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Size-dependent and sex-specific negative effects of micro- and nano-sized polystyrene particles in the terrestrial invertebrate model *Drosophila melanogaster*

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ABSTRACT

Microplastic pollution is believed to be one of the most widespread and long-lasting changes on a global scale. Our understanding that microplastics significantly impact terrestrial systems and are a global change stressor continues to grow. In the present study, we investigated the negative effect of long-term (28 days of exposure in food) polystyrene particles of micro (1.0–1.9 μm, 0.4–0.6 μm) and nano (0.04–0.06 μm) scale, in low doses, on the fruit fly – representing a common, globally distributed terrestrial invertebrate, and a model species in many fields. Our observations involved such parameters as ingestion and transfer of particles, survival, reproduction, changes in ultrastructure and tissue and cell responses in midgut epithelium (the place of direct contact with plastic), ovary, and testis in adults, and transgenerational effects in larvae. These observations may indicate possible toxic effects of the tested substances, even in low doses, that can be expected in other taxa, in terrestrial ecosystems. We observed a negative impact of polystyrene particles on the fruit fly survival, midgut, ovary, and testis, involving ultrastructural alterations, such as autophagy and/or ultimately necrosis in the midgut, triggering oxidative stress and activating processes of antioxidative protection. Despite the changes, midgut function and reproduction were not altered – spermatogenesis and oogenesis proceeded normally. The effect was size-dependent – the smaller the polystyrene particles were, the more substantial was the impact they caused. Ultrastructural changes and studied parameters, i.e., generation of ROS (overproduction of which generates oxidative stress), total glutathione concentration (involved in defense against ROS, acting in distinct pathways), and total antioxidant concentration (the oxidative defense system) showed the highest levels after exposure to the smallest nanoparticles, and vice versa. The effect was also sex-dependent, with male flies being more sensitive. Negative effects in males were more substantial and more prominent, even after contact with larger particles, compared to females. The smaller particles (0.4–0.6 μm, 0.04–0.06 μm) were transferred to the ovary and accumulated in the oocytes. In this case, a transgenerational negative effect was detected in larvae. It was characterized by size-dependent alterations, with smaller particles triggering higher levels of ROS and cellular oxidative response. Only the largest particles (1.0–1.9 μm) did not pass into the gonad and did not alter the larvae. These observations together demonstrated that polystyrene particles of micro- and nanoscale, even in a low dose, can induce numerous negative effects on terrestrial invertebrates.

1. Introduction

A characteristic sign of the human environmental impact is the widespread presence of plastics, and plastic pollution is recognized as a severe global problem (de Souza Machado et al., 2018). Environmental plastic litter undergoes aging processes (i.e., degradation and disintegration) resulting from the action of physical, chemical, and biological drivers (Barnes et al., 2009). Recent reviews claim that most plastics, including many reported as biodegradable, are more prone to disintegration than degradation (de Souza Machado et al., 208). Under environmental conditions, larger plastic items degrade to so-called microplastics (microparticles – MP), usually fragments smaller than 5 mm in diameter. Further degradation and disintegration of microplastics generate particles with dimensions smaller than 0.1 μm, referred to as

Abbreviations: MP, microparticles; NP, nanoparticles; PS, polystyrene.

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nanoplastics (nanoparticles – NP). Extensive research on plastics has been carried out in aquatic systems, concentrating mainly on the marine environment (Duis and Coors, 2016). However, it should be noted that most plastic litter arriving in the oceans comes from the land, where it was produced, used, and disposed of. Research concerning plastic pollution in terrestrial ecosystems began, in fact, just a few years ago, and it has received far less scientific attention than its aquatic counterparts (de Souza Machado et al., 2018; Jambeck et al., 2015). It is surprising, given the high MP pollution established in the field. For example, MP contamination on land might be 4- to 23-fold greater than in the ocean (Horton et al., 2017). Although attempts to estimate terrestrial MP contamination are difficult, 355 MP/m^2/day fall-out in the Parisian metropolitan area was reported, corresponding to environmental exposure of 2–10 tons/year of fibers (de Souza Machado et al., 2018; Dris et al., 2016). Moreover, the use of NP in technical and medical applications is rising, and the input of primary NP into the environment is expected to increase (Cedervall et al., 2012).

A negative effect of plastics on different levels of organism functioning (mainly aquatic) was reported, and a broad spectrum of organisms can ingest especially smaller plastic particles. Moreover, the smaller the plastic particles are, the easier they can cross cell boundaries and enter the cells. Studies revealed several nanospecific outcomes, such as easy permeation of lipid membranes. NP inside the cells were observed to alter the membrane structure and decrease the molecular diffusion while softening it simultaneously. These changes in the membrane properties can severely affect cellular function, such as membrane protein sorting and functioning (Rossi et al., 2014). In addition to acute toxicity by disrupting membrane processes, NP can also be internalized by cells, which might increase their cytotoxicity targets (Syberg et al., 2015). Uptake of NP allows direct interaction between NP, genetic material, and cell organelles. Indeed, changes in gene expression, inflammatory and biochemical responses, and carcinogenesis have been reported after NP exposure in human and non-human toxicological models (Forte et al., 2016; Kato et al., 2003). NP uptake and toxicity mechanisms seem to depend on their specific properties (size, surface area, electric charge, surface modification, and hydrophobic properties). Generally speaking, the analysis of different cultured cell lines has revealed that NP can induce damage to the mitochondria, lysosomal disruption and release of potent lysosomal enzymes, cellular oxidative stress, oxidative damage, and high ROS production, or increase in the ratio of oxidized glutathione to reduced glutathione (GSSG:GSH), and can lead to apoptotic or necrotic cell death. The level of such bioreactivity was found to vary depending on the cell type and properties of NP (Ruenraroengsak and Tetley, 2015).

