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New insights into the reproductive hazards posed by polystyrene nanoplastics

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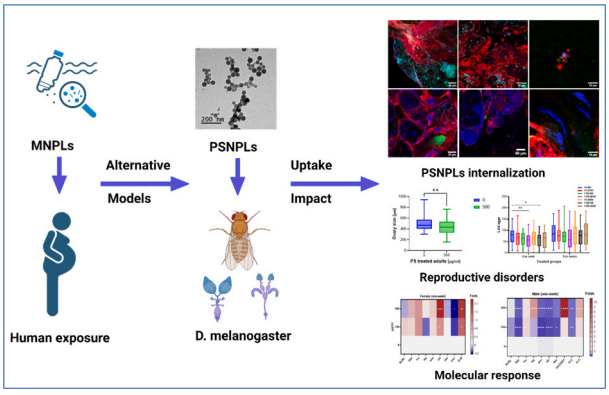
HIGHLIGHTS

- Reproductive toxicity of polystyrene nanoplastics has been determined in *Drosophila*.
- Internalization in ovaries, testes, and gametes (ova and sperm) was demonstrated.
- Reduced fecundity, lower fertility, and a skewed sex ratio were observed.
- Disruptions in the expression of reproductive and developmental genes was induced.
- Results highlights the potential for nanoplastics to cause reproductive damage.

GRAPHICAL ABSTRACT



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ABSTRACT

Reproductive toxicity from micro/nanoplastics (MNPLs) is an emerging concern requiring further investigation to close existing knowledge gaps. This study explores the reproductive toxicity of polystyrene nanoplastics (PSNPLs) using *Drosophila* as an *in vivo* model. Males and females were exposed to PSNPLs (100 and 500 μg/mL) for one/two weeks. Confocal and transmission electron microscopy revealed widespread distribution of PSNPLs across various tissues, including ovaries, testes, and gametes (ova and sperm). Structural damage, such as hole formation in the seminiferous tubules and ovarian atrophy, was observed following exposure. The physical presence of PSNPLs in reproductive organs disrupted reproductive outcomes, particularly in matings between exposed males and females. Key impairments included reduced fecundity, lower fertility, and a skewed sex ratio, especially after one week of exposure, with minimal effects after two weeks. Significant disruptions in the expression of reproductive and developmental genes were observed in both sexes, with males exhibiting greater sensitivity to PSNPLs, regardless of exposure concentration or duration. These findings provide critical insights into the reproductive toxicity of PSNPLs, underscoring both physical disruptions in reproductive tissues and

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molecular alterations. This highlights the potential for MNPLs to cause hidden reproductive damage and emphasizes the sex-dependent nature of these toxic effects.

1. Introduction

There is growing global concern about the potential health hazards posed by emerging contaminants, including micro- and nanoplastics (MNPLs). While research on the harmful effects of MNPL exposure has increased exponentially, certain aspects, such as the potential reproductive toxicity of these particles require further investigation. Interestingly, reported data using mammalian models has shown sperm damage in male mice exposed to polystyrene NPLs [20,73]. When comparing males and females' effects, although both testes and ovary's function was altered, the effects induced in females were more drastic [70]. A recent study shows that the effects of MPLs in females were potentiated when co-exposed with phthalates [74]. The presence of MNPLs in various organs, including the human reproductive system, has been documented, underscoring the need for a deeper understanding of their impacts on reproductive health and the underlying molecular mechanisms [69]. This highlights an urgent need for increased awareness, targeted research, and robust regulatory measures to manage this emerging threat.

The small size of nanoplastics (NPLs), typically less than 1 μm, allows them to enter the human body through ingestion, inhalation, or dermal exposure [56]. Once internalized, these particles can translocate to various organs, including the reproductive system [81]. Notably, MNPLs have been detected in the human placenta [54], chorion-amniotic membranes [19], and even within fetuses [77]. Although research on the reproductive toxicity of MNPLs is still limited, recent studies in mammals, including humans, suggest that these particles can induce miscarriage, stillbirth, reproductive dysfunction, and developmental disturbances in offspring [24,66,75]. Addressing concerns about MNPL reproductive toxicity requires the use of both *in vitro* and *in vivo* models. Considering the ethical and economic constraints of higher-order animal models [47] and the limitations of *in vitro* studies —which often involve only a few cell types and fail to capture the complexity of entire organ systems— *Drosophila melanogaster* stands out as an effective and reliable *in vivo* alternative for these studies.

D. melanogaster has emerged as a valuable model organism for studying the potential effects of nanoscale materials, including MNPLs [5,6,57,68]. Its efficacy stems from several key advantages: a short life cycle, genetic tractability, well-characterized biology, ease of maintenance, low cost, and significant genetic homology with humans [62]. These characteristics make *Drosophila* a powerful tool for uncovering the biological effects of MNPLs and generating insights relevant to human biology [8]. Although there are physiological and anatomical differences between *Drosophila* and humans, many fundamental biological mechanisms and pathways governing development and survival are evolutionarily conserved [65].

Accordingly, this study aims to investigate the potential reproductive toxicity of MNPLs. *D. melanogaster* has been selected as the *in vivo* model to be exposed, and commercial polystyrene nanoplastics (PSNPLs) were used as a representative model for MNPLs. Environmental MNPLs can originate from two sources: secondary MNPLs, which result from the degradation of larger plastic items, and primary MNPLs, which are engineered at micro/nano scales for specific uses, such as the polystyrene microbeads found in cosmetics. Both types eventually end up in the environment. The use of PSNPLs in this study is particularly relevant due to the extensive production and application of this polymer. In Europe alone, polystyrene accounts for 5.4 % of the total 58.7 million tons of plastic produced annually [51].

While the hazardous effects of PSNPLs have been widely studied in other contexts, their potential reproductive toxicity remains poorly understood [26]. To address this gap, the main objectives of this study are: (i) to chronically feed *Drosophila* with PSNPLs for 1 and 2 weeks, (ii) to assess the presence of PSNPLs in the reproductive organs of both male and female flies, (iii) to evaluate fecundity over a 3-week period, covering nearly the entire oviposition cycle, (iv) to measure fertility, defined as the percentage of adult flies that emerge from the eggs, and (v) to investigate changes in the expression of a set of genes known to be involved in *Drosophila* reproduction in response to PSNPL exposure.

2. Material and methods

2.1. Polystyrene nanoparticles PSNPLs characterization

Polystyrene nanoparticles (PSNPLs, with a diameter 80 nm) were delivered from Spherotech (Lake Forest, IL, USA, reference SPH-PP-008–10). According to the manufacturer the PSNPLs were prepared through polymerization of styrene monomer using potassium persulfate as a polymerization initiator, resuspended in deionized water, and sodium azide (0.02 %) was added as a bacteriostatic. To further characterize PSNPLs, a Malvern Zetasizer Nano-ZS zen3600 instrument (Malvern, UK) was used to determine the hydrodynamic size and zeta potential, while transmission electron microscopy (TEM; Jeol 1400, Tokyo, Japan) was applied to underscore size, morphology, and degree of aggregation of PS particles. The functional groups of PSNPLs were determined by Fourier-transform infrared spectroscopy (FTIR) using a Hyperion 2000 micro-spectrometer (Bruker, Billerica MA, USA). These analyses were done at the Molecular Spectroscopy and Optical Microscopy facility at the Institut Català de Nanociència i Nanotecnologia (ICN2). To achieve the desired concentrations, PSNPLs were dispersed in Milli-Q water.

2.2. Exposure/feeding

The Canton-S strain of *Drosophila melanogaster* was employed to define the reproductive toxicity of PSNPLs exposure. The newly emerged adults were collected and separated according to their sex (males and females) into groups of 50 individuals. For treatments, PSNPLs were mixed with peach-grape juice, while unexposed flies were fed on PSNPLs-free juice. Two concentrations of PSNPLs were selected to treat *Drosophila* flies (100 and 500 μg/mL). PSNPLs-juice stock mixture solutions were prepared according to Nanogenotox dispersion protocol [46]. Thus, 40 mL of each PSNPL concentration (100 and 500 μg/mL) were sonicated at 10 % amplitude using an SSE-1 Branson sonicator, for 16 min. Further, the PSNPLs-juice solutions were aliquoted into suitable tubes (5 mL). To guarantee well dispersion of PSNPLs, all tubes were directly frozen in liquid nitrogen and kept in −80 °C for further use. To feed/expose flies, 1 mL of juice (mixed/unmixed with PSNPLs) was used to wet the bottom side of the sponge plug of the flies' tubes (falcon tubes, 50 mL size). Each tube contains a piece of paper to regulate the file's activity-relax regime. Flies were allowed to feed on juice for two days, then transferred into new tubes with freshly juice-wetted plugs. The last step was repeated till the end of the experiment. This protocol of feeding/exposure was applied for the first time in this study (Fig. S1).

