression levels did not differ significantly among experimental treatments (P>0.05). However, on day 60, cyp19a expression in T2 and T3 (with higher concentrations of polystyrene microplastics) was significantly reduced compared to the control (P<0.05), and this reduction intensified with increasing microplastic concentration. Overall, in the higher-concentration treatments, cyp19a gene expression on day 60 was significantly lower than on day 21 (Fig. 2C).

Reproductive performance indices: The results for reproductive performance indicators showed that exposure to polystyrene microplastics adversely affected fertilization, hatching, and larval survival rates, particularly in treatments with higher concentrations and longer durations. On day 21, only the highest-concentration group (T3) showed a significant reduction in fertilization rate compared with the control and other treatments (P<0.05). However, on day 60, this reduction was more pronounced and significant in T2 and T3 (P<0.05) (Fig. 3A). On day 21, only the highest concentration (T3) showed a significant decrease in hatching rate compared with the control (P<0.05). However, by day 60, both T2 and T3 treatments showed significantly lower hatching rates than the control (P<0.05) (Fig. 3B). Similarly, larval survival showed a significant reduction only in T3 on day 21 (P<0.05). But on day 60, T2 and T3 showed a significant decrease in larval survival compared to the control, with the decline more pronounced in T3 (Fig. 3C). Overall, the negative impact of microplastics on reproductive indices intensified with increasing exposure concentration and duration.

Discussions

Microplastics are a significant and emerging environmental challenge and have attracted researchers' attention in recent years because of their

high consumption and widespread presence in aquatic environments. As they enter the aquatic food web, microplastics pose a serious threat to aquatic organisms' health and can disrupt vital biological processes, particularly reproduction. These impacts may lead to far-reaching ecological consequences (Sharma and Chatterjee, 2017). In this regard, the current study investigated the effects of polystyrene microplastics on some physiological and reproductive factors in zebrafish, a well-established model organism in aquatic toxicology. This study aimed to elucidate the short- and long-term effects of polystyrene microplastic exposure on reproductive physiological pathways in zebrafish. The results showed that polystyrene microplastics exert multifaceted adverse effects on physiological, hormonal, and reproductive processes in fish, posing a serious threat to aquatic ecosystem health. In the first phase of the study, a comparison between microplastic concentrations in fish-free water and water containing fish showed a significant reduction in particle numbers in the presence of fish. This reduction may be attributed to the fish ingesting particles, their accumulation in body tissues, or their adhesion to the fish's external surface. In addition, the increased accumulation of particles in the fish over the long-term exposure period suggests the potential for bioaccumulation of polystyrene microplastics within body tissues. This pattern was particularly pronounced at higher concentrations. Our findings align with previous studies reporting that microplastic particles can accumulate in fish tissues and bodies (Yu et al., 2018; Lei et al., 2018).

Fish reproduction is highly sensitive to metabolic changes and is influenced by several factors, including the availability of body energy reserves. Successful reproduction relies on sufficient nutrient access to ensure the development and survival of the next generation (Scaramuzzi et al., 2006). Common consequences of microplastic ingestion include gastrointestinal obstruction, reduced food intake due to false satiety, and decreased caloric absorption. These effects are often attributed to ingesting non-edible particles that resemble food in size and shape, particularly at high concentrations (Galloway et al., 2017; Ogonowski et al., 2018; Mallik et al., 2021).

Key morphometric parameters used to assess fish reproductive health include the gonadosomatic index, fecundity, and oocyte diameter (Murua and Saborido, 2003). The present study found that, in the short term, these particles had no significant effect on fecundity, gonadosomatic index, or oocyte diameter. However, in the long term, these factors decreased in treatments exposed to polystyrene microplastics, with the effect more pronounced at higher concentrations. Consistent with our results, Wang et al. (2019) showed that polystyrene microplastics reduced long-term fecundity and gonadosomatic index in marine medaka (*Oryzias melastigma*). Previous studies have indicated that exposure to microplastics adversely affects organisms such as marine medaka, copepods, and daphnia, resulting in reduced feeding rates, diminished reproductive success, impaired growth, and lower fecundity (Tatarazako et al., 2007; Au et al., 2015; Ogonowski et al., 2016; Cong et al., 2019; Bai et al., 2021). Additionally, Cole et al. (2015) reported that exposure to polystyrene microplastics caused reduced feeding and subsequent energy deficiency in the marine copepod *Calanus helgolandicus*, which was associated with decreased yolk sac volume and smaller oocyte diameter (Cole et al., 2015).

Numerous environmental stressors have been shown to act through the lipid metabolism pathway (Lee et al., 2018). Previous studies have also shown that polystyrene has an affinity for lipids and becomes trapped in fat-rich organs, such as the yolk sac (Mattsson et al., 2015; Lee et al., 2019). Because lipids support energy homeostasis, structural development, and other processes in zebrafish, disrupting lipid metabolism may have detrimental effects, including reduced nutritional status, growth, reproductive success, and fecundity (Fraher et al., 2016). Thus, decreased fecundity and gonadosomatic index may result from a shift in energy allocation toward growth rather than reproduction. Furthermore, since the size of an oocyte is proportional to the volume of its yolk sac, we conclude that the significant reduction in oocyte diameter is due to the decrease in yolk sac

volume.