Reviewing the world literature, we identified a gap in knowledge that we intended to fill, to better understand the complex nature of this problem and the scope of the impact of NP. As the research on the potentially deleterious impacts of NP has focused on marine organisms and in vitro cultured cell lines, we decided to investigate the impact of MP and NP on terrestrial organisms, as well as on their tissues and cells, in vivo. We chose the fruit fly *Drosophila melanogaster*, as a representative of common and globally distributed terrestrial invertebrates that is also a model species in biological studies. We studied the viability and transfer and determined whether exposure to MP and NP changes the morphological structure, ultrastructure, and functioning of selected tissues and organs in fruit fly adults and whether it influences their physiological parameters. We also focused on reproduction and potential threats to the next generations.

For our study, we chose polystyrene (PS) due to its large production volume. PS is a synthetic aromatic polymer made from monomer styrene in a polymerization process. General purpose PS is a clear, hard, and brittle material that is easy to color. It is used for protective packaging, containers, lids, bottles, and trays, and it is also licensed for food packaging and modified atmosphere food packaging. As a result, PS is the fourth most often produced plastic type worldwide after polyethylene, polypropylene, and polyvinylchloride, with several billion kilograms produced per year (Maul et al., 2007; Ng et al., 2018). This, together with the fact that the PS degradation process leads to the generation of micro- and nanoplastics, as demonstrated recently, means that PS constitutes a vast amount of potentially dangerous contamination (Ng et al., 2018). In the present study, we elected to analyze three different sizes of PS MP and NP, selected by us as model plastic particles: 1.0–1.9 μm, 0.4–0.6 μm, and 0.04–0.06 μm.

2. Material and Methods

The material for the study was adult specimens and larvae of the fruit fly *D. melanogaster* of variation y,w (I); 43.4, y + , {w+ } (II). Flies were obtained from Vienna Drosophila Resource Center. They were bred on a simple cornmeal-molasses-yeast-agar medium in 50 ml vials or scaled up to 250 ml bottles. After several weeks of adaptation to laboratory conditions, the animals were divided into the following experimental groups: **C** – a control group – animals cultured under laboratory conditions and fed ad libitum with yeast paste devoid of PS particles; **NPred** – animals cultured under laboratory conditions and fed ad libitum with yeast paste supplemented with 0.04–0.06 μm fluorescent Nile red PS particles (0.001 μL/ml final concentration; Spherotech); **NPpink** – animals cultured under laboratory conditions and fed ad libitum with yeast paste supplemented with 0.4–0.6 μm fluorescent pink PS particles (0.001 μL/ml final concentration; Spherotech); **MPyellow** – animals cultured under laboratory conditions and fed ad libitum with yeast paste supplemented with 1.0–1.9 μm fluorescent yellow PS particles (0.001 μL/ml final concentration; Spherotech).

Each experimental and control group containing 100 adult flies (males and females) was kept for 28 days in embryo collection cages. At the bottom of the cages, grape-agar plates were affixed. The grape-agar plates were changed every two days, with the addition at each change of 1 ml of yeast paste and an appraisal of the condition of the culture.

After the established exposure time for plastic, **adult female and male specimens** were anesthetized using a CO_2 anesthetizing station with a block and blowgun, sectioned for isolation of midguts (the middle region of the digestive system), ovaries, and testes, and prepared for analysis, according to the methods described below. To collect the **larvae**, fresh grape plates were placed on the second to last day of the experiment (~5 PM) and incubated at 25 °C overnight. Next, plates were collected in the morning (~9 AM), and covered with a lid. The embryos were aged 24–30 h to establish plates full of 1st instar larvae that were collected for analysis. Finally, specimens cultivated and used for analysis, in all stages of the developmental cycle (adults, larvae, embryos, and eggs), after use in experiments, were frozen and then disposed of.

2.1. Survival probability

Individuals were taken from the fly stock colony as above. They were divided into a control and three experimental groups of 100 individuals each. The survival rate was checked every two days, and dead individuals were removed. The mortality of males and females was counted separately. The breeding conditions remained the same as described above.

2.2. Fluorescence microscopy

Isolated organs of adult specimens and entire larvae were fixed in 4% formaldehyde (freshly prepared from paraformaldehyde) in PBS (phosphate buffered saline, NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4 8 mM; KH_2PO_4, 1.5 mM, pH 7.4) for 40 min at room temperature and washed in PBS, mounted onto microscopic slides and analyzed under an Olympus BX60 microscope equipped with appropriate filters and an XC50 digital camera (Olympus, Tokyo, Japan) and cellSens Standard software (Olympus, ver. 1.8.1). Another way of analysis was embedding isolated organs in an embedding medium and freezing them. Afterward, the material was sectioned using an MNT cryostat (SLEE medical GmbH)

and analyzed under the same Olympus BX60 microscope.

2.3. Light microscopy and transmission electron microscopy

Isolated organs were prepared using the protocol described in our previous work (Dreszer et al., 2023; Urbisz et al., 2021), which was: fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for one week and then washed several times in the phosphate buffer and postfixed for 2 h in 1% OsO_4 in the same phosphate buffer; washed in phosphate buffer and dehydrated in a graded series of ethanol, replaced with acetone; and embedded in Epoxy Embedding Medium (Sigma-Aldrich, St. Louis, MO, USA). Semi-thin sections (0.8 μm thick) were cut on a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany), stained with 1% methylene blue in 0.5% borax, and examined under an Olympus BX60 microscope equipped with an XC50 digital camera (Olympus) and cellSens Standard software (Olympus, ver. 1.8.1). Ultrathin sections (80 nm) were cut on an RMC Power XT ultramicrotome (RMC Boeckeler, Tucson, AZ, USA). The sections were contrasted for 15 min in uranyl acetate (prepared as saturated solutions in 50% ethanol) and 20 min in lead citrate (Reynolds, 1963) and examined under a Hitachi H500 transmission electron microscope (Hitachi, Tokyo, Japan) at 75 kV.