2.3. Fecundity and fertility

To keep the virginity of females, all new adults were discarded, and pupae were allowed to give new adults for eight hours. Immediately, flies were sorted into males and females and kept in separated suitable flasks containing standard feeding media. This procedure was repeated till got the desired fly's number. To proceed flies (males or females) were divided into groups of fifty individuals. Then, the flies were transferred

into falcon tubes plugged with sponge plugs wetted with juice (0, 100, and 500 μg/mL PSNPLs), as described previously. The exposure lasted for one week or two weeks. At the end of treatment, the males and females of one-week exposed flies were divided into seven subgroups; each group consisted of 20 individuals (10 males with 10 females) in triplicates. While the negative control group was established by mixing nontreated flies (males and females), the remaining six groups included one or both sexes treated with 100 or 500 μg/mL of PSNPLs as schematized in Supplementary Materials (Fig. S2). For flies' adaptation, all the groups were left for one day and allowed to feed on a normal agar-gelled medium at suitable conditions of temperature (24 ± 0.5 ºC), humidity (65 ± 5 %), and light/dark cycle (12 h interval). To determine flies' fecundity, the laid eggs on the surface of the black medium (facilitate egg counting) were counted, while adult flies were transferred to new flasks. This step was repeated every day until all flies stopped laying eggs. The laid eggs were left in incubation (24 ± 0.5 ºC) and were observed till adults emerged. The emerged adults were counted in proportion to the number of laid eggs to calculate fertility. In addition, the percentage of males to females was determined to evaluate gender ratio. The ovary sizes of exposed flies to 500 μg/mL of PSNPLs in comparison to those of unexposed flies were measured to highlight if nanoplastic exposure could induce ovary atrophy. To conduct that, PSNPLs-treated females were dissected, and their ovaries (100 ovaries for each group) were extracted, photographed, and measured with the ImageJ program.

2.4. PSNPLs internalization

After one week of exposure, the distribution of PSNPLs inside various flies' organs including the gastrointestinal tract, hemolymph, ovaries, testes, and even feces after exposures to 500 μg/mL of PSNPLs was investigated. Transmission and confocal microscopies were employed to underscore PSNPLs uptake.

2.4.1. TEM investigation

- *Ultrathin sections*: Transmission electron microscopy has been used to investigate ultrathin sections of various organs of PSNPLs-exposed flies, following our previous protocol [3]. Treated adults were dissected in buffer solution (PB; 0.1 M, pH 7.4), and immediately the dissected organs (intestinal tracts, ovaries, and testes) were fixed in 0.15 M phosphate buffer containing 4 % paraformaldehyde and 1 % glutaraldehyde, pH 7.4. Then, the fixed samples were further manipulated at the UAB-TEM Services, where they post-fixed and stained with a mixture of 1 % (w/v) osmium tetroxide and 0.8 % (w/v) potassium hexacyanoferrate for 2 h, in a cold environment (4 ºC). To proceed, the samples were washed (in deionized water), dehydrated (in a graded series of acetone), and embedded in Eponate 12TM resin (Ted Pella Inc., Redding, CA). The embedded organs were left to polymerize for 48 h at 60 ºC. The ultrathin sections were prepared by cutting the organ's blocks with a diamond knife (450, Diatome, Biel, Switzerland). In the last step, the sections were mounted on copper grids and contrasted with uranyl acetate (30 min) and Reynolds lead citrate (5 min) solutions. TEM (HITACHI H-7000, 125 kV) was employed to underscore PSNPLs' internalization in different flies' tissues.

-*Hemolymph*: Hemolymph samples were obtained by dissecting treated flies in PBS buffer solutions. Then the samples were directly mounted on a carbon grid and left to dry in a clean area to be ready for TEM investigations.

-*Ovaries and feces digestion*. For ovaries, samples of exposed female ovaries were allowed to digest in 500 μL H_2O_2 (30 %) at 60 ºC for 24 h and left for another 24 h at 4 ºC to dissolute. The digested ovaries were filtered through a 0.22 μm filter and then centrifuged at 16,000 rcf for 15 min. Supernatants were removed, and drops of bottom solution were placed on TEM grids to investigate. To detect PSNPLs in feces, paper pieces containing feces of the exposed adults were collected, stirred (700 rpm for 1 h), and filtered to remove paper remnants. Then, the solutions were aliquoted in various tubes and centrifuged at 10,000 rpm for 30 min. The supernatants were discarded except the bottom 1 mL of each tube, which was collected and centrifuged. Finally, after the supernatant was removed, drops of the bottom solution were dropped on TEM grids.

2.4.2. Confocal microscopy

Organ samples of the exposed flies to labeled PSNPLs were dissected in 1 % PBS and assessed with confocal microscopy (Leica TCS SP5). To visualize cellular membranes (in red) and nuclei (in blue), Cellmask™ Deep Red plasma (ThermoFisher Scientific, Carlsbad, CA, USA) and Hoechst 33342 (ThermoFisher Scientific, Carlsbad, CA, USA) were applied (1 μL:1 mL samples). PSNPLs were detected at emission wavelengths of 585 nm. Various images at randomly selected fields for each sample were picked up, and further manipulated by ImageJ software having Fiji extension.

2.5. Molecular response (Real-Time PCR)

To unveil molecular response that could be induced with PS-NPLs exposure, the expression of various reproductive and developmental-related genes (*arm, Buffy, dpp, dsx, EcR, fru, K81 (ms(3)K81), shi, emc, kl-2, kl-5,* and *wg*) was determined in whole flies (female and male). Supplementary Table S1 shows the genes primers used. The primers were designed according to the National Centre for Biotechnology Information NCBI and Tu et al. [61]. To normalize the gene expression, the housekeeping *actine 5 C* (*Act5C*) gene was used. To proceed, flies were treated with PSNPLs (0, 100, and 500 μg/mL) for one or two weeks and frozen at −80 °C. RNA was extracted using TRItidy G™ (PanReac AppliChem) and quantified with a NanoDrop 1000 spectrophotometer. To discard DNA contamination, RNA samples were further treated with RNase-free DNase Kits. To get cDNA, RNA samples were reversely transcribed using a transcriptor first-strand cDNA synthesis Roche kit. The real-time PCR using a LightCycler® 480 SYBR Green I Master (Roche, Mannheim, Germany) was applied to quantitatively polymerize cDNA samples. RT-PCR involved sequenced procedures, i) pre-incubation (95 °C for 5 min, one cycle), ii) amplification (45 cycles of 95 °C for 10 s, 63 °C for 15 s, 72 °C for 25 s and 78 °C for 5 s), and iii) cooling. The values of gene expression were calculated by the LightCycler software.

2.6. Statistical analysis

Data were analyzed using GraphPad Prism 9 software (GraphPad Software Inc., CA, USA). The normal distribution of data was checked using the Shapiro-Wilk test. Correlation (Pearson r) and linear regression analyses were performed to highlight potential relationships with different variables. The one-way ANOVA or two-way ANOVA (parametric tests) with Tukey's multiple comparison test was applied for normally distributed data. The Kruskal-Wallis (non-parametric test) with Dunns multiple comparison test was used for skewed normality data. Normally distributed data were represented as mean ± standard deviation, while the median and interquartile range (Box and violin) represented the nonparametric data. Chi-square test was used to detect changes in sex ratio. *P* values, $*p \leq 0.05$, $* *p \leq 0.01$, $* **p \leq 0.001$, and $* ** *p \leq 0.0001$ were used to define statistical significance between independent variables or in comparison with controls.