Measuring sex hormones has been proposed as one of the most practical tools for evaluating fish reproduction and correlates well with transcriptional changes in steroidogenic genes in fish (Ma et al., 2012). Sex hormones (E2 and T) play a crucial role in gonadal development and function. In the ovaries, E2 is synthesized and transported to the liver, where it induces the production of Vitellogenin (Vtg) and Choriogenin (Chg), which are used for oogenesis (Bourguiba et al., 2003). The aromatase enzyme (cyp19a) catalyzes the conversion of androgens to estrogens; thus, alterations in its activity can affect the concentration and balance of sex hormones in fish (Uchida et al., 2004). In this study, short-term exposure to polystyrene microplastics did not significantly affect sex hormone levels or cyp19a gene expression. However, long-term exposure significantly reduced E2 and T levels and cyp19a gene transcription in T2 and T3. In a study by Hanachi et al. (2021), microplastics reduced aromatase gene expression in zebrafish. Also, in line with the current study, Wang et al. (2019) showed that polystyrene microplastics, in the long term, reduce the levels of E2 and T hormones, as well as the expression of the cyp19a gene in medaka fish (Wang et al., 2019). Steroid hormones regulate gonadal development, and lower steroid levels correlate with a decreased gonadosomatic index (Liu et al., 2011; Wang et al., 2015).

In line with the decrease in the gonadosomatic index, sex hormone levels decreased over the long term in this study. The reproductive process in fish is regulated by coordinated interactions between sex hormones along the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropin-releasing hormone (GnRH), released by the hypothalamus, promotes the synthesis and secretion of gonadotropin hormones in the pituitary gland. It also stimulates pathways involved in steroidogenesis, gametogenesis, and gonadal maturation. Disruption of this axis can impair sex hormone synthesis (Sofikitis et al., 2008; Ji et al., 2013). Microplastics negatively regulate the HPG axis, thereby decreasing sex hormone levels (Wang et al., 2019). In addition, previous studies have shown that reduced cyp19a gene expression leads to lower sex hormone levels (Kinoshita and Chen, 2003; Wang et al., 2019). Our study showed that exposure to polystyrene microplastics suppresses the steroidogenesis pathway by reducing cyp19a transcription in zebrafish. Because the steroidogenesis pathway produces sex hormones, our findings indicate that long-term exposure to polystyrene microplastics reduces sex hormone levels in zebrafish by inhibiting their synthesis. Based on these results, we conclude that long-term exposure to polystyrene microplastics disrupts sex hormone balance and reduces cyp19a expression.

Changes in HPG axis hormone levels and gene transcription are often associated with variations in reproductive processes, such as fertilization and hatching rates (Zhang et al., 2008; Ma et al., 2012). In assessing fertilization, hatching, and survival rates, the results showed significant decreases in these indices in the higher-concentration treatments, particularly during long-term exposure. This decrease may reflect disruptions in sexual maturation and oocyte and embryonic development, delayed hatching, and increased cellular damage from microplastic accumulation in the body, suggesting that microplastics have intergenerational effects on zebrafish. Exposure to various environmental stressors can lead to persistent physiological changes in zebrafish (Chen et al., 2021). These changes can even continue into subsequent generations without exposure (Anway et al., 2006; Knecht et al., 2017; Vera-Chang et al., 2018; Blanc et al., 2020). Previous studies have shown that exposure to microplastics reduces heart rate, delays hatching time, and decreases fertilization and hatching rates in fish (Wang et al., 2019; Duan et al., 2020; De Marco et al., 2022; Rahimi et al., 2024). Also, in another study, Zhao et al. (2020) showed that microplastics reduce the hatching rate in zebrafish (Zhao et al., 2020). Microplastics are recognized by the immune system as foreign substances that stimulate or suppress immune function, so interaction with microplastics may disrupt the immune system's function, potentially leading to

increased mortality, reduced fertilization and hatching rates, and ultimately lower larval survival (Luo et al., 2019; Liu et al., 2022). Furthermore, microplastic exposure induces oxidative stress and impairs fish growth and reproduction (Prokić et al., 2019). Oxidative stress during embryonic stages also significantly affects fish embryos, potentially reducing hatching rates and larval survival (Scholz et al., 2008). Consistent with our results, a study by Assas et al. (2020) found that exposure to polystyrene microplastics for 21 days did not significantly affect survival rates in medaka fish (Assas et al., 2020). De Marco et al. (2022) reported that polystyrene microplastics did not affect survival rates in zebrafish larvae in the short-term (De Marco et al., 2022). The observed reductions in fertilization, hatching, and larval survival rates following parental exposure to polystyrene microplastics suggest potential intergenerational effects. The reproductive alterations observed in this study may be attributed to compromised gamete quality resulting from parental exposure.

Conclusion

The current study showed that polystyrene microplastic contamination in aquatic environments and long-term exposure of zebrafish to different concentrations of this microplastic lead to reproductive disruption and adverse effects on reproductive factors. Furthermore, it disrupts the balance of sex hormones and the expression of genes involved in reproduction, ultimately reducing key reproductive outcomes, including fertilization, hatching, and larval survival rates. This study also confirmed that exposure to polystyrene microplastics is a potential source of reproductive stress in freshwater fish and showed that long-term exposure may directly affect fish reproductive organs, impairing reproduction. Therefore, further research on other aquatic species, investigation of the synergistic effects of these particles with other pollutants, and monitoring of microplastic pollution in aquatic ecosystems will be needed to prevent long-term consequences for aquatic populations.

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