2.4. Oxidative stress assay

Oxidative stress parameters in adults and larvae of *D. melanogaster* were measured using a MUSE Cell analyzer (Millipore, Billerica, MA, USA) flow cytometer with 2000 events. *D. melanogaster* females and males from each experimental group were anesthetized on ice and the midguts, ovaries, and testis were isolated. Moreover, larvae in the 5th stage were selected. The number of tissues used for the preparation of one sample was determined during the preliminary studies; for the oxidative stress assay: midgut – 2, ovary – 1, testis – 1, larvae – 1; for the glutathione and antioxidant concentration assay: midgut – 5, ovary – 2, testis – 2, larvae – 5. All tissues and larvae were separately placed in 400 μL of a 0.1 M phosphate buffer PBS (pH 7.4). Next, they were gently homogenized in a homogenizer (Minilys, Bertin Technologies) to obtain a cell suspension. According to the manufacturer's recommendations, measurements were taken using the Muse Oxidative Stress Kit (Luminex Corporation, Austin, USA). The ROS (cells exhibiting ROS, ROS(+)) level was measured as the first stage of microplastic-induced redox homeostasis disruption. Measurements were performed after mixing 10 μL of cell suspension and 190 μL of Muse Oxidative Stress working solution incubated for 30 min at 37 °C.

2.5. Glutathione and antioxidant concentration assay

Each of the oxidative stress parameters was measured on a UV–vis spectrometer (TECAN Infinite M200, Austria) on the basis of colorimetric test kits from Sigma-Aldrich (Merck, Darmstadt, Germany). Total glutathione (GSH + GSSH) was tested according to the instructions of the reagent company (Sigma-Aldrich, Germany, Cat.# CS0260). Tissues from *D. melanogaster* males and females and larvae in the 5th stage were frozen in liquid nitrogen, ground in a mortar and mixed with 5% 5-sulfosalicylic acid solution to deproteinize the samples. Samples were incubated at 4 °C for 10 min and then centrifuged at 10,000g for 10 min. The collected supernatant was used for further determinations. A reaction mixture was prepared by adding 150 μL of working mixture to 10 μL of the sample, incubating for 5 min and adding 50 μL of NADPH. Measurements of absorbance were taken at 412 nm for 5 min at 1-minute intervals. The total GSH concentration in every sample was determined using the standard curve based on glutathione standard solutions.

Antioxidant concentration was measured according to the manufacturer's protocols (Sigma-Aldrich, Germany, Cat.# CS0790). For every sample the following procedure was carried out: the midgut of males and females, ovaries, testis as well as the whole larvae were placed in Eppendorf tubes in 100 μL of a 0.1 M Sorensen buffer (pH 7.4). Next, all samples were homogenized and centrifuged (15,000g, 10 min, 4 °C). The submitochondrial fraction was collected and stored at − 70 °C for future analysis. Before analysis, the total protein concentration in every sample was determined according to the Bradford method (1976) using bovine serum albumin (BSA, protein content > 95%, Fluka) as the standard. The reaction mixture was prepared by adding 10 μL of sample, 20 μL of myoglobin working solution and 150 μL of ABTS substrate working solution. After 5 min, 100 μL of stop solution was also added. Absorbance was measured at 405 nm. The antioxidant concentration in every sample was determined using the Trolox standard curve.

2.6. Statistics

All assays for statistical analysis for every method were based on six replicates, performed in duplicate. The results are presented as mean ± SD values. The Kolmogorov-Smirnov test was used to check the normality of the distribution and Levene's test was used to test the equality of variance. If a significant difference was detected, Tukey's range test was used for a post hoc one-way analysis of variance (ANOVA). For each test, the significance level was 0.05.

The survival results were subjected to statistical analysis using a generalized Wilcoxon test according to Gehan. A significance level of $\alpha = 0.05$ was adopted. The graphical interpretation of the above results was presented using Kaplan-Meier probability plots.

The results and graphs were analyzed in GraphPad Prism ver. 9 and Statistica ver. 13 software.

3. Results

3.1. Survival probability and general behavior of flies in culture

Adult specimens of cultured fruit flies in all experimental groups fed with micro and nano PS particles and in the control group were in good condition throughout the experiment (Fig. 1). The individuals fed, were active, and had good motor skills – they flew and climbed up the walls of breeding cages. The females laid eggs intensively. Notably, the number of laid eggs was much smaller in the MPyellow group than in the other experimental and control groups (Fig. 1). Individuals took the feed the same way as those in the control group fed with pure yeast paste. No reduced food intake was noticed in the experimental groups compared to the control.

The probability of survival in the analyzed groups differed statistically significantly between females and males (Table 1). Therefore, separate analyses were conducted for both genders (Fig. 2). Survival curves exhibit a different pattern between males and females. Mortality among males from all study groups was confirmed as early as 24 h into the observation (Fig. 2B), whereas all females survived until the 10th day of the experiment (Fig. 2A).

The highest probability of survival for both genders was observed in individuals from the experimental group MPpink, while the lowest was in the MPyellow group. In the NPred group, the survival curve was similar to the results obtained in the control group.

3.2. Plastic intake

Fluorescence microscopy analysis showed the presence of PS MP and NP in the intestinal lumen (Fig. 3). In the lumen of the midguts of all experimental groups, there were signals emitted by fluorescent PS particles. Strong signals were observed in the case of bigger particles (MPyellow, MPpink) (Fig. 3D-E). In contrast, in the case of the smallest PS particles (NPred), the signal was dispersed and gave off a faint reddish glow and tint in the entire midgut lumen (Fig. 3F).