3. Results and discussion

3.1. PSNPL characteristics and exposure

The ubiquitous presence of micro- and nanoplastics (MNPLs) in the environment makes human exposure unavoidable, raising concerns about their potential health effects [83]. Despite this, few studies have directly addressed the impact of MNPLs on human fertility and reproduction due to ethical and practical limitations [71]. To bridge this gap,

D. melanogaster was used as a model organism in this study to investigate the reproductive toxicity of MNPLs. As a lower-ranking organism, *Drosophila* bypasses complex ethical considerations and is widely accepted for reproductive toxicity studies [9].

The physicochemical properties of MNPLs, such as size, shape, surface charge, and stability, play a critical role in their biological impact [28]. Thus, detailed characterization of the polystyrene nanoplastics (PSNPLs) used in this study is essential. The PSNPLs were analyzed, and the results are shown in Supplementary Materials (Fig. S3). Transmission electron microscopy (TEM) images reveal that the PSNPLs are spherical in shape and generally well-dispersed, although some aggregation was noted. The average particle diameter measured by TEM was 45.1 ± 10.42 nm (Fig. S3A, B). Zetasizer analysis further showed that the PSNPLs have a negative surface charge, with a zeta potential of −49.70 ± 0.66 mV, confirming good dispersion despite the larger hydrodynamic diameter of 93.18 ± 1.84 nm due to slight aggregation (Fig. S3C). Fourier-transform infrared spectroscopy (FTIR) confirmed the chemical structure of the PSNPLs (Fig. S3D).

Although MNPLs can enter the body through various exposure routes such as inhalation and dermal absorption, ingestion is considered the primary pathway [75]. In this study, adult *Drosophila* were exposed to 0, 100, and 500 μg/mL concentrations of PSNPLs, delivered through peach juice for one or two weeks. To optimize exposure, a novel method was employed, improving on traditional solid-food media approaches. This method involved wetting the sponge plugs of rearing vials with peach-grape juice, providing a consistent food source that mimicked natural feeding behavior, as flies typically suck liquids through their proboscis [72]. This feeding approach has been shown to be useful when testing AgNPs exposure, permitting to follow up and quantify their intake [7]. This approach also minimized the risk of drowning, a potential issue in liquid-based exposures. The effectiveness of this exposure protocol was confirmed through morphological observation of the crop and the presence of feces, which appeared as black spots on paper. In contrast, flies housed with dry sponge plugs died within 2–3 days, demonstrating the importance of the moist food source.

Since the real concentrations of MNPLs in the environment remain uncertain, due to limitations in current detection methodologies and inconsistencies across reported findings, we have chosen two concentration (100 and 500 μg/mL) both reported in several *in vivo/in vitro* studies. The low concentration (100 μg/mL) used is widely repeated among studies. The highest one (500 μg/mL) was used to force the system and better visualize potential reproductive effects. In this context it must be indicated that in humans, MNPL exposure levels have been estimated to reach up to 5.8 μg/kg-bw/day for adults and 83 μg/kg-bw/day for infants [80]. In addition, if we consider the recommendations of EFSA (European Food Safety Authority) for nanomaterials, the tested exposures must reach 100 μg/mL in *in vitro* and 100 mg/Kg bw in *in vivo* studies [17]. Accordingly, the used exposure to 500 μg/mL correspond to 1 μg/g, which is 100 times lower that the proposal of EFSA.

3.2. Internalization

Drosophila adults exhibit varying susceptibilities to environmental stressors compared to larvae, due to differences in physiology, metabolism, and tissue sensitivity, particularly in the reproductive system. This research gap is significant, as adult reproductive organs may be especially vulnerable to environmental contaminants like MNPLs [37]. To address this, our study systematically examines PSNPL internalization in adult *Drosophila* and its potential effects on reproductive function. This includes evaluating PSNPLs' presence in various organs and assessing their impacts on fecundity, fertility, and molecular responses. While PSNPL internalization has been extensively studied in larvae [3,4,41], limited knowledge exists regarding this process in adult flies, particularly in relation to reproductive organs.

The accumulation of PSNPLs in reproductive and digestive tissues was analyzed using confocal microscopy and transmission electron microscopy (TEM). Confocal microscopy confirmed the internalization of PSNPLs in various *Drosophila* organs after one and two weeks of exposure to 500 μg/mL PSNPLs. Since no significant differences were observed between one and two weeks of exposure, only the results after one week are presented here (Fig. 1), while the two-week results are included in Fig. S4. Fluorescent PSNPLs (with a green spectrum) were widely distributed throughout the body, appearing inside enterocytes of the intestine, close to the nuclei (Fig. 1A), within hemocytes in the hemolymph (Fig. 1B), on the outer walls of ovarioles (Fig. 1C and D), and within the testes and seminal vesicles (Fig. 1E and F). Similar observations were made after two weeks of exposure, showing extensive PSNPL accumulation in the crop, intestine, hemolymph, ovary, and testes.

It has been proposed that the proximity of the ovary to the gut allows PSNPLs leaking from the gut to affect ovarian development [76]. Although previous studies have reported the presence of PSNPLs (100 nm) in the gut, crop, and ovary of fruit flies using fluorescence microscopy based on intensity changes [61], the precise localization of PSNPLs was not fully explored. Our study provides detailed insights into this localization, highlighting the potential reproductive risks of PSNPL exposure.

To further confirm the presence of PSNPLs, ultrathin sections of the gut (Fig. 2), ovary (Fig. 3), and testes (Fig. 4) from flies exposed to 500 μg/mL of PSNPLs were examined using transmission electron microscopy (TEM) after one week of exposure. The results revealed an extensive distribution of PSNPLs throughout the digestive tract, particularly within the midgut lumen, near the peritrophic membrane, and attached to bacterial cell walls. PSNPLs were also observed crossing the intestinal barrier and infiltrating enterocytes.

In the ovaries, PSNPLs were visualized near mitochondria (Fig. 3A-E) and distributed along the outer ovary wall (Fig. 3F). The proximity of PSNPLs to ovarian mitochondria may have contributed to disturbances in mitochondrial membrane potential. This aligns with findings by Wan et al. [67], who reported that exposure to PSNPs (50 nm) at concentrations of 50–200 μg/mL increased oxidative stress, reduced mitochondrial membrane potential, and elevated apoptosis in human trophoblast cells.

In male flies, PSNPLs were detected in various regions of the testes, including the extracellular matrix (Fig. 4A) and near spermatid cysts (Fig. 4B-F). Their presence in the testes led to the formation of small transparent cavities within the seminiferous tubules and spermatids.

Additionally, Supplementary Fig. S5 shows polystyrene particles in hemolymph samples (Fig. S5A-B), H_2O_2-digested feces (Fig. S5C-D), and ovaries (Fig. S5E-F) of adults exposed to 500 μg/mL PSNPLs for one week, as observed directly through TEM. Importantly, PSNPL exposure induced ovarian atrophy, as indicated by a significant reduction in ovary size (Fig. 3G).

Previous studies in *Drosophila* have suggested that the accumulation of micro- or nanosized PS particles in tissues can damage the intestinal tract, increasing necrosis and apoptosis in midgut cells [18] as well as in oocytes [61]. Notably, the most severe impact was observed with smaller PS particles [63], emphasizing the reproductive risks posed by nanoplastics. These findings raise significant concerns regarding the potential effects of MNPLs on fecundity and fertility in various organisms, including humans. Given that PSNPL exposure has been associated with granulosa cell apoptosis in mouse ovaries, which may reduce female fertility [78], this study aims to further investigate these reproductive effects following both short-term (one week) and long-term (two weeks) exposure.

3.3. Fecundity, fertility, and molecular analysis

Several key endpoints are frequently used to identify reproductive toxicity, including fertility, fecundity, histopathological and hormonal changes, sexual development, and reproductive performance. These adverse effects often result from apoptosis, necrosis, and inflammation caused by exposure to nanoscale materials [10]. For instance, PS-NPLs

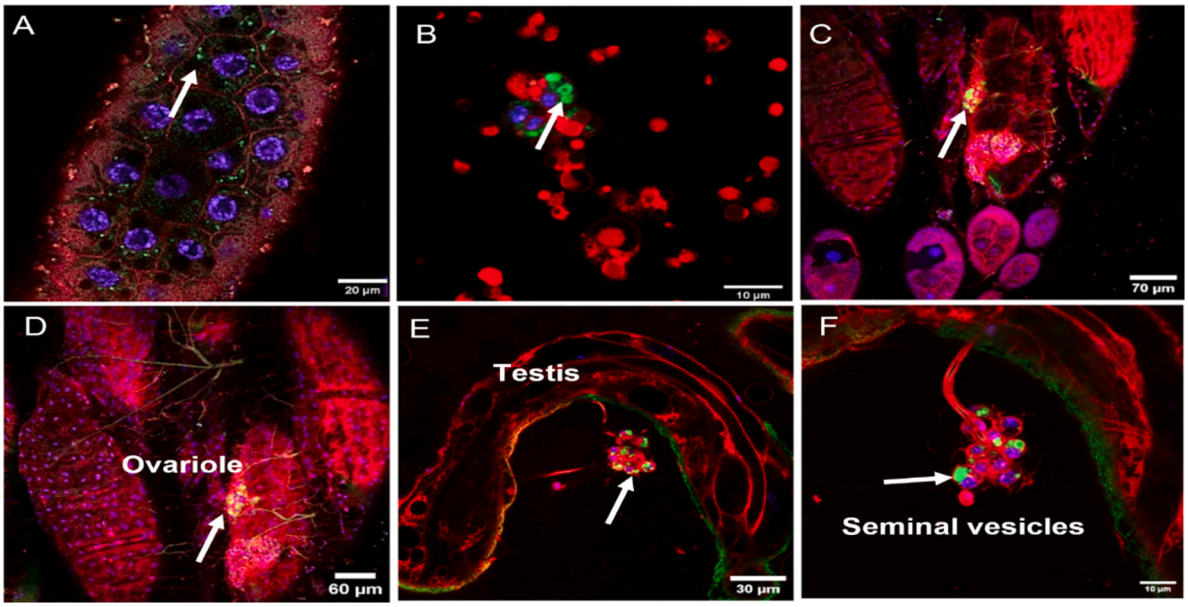


Fig. 1. Confocal fluorescent images of PS-NPLs in different body organs of Drosophila' adults after one week exposure. A: intestine, B: hemolymph, C and D: ovary, E and F: testis. Arrows indicate the presence of PS-NPLs.

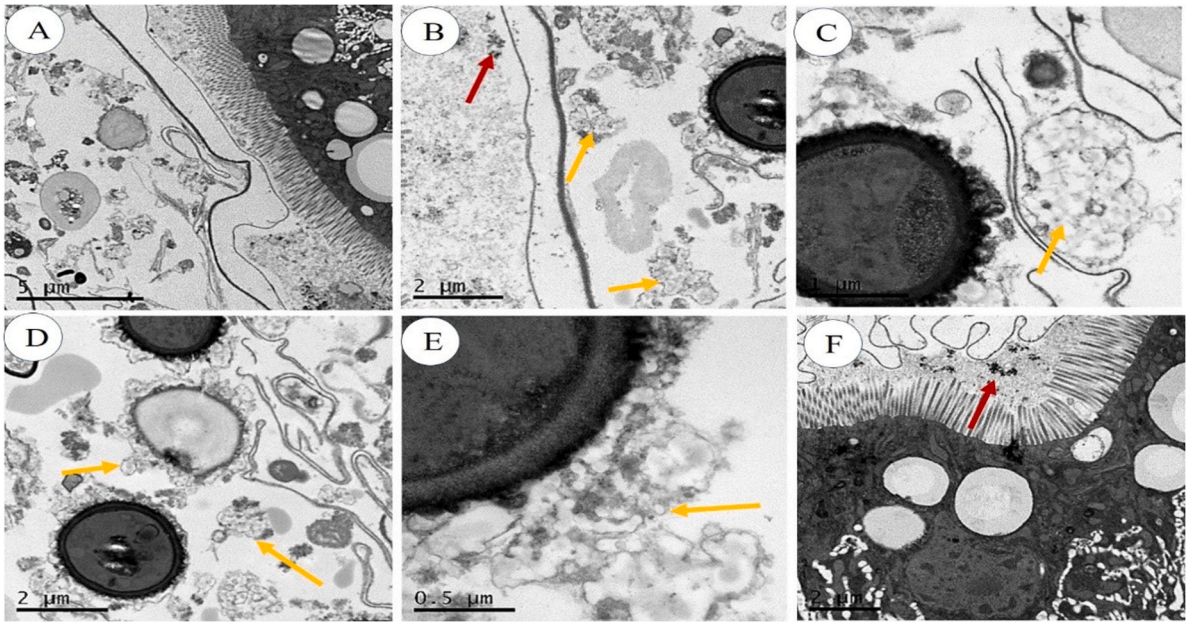
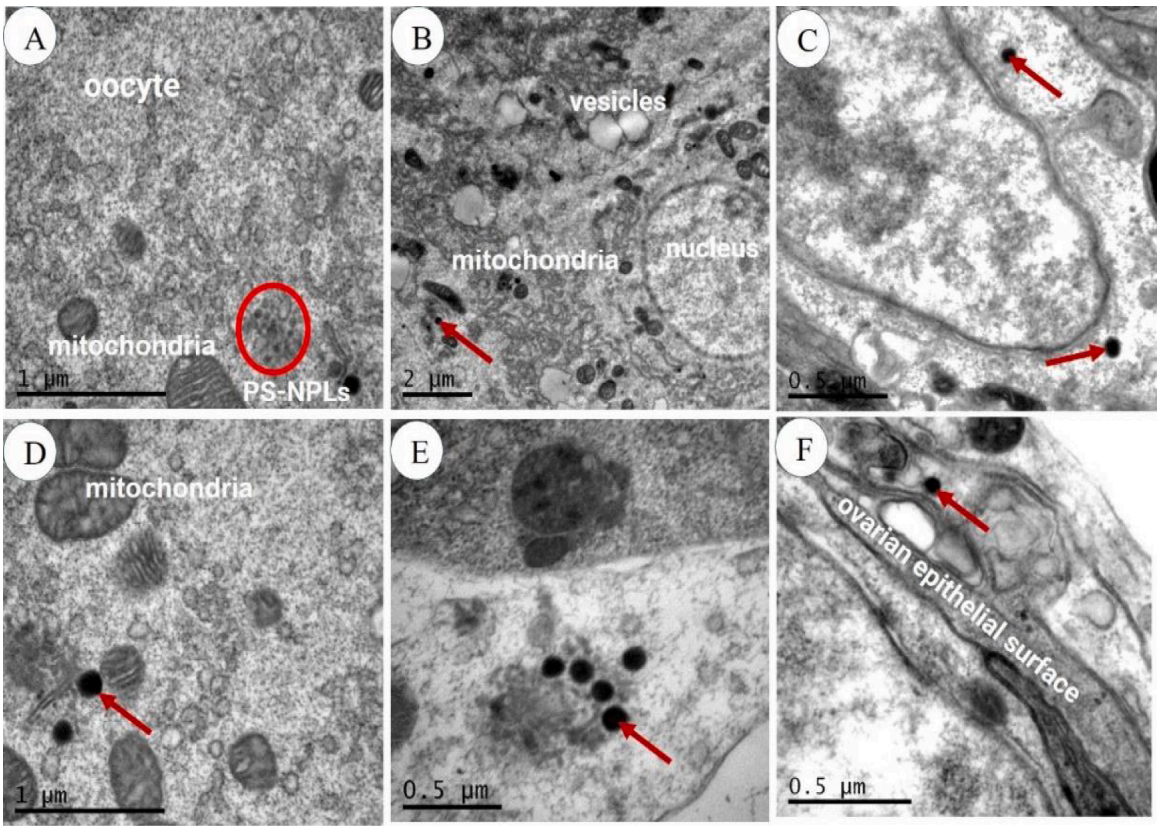


Fig. 2. TEM images of the ultrastructural of the intestine of one week-exposed flies to PSNPLs. PSNPLs appeared in large faint cloudy batches. A: general view of intestine. B: PSNPLs inside midgut lumen (orange arow) or in enterocytes cytoplasm (red arrow), C: PSNPLs besieged with intestinal membranes, D&E: PSNPLs around midgut' bacteria, F: PSNPLs inside enterocytes' cytoplasm.