Additionally, signals of the fluorescent NPpink and NPred particles were noted within the ovaries, specifically inside the cytoplasm of the oocytes and nurse cells, that were developing within the egg chambers,

Fig. 1. Fruit fly culture during the experiment. **A, B)** Adult specimens of cultured flies in the control group and all experimental groups fed with micro and nano PS particles. Note the activity of flies in the culture. **C)** Eggs laid after 28 days of culture. Note the lower number of eggs in the MPyellow group than in the other experimental and control groups.

Table 1
Analysis of survival probability between males and females within the studied control and all experimental groups by Gehan-Wilcoxon test.

	Control	MPyellow	MPpink	NPred
Males – Females	0.00005	0.00000	0.00002	0.00018

Data marked in red are statistically significant p-values ($p \leq 0.05$).

as well as more externally, that is, in the follicular cells that make up the egg chamber envelope (Fig. 3H-J). The largest particles (MPyellow) did not accumulate within the ovaries, and no signals from them were observed by fluorescence microscopy (Fig. 3G).

3.3. Transmission electron microscopy analysis

Transmission electron microscopy (TEM) analysis was conducted to show the exact localization of micro and nano PS particles and to analyze the ultrastructure of the midgut of female (Figs. 4, 5) and male (Figs. 6, 7) specimens of the fruit fly and the ovary (Fig. 8) and testis (Fig. 9). This analysis was conducted to check the proper ultrastructure, capture potential changes after exposure on PS MP and NP particles, and trace and figure out whether the process of forming egg cells and sperm cells occurred appropriately. Because there were no significant differences in the cellular organization of the analyzed organs, i.e., midguts, ovaries, and testes, in all experimental and control groups, the main part of the description below applies to all studied groups. Any ultrastructural differences noted during the analysis in TEM, regarding studied groups, are indicated in the text correspondingly.

3.3.1. Ultrastructure of the midgut

The midgut constitutes the middle region of the digestive system. It is lined with pseudostratified epithelium that rests on a non-cellular basal lamina. The epithelium is surrounded by visceral muscles (Figs. 4–7). In the vicinity of the muscles, the tracheas were also located. Because there were no differences in the ultrastructure of cells in male and female flies, unless otherwise indicated, the following description applies to both sexes. In the midgut epithelium, three types of cells can be distinguished: digestive (enterocytes), secretory (enteroendocrine), and regenerative cells. The most abundant cells were digestive cells (Figs. 4–7). They constitute the main part of the midgut. Cells of other types were few and scattered among digestive cells. Digestive cells were cylindrical in shape, and in their cytoplasm, three regions –basal, perinuclear, and apical – were distinguished. As a rule, the cytoplasm was filled with numerous cell organelles, such as mitochondria, cisterns of the rough endoplasmic reticulum, Golgi complexes, lysosomal granules with different electron densities, and small endosomal vacuoles with electron-lucent content (Figs. 4–7). Autophagosomes, autolysosomes, and lipid droplets were also noted (Figs. 4A,B,D, 5B-E,H,I, 6A,C,D, 7). The cell nucleus of the digestive cells was elongated and oval in shape, with a distinctly electron-dense nucleolus and patches of heterochromatin. The basal cell membranes of digestive cells formed folds and a distinct basal labyrinth in the cytoplasm. The apical cell membrane formed long microvilli (Figs. 4–7). The regenerative and enteroendocrine cells were randomly distributed in the midgut epithelium, located between the digestive cells' basal regions, and rested on the basal lamina. They did not come into contact with the midgut lumen. Their cytoplasm did not show any regionalization in organelle distribution (Figs. 5E, 7B,C).

In the specimens fed with PS particles of all sizes studied (MPyellow, MPpink, NPred) within the midgut epithelium with the proper ultrastructure, as described above, numerous additional structures were observed in the cell cytoplasm, i.e., lamellar bodies (Fig. 4D), autophagosomes with remnants of organelles and electron-dense granules (MPyellow female (Fig. 4D) and male (Fig. 6C,D)). Considerably more of these types of structures were found in the midgut in males of all experimental groups. In MPpink females, autophagosomes with remnants of organelles and electron-dense granules were also noted outside the midgut epithelium between muscles lining the basal lamina (Fig. 5D). In the male of MPpink, lamellar bodies were also visible in the

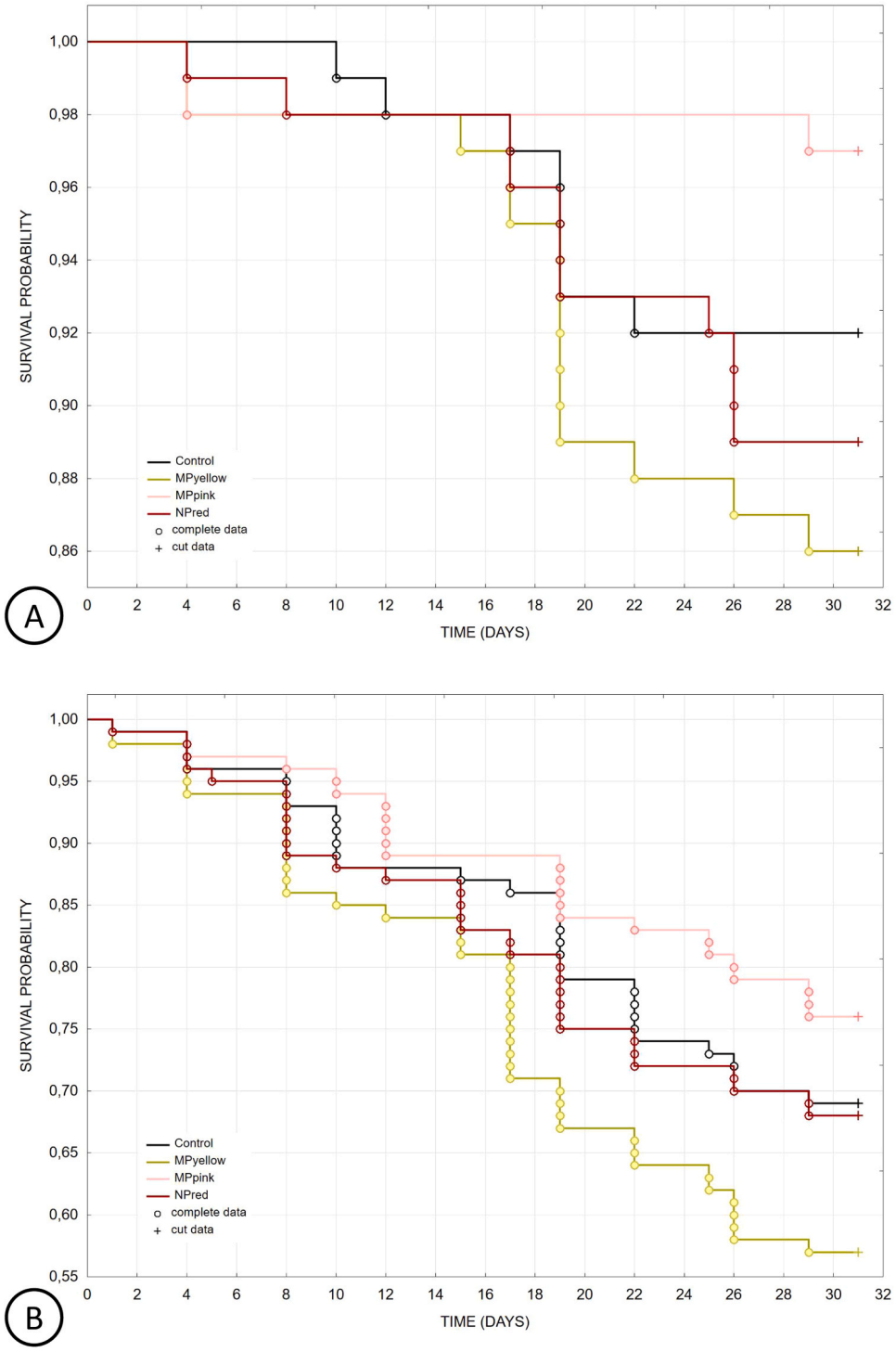

Fig. 2. Probability of imago survival of *D. melanogaster* females (**A**) and males (**B**) depending on the food consumed (control group, with MPyellow, MPpink, NPred) by day, presented in the form of a Kaplan-Meier plot.

cytoplasm of regenerative cells (Fig. 7B). In MPpink males and NPred females, in some enterocytes, the mitochondria moved toward the apical part of the cell and entered the microvilli. Such mitochondria were elongated and filamentous parts filled the microvilli cytoplasm, while their more extensive parts remained in the cytoplasm of the apical part of the cell (Figs. 5F,G, 7D). In NPred females, high and medium electron density granules were visible in the cytoplasm and within the nuclei (Fig. 5J). Cells in the process of degeneration (necrosis) were also noted. Such cells were washed out of the cytoplasm without the organelles, mainly in the apical part (Figs. 5H,I, 7A). Further, in NPred males, numerous autophagosomes with electron-dense grains were located in such cells (Fig. 7E-G). In summary, we observed degenerative changes in the midgut cells, which were more numerous the smaller were the particles fed to the flies, and also more numerous in males than in females of a given experimental group.

3.3.2. *Ultrastructure of the ovary and the course of oogenesis*

Ovaries of all experimental groups showed the proper ultrastructure (Fig. 8C-J) similarly to the control group (Fig. 8A,B). Several ovarioles comprised the single ovary, in which the process of oogenesis occurred,