(50 nm) have been shown to reduce fertility and fecundity in mice [29, 75] and can induce miscarriage in pregnant mice [67]. Considering the current miscarriage rates (15–25 %) in pregnancies worldwide [34], there are raising concerns of MNPLs exposure that could further threaten human reproduction. In this context, the present study aimed to clarify the reproductive disorders of PSNPLs by investigating the effects on fly fecundity (Figs. 5 and 6), fertility (Figs. 7 and 8), and the associated alteration in relevant gene expression (Fig. 9) following one or two weeks of exposure to PSNPLs. Genes are the functional units of heredity, playing a crucial role in regulating cell fate, differentiation, stress adaptation, and metabolic control. Beyond their intrinsic functions under normal conditions, genes are also responsible for correcting internal errors and maintaining homeostasis in response to environmental changes. Thus, changes in the expression of specific genes have been widely utilized in toxicological studies to explore the potential impacts of contaminants [42].

Regarding MNPL effects, one week of PSMPL exposure has been reported to mediate ovary atrophy and inhibit oviposition associated with altered genes in exposed females [76]. To further investigate reproductive disorders at the molecular level associated with MNPLs exposure after one- or two weeks, the expression of several relevant genes in both genders of *Drosophila* were analyzed. The studied genes included *Buffy, dpp, fru, wg, arm, shi,* and *dsx* for both sexes, while *emc* and *EcR* were studied only in females and *ms(3)K81, kl-2,* and *kl-5* were studied only in males. Fig. 9 summarizes the gene expression fluctuations for male and female flies exposed to varying PSNPLs concentrations (100 µg/mL and



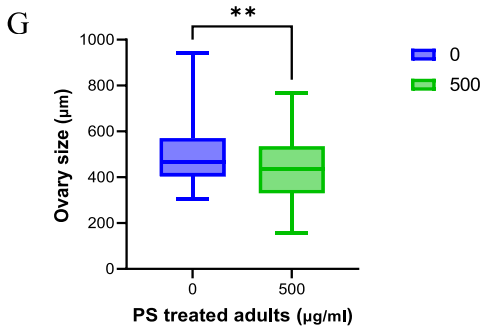


Fig. 3. TEM micrograph of the ultrastructural of *Drosophila* ovary (A to F) showing wide distribution of PSNPLs. Adults were treated with PSNPLs for one week. A-D: PSNPLs close to mitochondria, E&F: PSNPLs close to the outer wall of the ovary. The ovary size of *Drosophila melanogaster* exposed to PSNPLs in comparison to non-treated flies (G). Multiple pairwise comparisons (Dunn's test) were performed using Kruskal-Wallis. * $*p \leq 0.01$.

500 µg/mL).

In our study, fecundity, typically measured by the number of offspring produced or eggs laid, was notably affected by exposure to PSNPLs. Since unexposed adult flies (male-female pairs; F_0-M_0) produced a total of 4806 eggs, but this number was dropped significantly to 3527 and 3717 eggs following exposure flies to 100 and 500 µg/mL of PSNPLs, respectively. This would agree with those showing that PET microplastics (1–2 µm) exposure induced a reduction in the egg production of *Drosophila melanogaster* females [57]. When only one gender was exposed in various fly groups (F_0-M_{100}, F_{100}-M_0, F_0-M_{500}, and F_{500}-M_0), the egg production also decreased to 4101, 3812, 4019, and 3521 eggs, respectively. These results indicate that PSNPLs exposure affects egg production, with more pronounced effects when both exposed sexes are mated. In the two-week experiment, a similar trend was observed, with treated groups (F_0-M_{100}, F_{100}-M_0, F_{100}-M_{100}, F_0-M_{500}, F_{500}-M_0, and F_{500}-M_{500}) showing little reductions in oviposition (4909, 4591, 4275, 4981, 4683, and 5366 eggs, respectively) compared to unexposed flies (F_0-M_0) with 5382 eggs. Of note, the relatively high number of eggs in the two-week experiment compared to the one-week experiment may be attributed to the extended period of nutritional intake without mating, which allowed females to accumulate and produce more eggs. In terms of significance, the statistical analysis showed that reduction in egg production only attain significant differences in the F_{100}-M_{100} and F_{500}-M_0 groups of the one-week experiment (Fig. 5). Notably, the significant differences involving exposed females, highlighting the potential reproductive hazards on this sex. This would agree with a recent report showing that exposure to nanoplastics poses a substantial reproductive health risk to female mice (Wan et al., 2024). Conversely to the results of the one one-week experiment, no significant differences were observed in the fecundity among the various groups of the two-week experiment, which may be associated with epigenetic changes that facilitate rapid adaptations in insects [22]. While females typically prioritize mate selectivity

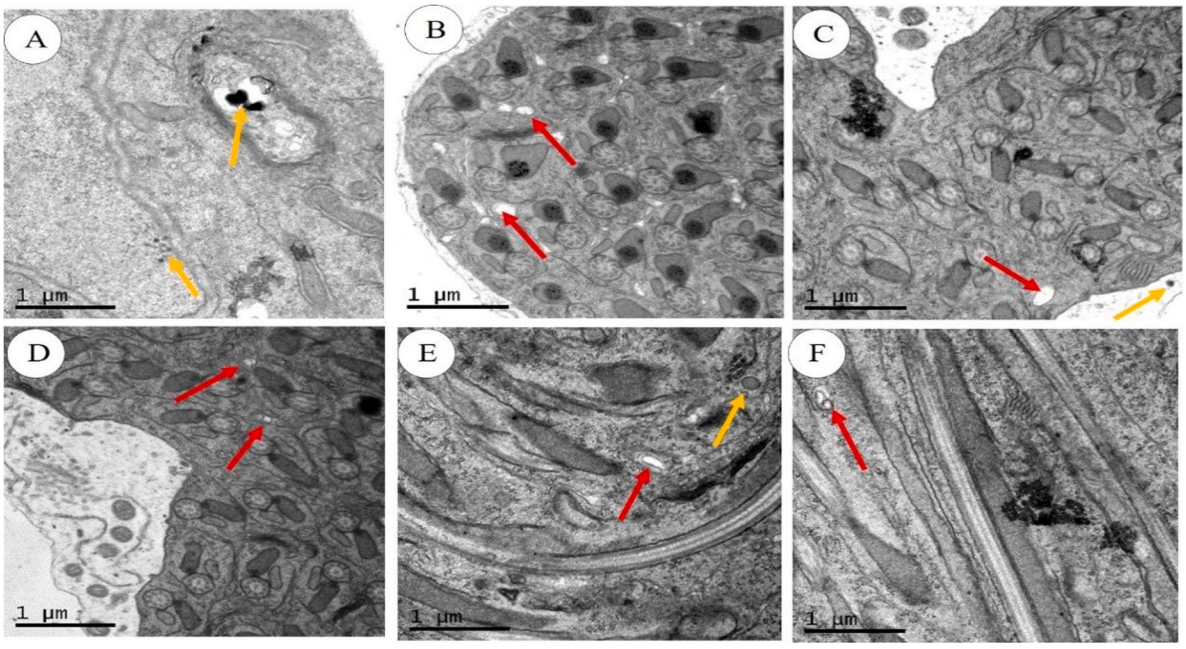


Fig. 4. TEM micrograph of ultra-sections of *Drosophila* testis (A to F) after one of exposure to PSNPLs. A: PSNPLs inside extracellular matrix, B-F: PSNPLs inside spermatids cysts. PS-NPLs are distributed as dense black dots (orange arrow) forming small transparent holes in the seminiferous tubules and spermatids (red arrows).