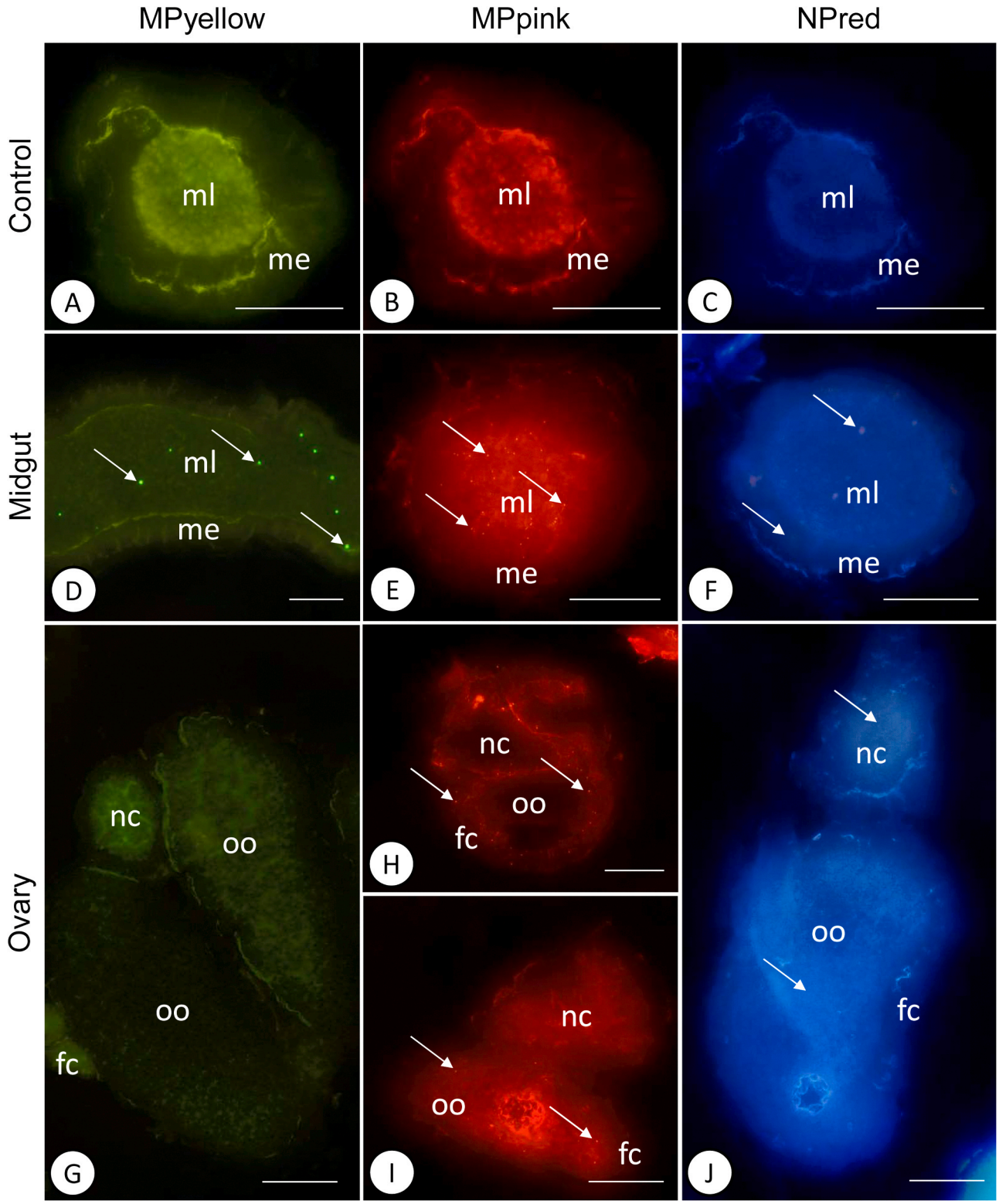

Fig. 3. Localization of fluorescent PS particles in the midgut and ovaries of fruit flies. **A-C)** Midgut of the control group with no fluorescent particles inside. The midgut epithelium and the lumen filled with digestive content show autofluorescence. **D-F)** Midgut of experimental groups feed with PS fluorescent particles. Arrows show the localization of MPyellow (**in D**), NPpink (**in E**), and NPred (**in F**) particles in the midgut lumen (*ml*) and epithelium (*me*). Note: in the smallest NPred particles, the signal was dispersed and is visible as a faint pinkish glow and tint in the entire midgut lumen and epithelium. **G-J)** Ovarian egg chambers of experimental groups. Arrows show the localization of NPpink and NPred particles in the oocytes (*oo*), nurse cells (*nc*), and follicular cells (*fc*). Note the lack of fluorescence in the case of MPyellow particles and dispersed signals as a faint pinkish glow and tint in NPred particles. Fluorescence microscopy, whole-mounted preparation, images in the first column (green fluorescence) were taken using the 488 nm laser and FITC filter, in the second column (red fluorescence) using the 635 nm laser and PE Texas Red filter, and in the third column (blue fluorescence) using the 405 nm laser and DAPI filter, scale bar = 50 μm.