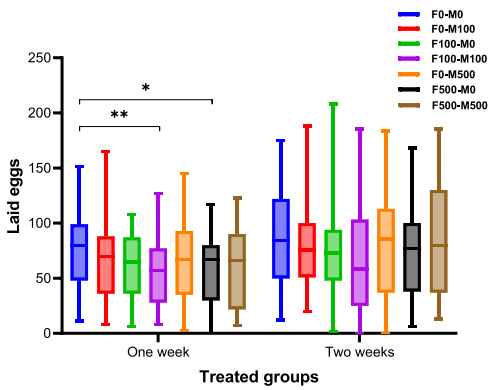


Fig. 5. Total fecundity of PSNPLs treated groups for one week or two weeks in comparison with non-treated flies. Multiple pairwise comparisons (Dunn's test) were performed using Kruskal-Wallis. $*p \leq 0.05$, $**p \leq 0.01$. *F; females, M; males, 0; nontreated flies; 100; flies treated with 100 µg/mL, and 500; flies treated with 500 µg/mL.*

and invest in offspring quality under normal conditions, adverse environments may drive them to adopt alternative strategies, such as increased mating frequency and egg production. This shift may represent an adaptive response or a loss of reproductive control, potentially resulting in indiscriminate mating and substandard eggs [25]. Consistent with this, Yan et al. [76] found no significant changes in the egg-laying of *Drosophila* exposed to MPLs. Our findings support earlier research showing that the fecundity of *Drosophila* returns to normal levels following long-term exposure to AgNPs [48]. Under a multi-generational PSNPLs exposure regimen, significant oviposition inhibition was observed in flies exposed in the F3-F5 generations, although no significant reduction was noted in the F1 and F2 generations [61]. Nevertheless, in that study counted eggs were only counted during the first five days of oviposition across all sequential generations, which does not capture the potential effects of PSNPLs on fecundity during the mid or late ages of the reproductive lifespan.

To understand oviposition kinetics, egg counting was done throughout the entire spawning period after a one- or two-week experiment. The oviposition period was divided into three intervals, each representing one week, to assess fecundity at early, middle, and late periods of the reproductive lifespan (Fig. 6). Interestingly, after one week of exposure, PSNPLs did not significantly affect oviposition during the young period. However, significant suppression was observed in the F_{100}-M_{100} and F_{500}-M_0 groups during both the middle and late stages, and in the F_{500}-M_{500} group during the late period of reproductive lifespan. Also, mated males and females (F_{100}-M_{100}) treated with 100 µg/mL PSNPL for two weeks showed a significant inhibition in oviposition compared to untreated flies during the late reproductive phase. This would indicate that fecundity is highly susceptible to the MNPLs accumulation, particularly in older individuals [63,76].

These results suggest that PSNPLs exposure, and by extension potentially other MNPLs, poses a risk both in the medium and long term. Notably, it has been reported that PSNPLs accumulate in human ovarian granulosa tumor cells, resulting in proliferation inhibition, apoptosis, oxidative stress, activation of key regulators of the Hippo signaling pathway, and downregulation of CTGF and Cyr61 [78].

It is important to note that the decrease in fecundity rate might cause deleterious effects on fertilization as observed in zebrafish [23], although increased embryo hatching rate has been also observed [82]. In *Drosophila,* and as contrast, a significant inhibition of the eclosion rate in fruit flies was reported [61]. To address these discrepancies, the percentage of emerged adults relative to the number of produced eggs in each group was calculated to assess the fertility of the flies after PSNPLs exposure during the entire period (Fig. 7) and dividing the fecundity lifetime into three periods (Fig. 8). Results demonstrated a negative effect of PSNPLs exposure on the average total fertility of flies treated for one week. The percentage of emerged adults from laid eggs showed evident decreases among various groups, reaching statistical significance when both sexes were treated with 100 µg/mL of PSNPLs.

When the effects on the fertility at different stages of the reproductive lifespan were determined, a dramatic decrease was observed in the late period, and these effects were observed for flies exposed for both one and two weeks, indicating a negative impact of PSNPLs on fertility, particularly with late reproductive life span. It should be noted that the observable decrease in the rate of emerged adults in the late period may be attributed to the aging of the flies [44].

The impact of PSNPLs exposure on sex ratio of the emerged flies was

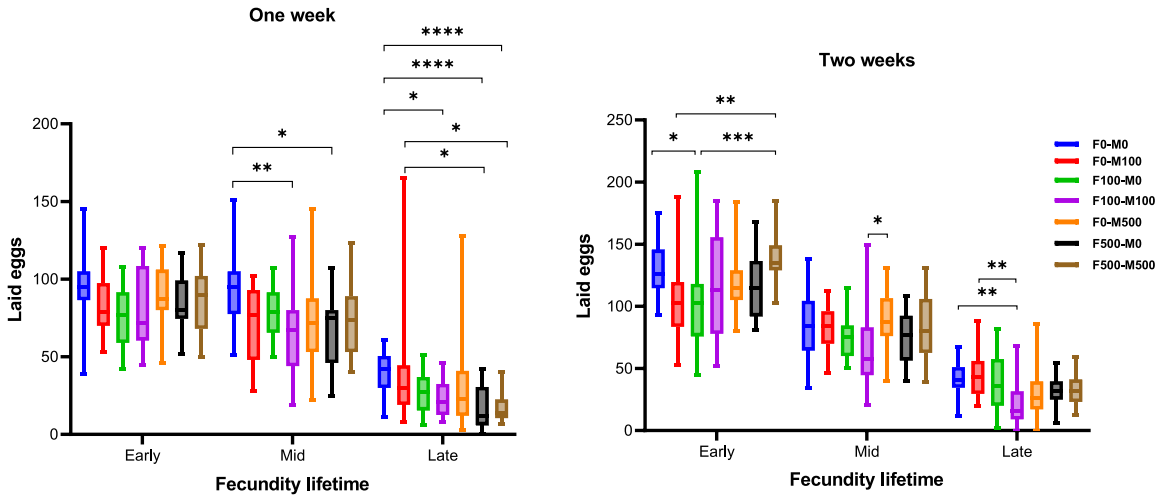


Fig. 6. Weekley fecundity of PSNPLs treated groups for one week or two weeks in comparison with non-treated flies. The experiment lasted for 21 days, fecundity lifetime divided into three periods, each one representing one week. Multiple pairwise comparisons (Dunn's test) were performed using Kruskal-Wallis. *$p \leq 0.05$, * *$p \leq 0.01$, * **$p \leq 0.00$, and * ** *$p \leq 0.0001$. *F; females, M; males, 0; nontreated flies; 100; flies treated with 100 μg/mL, and 500; flies treated with 500 μg/mL.*

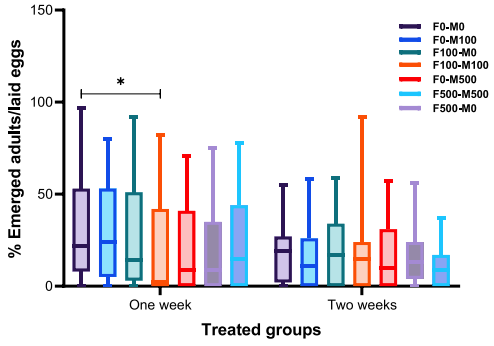


Fig. 7. Total fertility of PSNPLs treated groups for one week or two weeks in comparison with non-treated flies. Multiple pairwise comparisons (Tukey's test) were performed using two-way ANOVA. *$p \leq 0.05$, * *$p \leq <0.01$. *F; females, M; males, 0; nontreated flies; 100; flies treated with 100 μg/mL, and 500; flies treated with 500 μg/mL.*

also determined and the results are presented in supplementary figures (Fig. S4 and Fig. S5). A significant disturbance in the overall sex ratio was observed in flies exposed to 100 μg/mL (F_{100}-M_{100}) after one week of exposure (Fig. S4), and this effect was noted in the same exposure group (F_{100}-M_{100}) during the middle and late reproductive lifespan (after one week of exposure) and at the early stage in the two-weeks exposed flies (Fig. S5). Effects on sex ratio have been reported after exposures to PE- and PVCMPLs (23–500 μm) [31]. Park et al. [49] observed that polyethylene microplastics (PEMPLs) exposure altered the male-to-female sex ratio in mouse offspring, though the effect was not dose-dependent, consistent with our findings. Similarly, intravenous exposure to MPLs resulted in reduced fertility and an abnormal sex ratio in mouse offspring [52]. In another study, microfibers were shown to negatively affect the sex ratio in the water flea *Daphnia magna*, potentially due to the production of more males and a shift toward sexual reproduction [36]. However, deviations in sex ratio may be linked to increased fetal deaths in one gender, leading to a decline in its birth ratio [55].