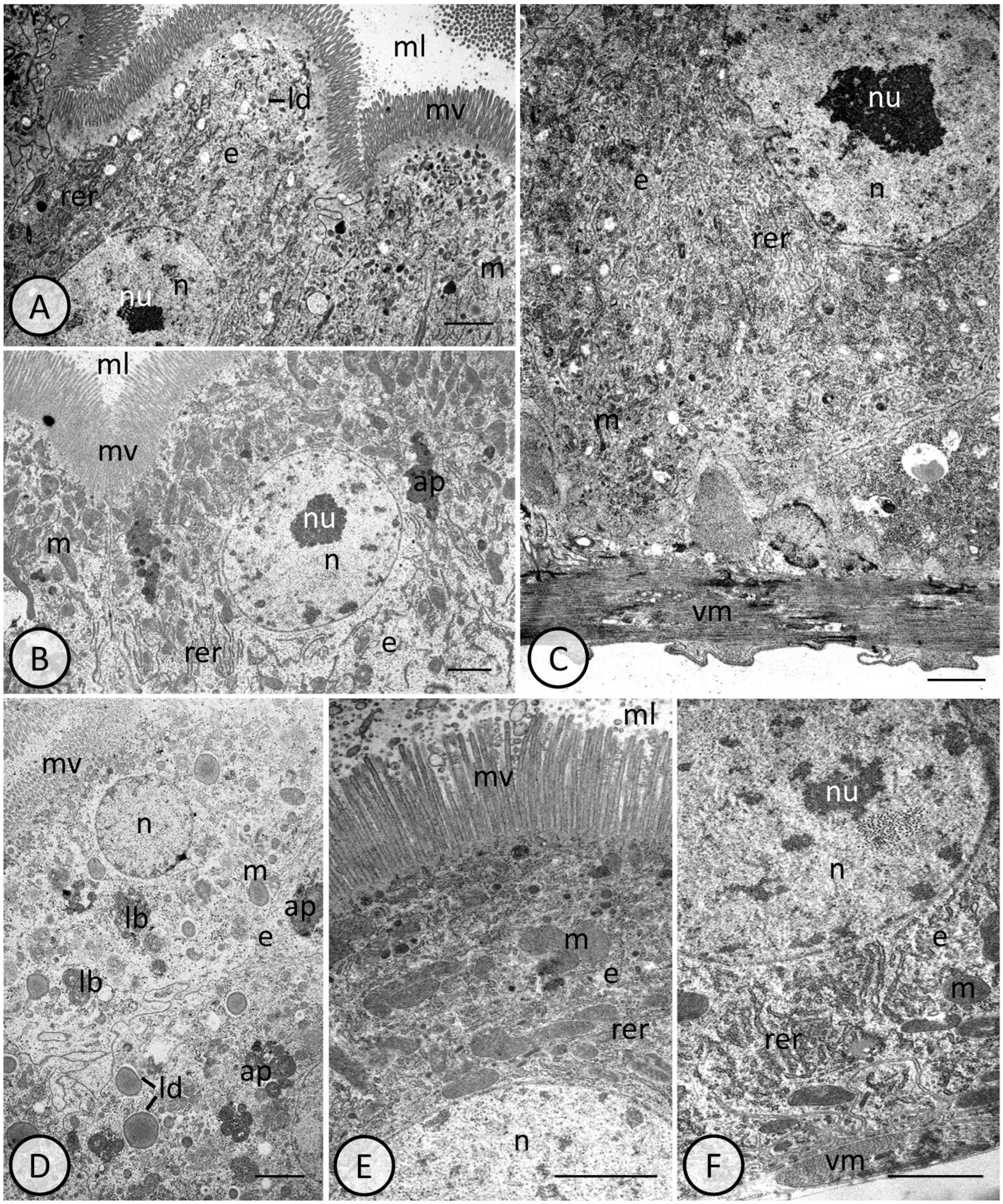

Fig. 4. Ultrastructure of the midgut in the female control group (**A-C**) and MPyellow experimental group (**D-F**). Enterocyte (*e*), nucleus (*n*), nucleolus (*nu*), mitochondria (*m*), microvilli (*mv*) in the apical cytoplasm, cisterns of the rough endoplasmic reticulum (*rer*), lipid droplets (*ld*), autophagosome (*ap*), lamellar bodies (*lb*), midgut lumen (*ml*), visceral muscles (*vm*). Transmission electron microscopy, scale bar = 2.0 μm.

and the egg cells were formed (not shown). Ovarioles consisted of egg chambers in a linear arrangement. As oogenesis progressed, in each egg chamber, one oocyte developed. This cell became large and the cytoplasm became filled with numerous cell organelles such as mitochondria, cisterns of endoplasmic reticulum, Golgi complexes, small micropinocytotic vesicles, and electron-dense storage material (yolk) (Fig. 8B, D, E, G). In large vitellogenic oocytes, the first egg envelope (called the vitelline envelope) was deposited outside the oolemma in the form of a continuous and smooth layer of medium electron density (Fig. 8E). The rest of the cells in the egg chamber developed and functioned as nurse cells. Nurse cells showed characteristics of very active cells. They possessed large nuclei with irregular outlines and prominent