It has been suggested that under chronic exposure scenarios populations adapted to contaminants investing more in reproductive efforts, including higher viability, increased fecundity, and faster egg-to-adult development, as a form of preadaptation to contamination [32]. Nonetheless, while the adaptation might reduce immediate toxic effects, possibly long-term exposure could lead to cumulative effects or new forms of stress. Therefore, this study unveiled the hidden reproductive toxicity effects of PSNPLs via studying the expression of battery of reproductive related genes.

As observed, PSNPLs exposure caused widespread molecular disturbances, leading to interference in the expression of all the studied genes, regardless of concentration, exposure duration, or individual sex. While *shi* gene was significantly increased in females under PSNPLs

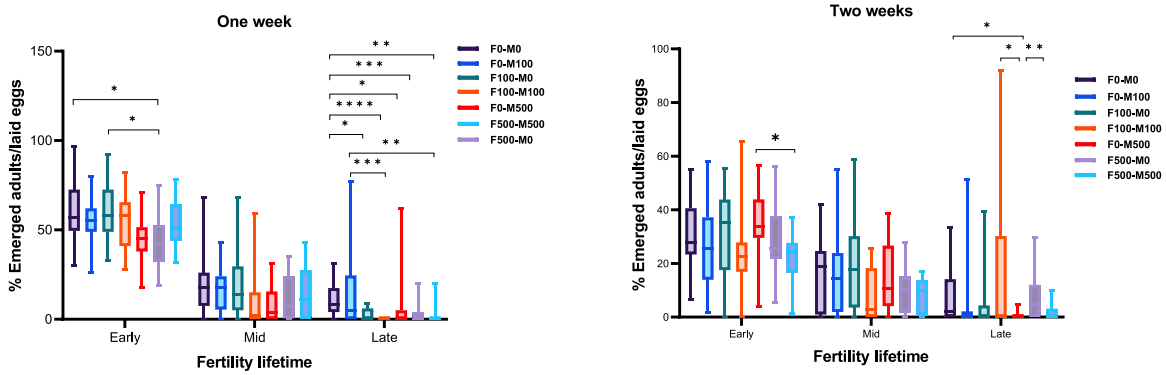


Fig. 8. Weekley fertility of PSPLs treated groups for one week or two weeks in comparison with non-treated flies. The experiment lasted for 21 days, fecundity lifetime divided into three periods, each one representing one week. Multiple pairwise comparisons (Dunn's test) were performed using Kruskal-Wallis. *$p \leq 0.05$, * *$p \leq 0.01$, * **$p \leq <0.00$, and * ** *$p \leq <0.0001$. *F; females, M; males, 0; nontreated flies; 100; flies treated with 100 μg/mL, and 500; flies treated with 500 μg/mL.*

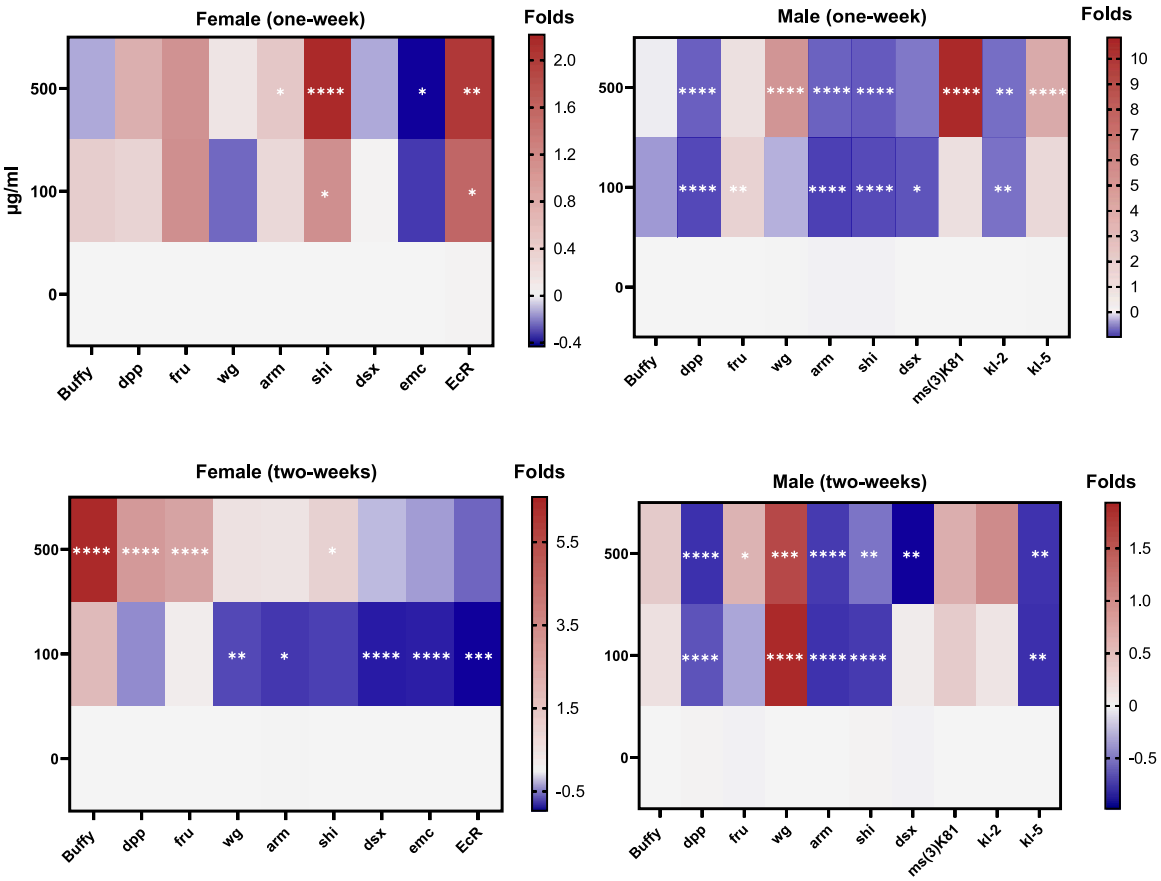


Fig. 9. Heat map of gene expression after PSNPLs exposure for one week and two weeks in both fly sexes. Two-way ANOVA (Dunnett's multiple comparisons test for parametric analysis), and Dunn's multiple comparisons test for non-parametric analysis. *$p \leq 0.05$, * *$p \leq 0.01$. * **$p \leq <0.001$.

exposure, regardless of concentration or duration, *buffy, dpp,* and *fru* are only expressed after prolonged exposure to high concentration. In males, genes showed different patterns, where *dpp, arm, shi, and dsx* were inhibited, while *wg* was significantly upregulated, regardless of concentration or duration. However, *EcR* in females and *ms(3)K81* and *kl-5* in males were significantly elevated following short-term exposure to high concentrations of PSNPLs.

Supplementary Figs. 6 and 7 highlight differential gene expression changes based on exposure duration (one week versus two weeks) and between sexes (males *vs* females) in *Drosophila* exposed to PSNPLs (100 and 500 µg/mL). In Supplementary Fig. 6, extending exposure to 100 µg/mL PSNPLs from one to two weeks led to significant changes in the expression of six genes in females (*arm, shi, fru, dsx, EcR,* and *emc*) and four genes in males (*buffy, wg, dsx,* and *kl-2*). Similarly, exposure to 500 µg/mL PSNPLs resulted in significant changes in five genes in females (*buffy, dpp, fru, shi,* and *EcR*) and four genes in males (*wg, ms(3)K81, kl-2,* and *kl-5*), when the exposure duration increased from one week to two weeks. Supplementary Fig. 7 highlights sex-based differences, where exposure to 100 µg/mL for one week led to significant differences in five genes (*buffy, dpp, arm, shi,* and *dsx*) between sexes, while only two genes (*dsx* and *wg*) were affected after two weeks. In contrast, exposure to 500 µg/mL showed an opposite trend, with four genes (*dpp, shi, wg,* and *arm*) affected after one week, and seven genes (*buffy, dpp, fru, wg, arm, dsx,* and *shi*) deregulated after two weeks. Therefore, the gene expression analysis showed that the hazardous impact of MNPLs exposure is sex-dependent, with lower concentrations inducing quicker molecular responses, while higher concentrations led to broader effects over a longer exposure period, depending on the sex of the organism. This would agree with the results showing that exposure to 100 mg/L of PSNPLs resulted in developmental and reproductive deterioration across the subsequent generations, accompanied by severe apoptotic and necrotic signals in the ovaries of exposed females [61].

The current study has identified several deregulated genes that could serve as molecular markers to elucidate the impairments in reproductive and developmental outcomes associated with MNPL exposure, linked to their internalization through the intestinal track and its translocation in the reproductive organs, as confirmed by TEM and confocal microscopy. Regarding the behavior of the different selected genes, *Armadillo (arm)* acts as an anchor protein that preserves the structural and functional integrity of the *Drosophila* gut and ensures proper epidermal cell polarization during embryogenesis [35]. Thereby, the broad downregulation of *arm*, especially in males might correlate with the presence of PSNPLs in the testes. Of note, *arm* expression has been shown to mitigate gut structural damage when Drosophila was exposed to bisphenol [11]. In addition, *Arm* also serves as a transducer in the Wingless (*wg*) signalling pathway during mitosis in *Drosophila* [39]. No studies have been found linking this gene to reproductive toxicity by environmental pollutants, including MNPLs.

The wingless (*wg*) and Decapentaplegic (*dpp*) signaling pathway are essential for germline stem cell recognition (GSC), reproductive development, and growth across species, from *Drosophila* to humans [14,15]. Overexpression of *dpp* blocks GSC differentiation, while its inhibition reduces proliferation [38] and the apoptotic stimuli activate *dpp/wg* signaling, leading to compensatory proliferation and tissue overgrowth [50]. In a recent study, MNPLs exposure likely disrupts different signaling pathways in the imaginal disc of *Drosophila* [58] and mediates negative effects on development and growth [16]. Accordingly, our finding of wide deregulation in *wg* and *dpp* in both genders of *Drosophila* after exposure to PSNPLs, particularly after two weeks of exposure, indicates the reproductive toxicity of PSNPLs. Furthermore, the disruption

of *Drosophila* female fecundity was associated with upregulating *dpp* [27]. Notably, these genes appear more sensitive to disruption in males, especially after short-term exposure.

Considering the broad downregulation of *shi* in males after one or two weeks of PSNPLs exposure suggests the negative effects of MNPLs on male fertility, where *shi* is crucial for maintaining actin cap integrity and sperm head compaction in *Drosophila* testes [13]. Regarding the *fruitless* (*fru)* and *doublesex* (*dsx)* genes, they are key determinants of courtship behavior, expressed in neurons that are scattered throughout the *Drosophila* central nervous system [33]. While the expression of both genes follows the same pattern in each sex, significant deregulation was observed in males at both exposure durations, while in females, deregulation occurred only after two weeks of exposure. Although *fru* is specific to males, it was detected in wild-type females, regulating with *dsx* some aspects of female sexual behaviors [64]. The expression of *fru* in females led to a failure to lay eggs [9], which reflects the reduced fecundity observed in flies following exposure to PSNPLs. Therefore, exposure to MNPLs disrupts genes related to reproductive effectiveness and courtship behavior.

Male fertility factors *kl-2* and *kl-5* reside in the long arm of the Y chromosome in *D. melanogaster* and were enriched in the proteome of pupal and adult testes [21]. Disruption and lacking *kl-2* and *kl-5* produced immotile sperm [79] that underscore the reduced fertility in flies after exposure to PSNPLs. Ecdysone is the major form of steroid hormone in *Drosophila melanogaster* playing various physiological roles including metabolism and reproduction, and ecdysone receptor (*EcR)* is highly expressed in the accessory gland of males [40]. In adult females, ecdysone is synthesized in the ovary and is involved in oogenesis [1]. Our results showed significant deregulation in female exposure to PSNPLs. Interestingly, *EcR* also plays a role in regeneration and its expression *EcR* drops in uninjured regions of wing discs, but simultaneously rises in cells around the injury-induced blastema together with tissue repair [60]. In that context, the ability of nanoscale materials to perforate tissue has been reported [2]. Induced reproductive abnormalities including reduced fecundity, delayed first brood, and reduced growth in *Daphnia*, have been recently associated with decreased expression levels of genes crucial for reproduction and development, especially *EcR* [12], highlighting our findings.

Buffy, a Bcl-2-like protein in *Drosophila melanogaster*, is essential for regulating cell survival and apoptosis in response to external stimuli [43]. The obtained results indicate that PSNPLs exposure produced significant upregulation of *buffy* in females exposed to 500 μg/mL for two weeks. It is important to mention that PSNPLs were visualized close to the mitochondria of *Drosophila* ovaries, highlighting the role of *Buffy* which resides in the mitochondria in controlling apoptosis through regulating mitochondrial changes associated with cell death [59]. On the other hand, elevated levels of *Buffy* can suppress apoptosis caused by DNA damage [53], and exposure to PSNPLs has been shown to induce DNA damage in *Drosophila* hemocytes [3]. Notably, this exposure also caused ovary atrophy, suggesting a link between environmental pollutants and mitochondrial dysfunction, impacting reproductive health. In that context, exposure to Bisphenol A significantly decreased mitochondrial and cellular metabolic activity *in D. melanogaster* which resulted in increased mortality of flies [45].

Our results on the molecular response to PSNPLs demonstrated a sex-dependent pattern, with males exhibiting greater sensitivity than females, particularly during short-term exposures. Our findings align with those reporting that male flies were more sensitive than females, showing more substantial and pronounced negative effects [63]. These differences may be attributed to the distinct metabolic levels and physiological characteristics in male and female flies, which influence their differential responses to environmental contaminants [30].

4. Conclusions

Given the growing concern regarding the reproductive effects of MNPL exposure, this study aimed to evaluate the reproductive impairments associated with PSNPL exposure, using *Drosophila melanogaster* as an *in vivo* model. While polystyrene particles had previously been reported in the intestinal tract and hemolymph, our study successfully demonstrated that reproductive organs are a significant target for MNPL accumulation. PSNPLs were visualized attached to sperm and ova, near ovarian mitochondria, and caused physical damage in the seminiferous tubules. These findings suggest that the presence of MNPLs in both ovaries and testes may directly contribute to reproductive toxicity.

In addition to ovarian atrophy, reductions in key reproductive parameters such as fecundity and fertility highlight the significance of our findings. Disruptions in sex ratios and changes in the expression of genes related to reproduction and development further underscore the effects of PSNPL exposure. These molecular responses could serve as sensitive markers of reproductive impairment, revealing that these effects are sex-dependent, with males exhibiting greater sensitivity. Future research should focus on transcriptomic analysis to uncover the interconnected response pathways and further investigate mitochondria as a target of MNPL toxicity. This is particularly important given the visualization of PSNPLs near ovarian mitochondria and the observed alterations in the expression of the Buffy gene, suggesting mitochondrial involvement in the toxic response.

It is important to note that while this study used pristine PSNPLs as a model of MNPLs, they may not fully represent secondary environmental MNPLs that result from the degradation of plastic goods. Therefore, future studies should expand reproductive toxicity research by using environmentally relevant (true-to-life) MNPLs to provide more accurate data for risk assessment.

Environmental implication

Reproductive toxicity is a major concern caused by environmental pollutants. To minimize the use of laboratory mammals, alternative *in vivo* models such as *Drosophila* are needed. In reproductive toxicology, detecting harmful agents in target organs is crucial for evaluating effects such as fertility and fecundity. Using polystyrene nanoplastics, we detected their presence in the testes, ovaries, sperm, and eggs, along with reductions in ovary size and fecundity. Alterations in the expression of genes related to reproduction were observed, highlighting the potential for nanoplastics to cause reproductive damage. This study underscores the reproductive toxicity risk posed by micro/nanoplastics

CRediT authorship contribution statement

Abass Doaa: Validation, Investigation. **Alaraby Mohamed:** Writing – original draft, Formal analysis, Conceptualization. **Hernández Alba:** Writing – review & editing, Supervision, Resources, Conceptualization. **Velázquez Antonia:** Supervision, Conceptualization. **Marcos Ricard:** Writing – review & editing, Supervision, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2025.138210.

Data availability

Data will be made available on request.

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