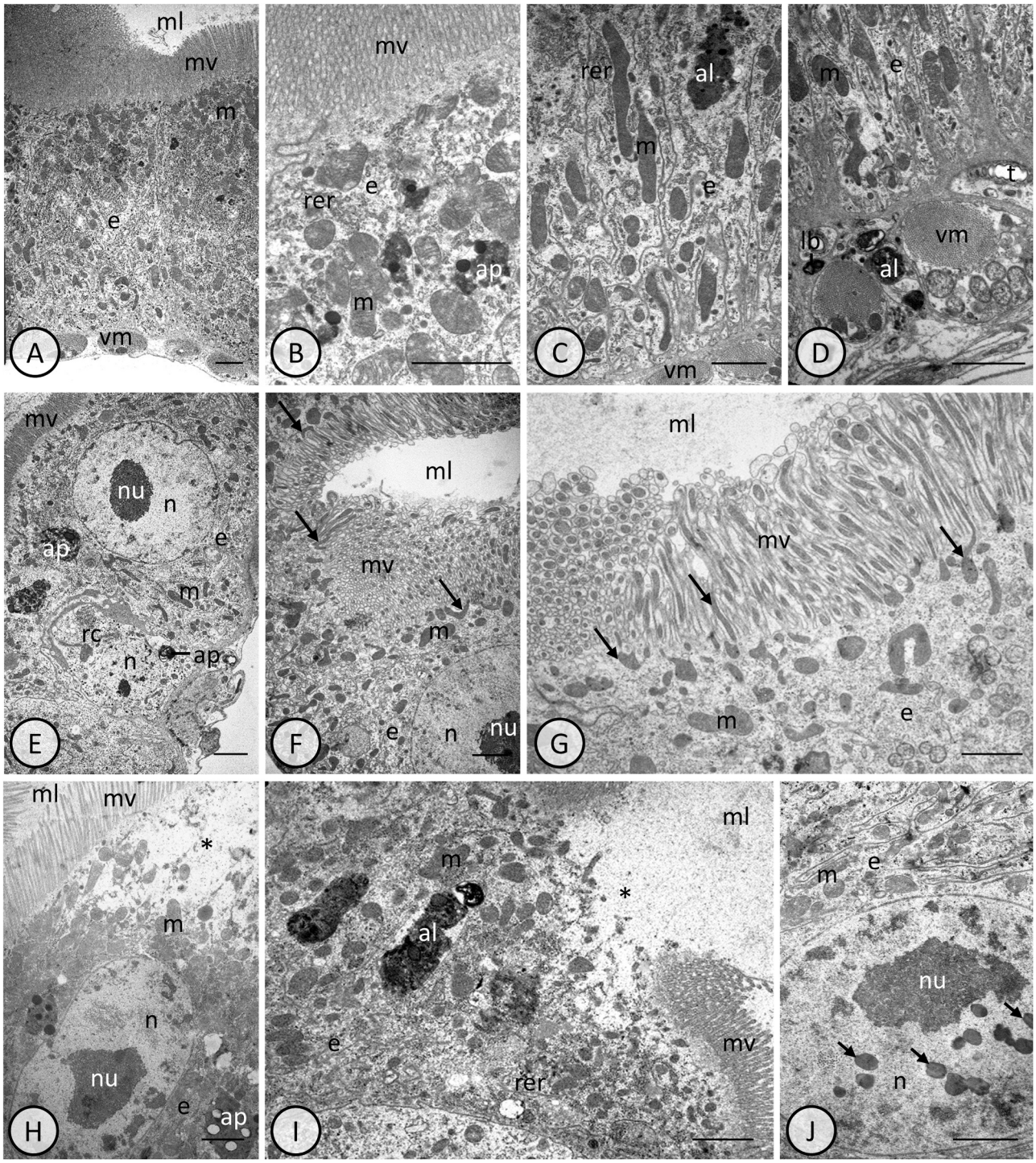

Fig. 5. Ultrastructure of the midgut in the female MPpink (**A-D**) and NPred experimental groups (**E-J**). Enterocyte (*e*), nucleus (*n*), nucleolus (*nu*), mitochondria (*m*), microvilli (*mv*) in the apical cytoplasm, cisterns of the rough endoplasmic reticulum (*rer*), autophagosome (*ap*), autolysosome (*al*), lamellar bodies (*lb*), midgut lumen (*ml*), visceral muscles (*vm*), trachea (*t*). Note also: autophagosomes and autolysosomes in muscle cells and regenerative cells (*rc*), mitochondria localized in the apical cytoplasm which entered the microvilli (*arrows*), degeneration of enterocytes indicated by their being washed out of the cytoplasm without the organelles, mainly in the apical part (*asterisks*), and medium electron density granules within the nuclei (*short arrows*). Transmission electron microscopy, scale bar = 2.0 μm.

electron-dense nucleoli. Numerous cell organelles, such as mitochondria, cisterns of endoplasmic reticulum, Golgi complexes, and small micropinocytotic vesicles, were observed in their cytoplasm (Fig. 8A-C, F,H). Ring canals connecting the neighboring cells within a syncytial cyst were observed. The ring canals were in the form of cytoplasmic channels and possessed an electron-dense inner rim (Fig. 8H). Both oocyte and nurse cells were enveloped by the epithelium made up of follicular cells (Fig. 8B-G). Follicular cells were cube-shaped with large spherical nuclei and prominent, usually spherical electron-dense nucleoli.

The ultrastructure of germ cells as well as follicular cells indicates the intensive and proper process of oogenesis. It was noted that in the NPred experimental group, lamellar bodies and autophagosomes/autolysosomes with electron-dense remnants were present in the oocyte and in

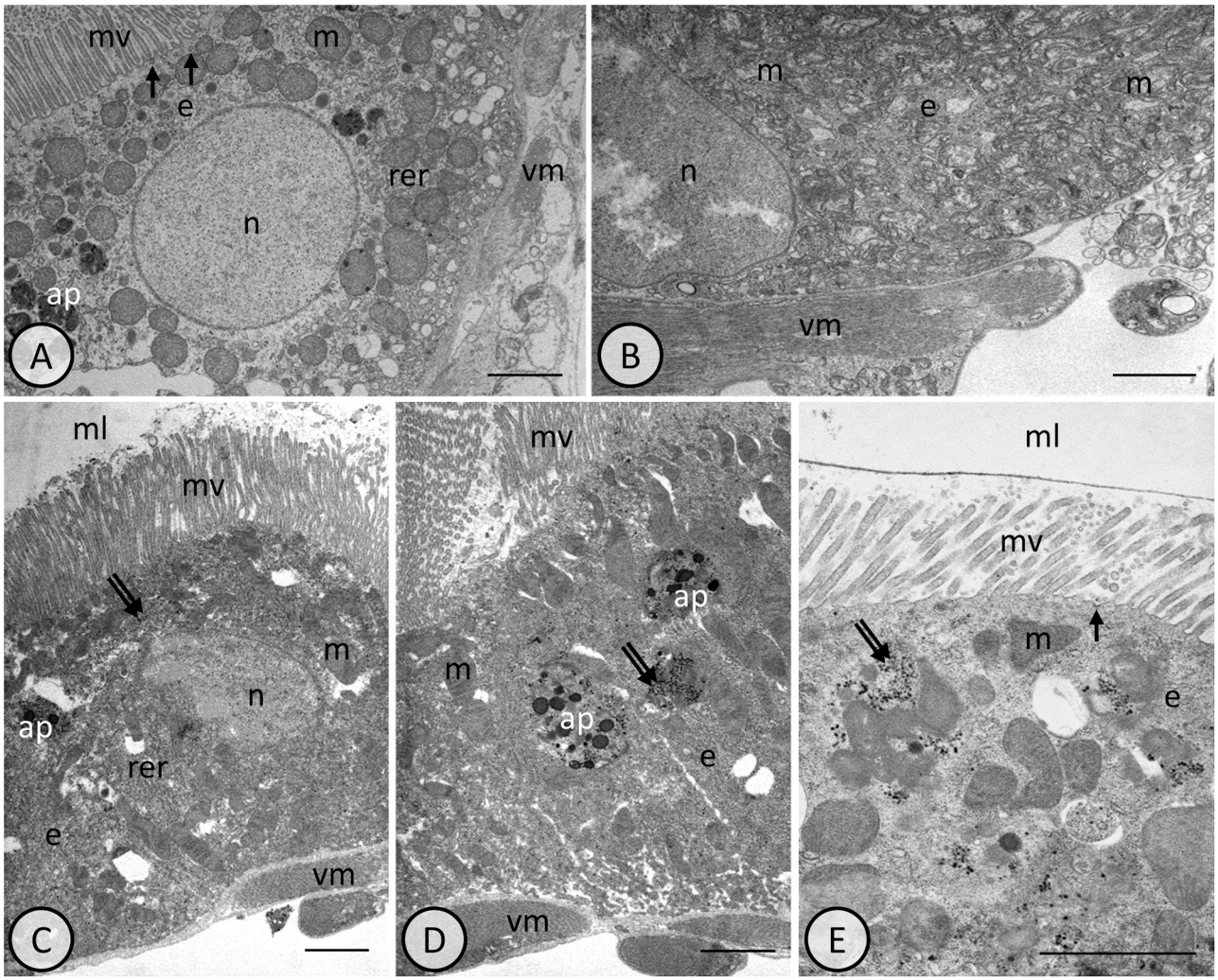

Fig. 6. Ultrastructure of the midgut in the male control group (**A-B**) and MPyellow experimental group (**C-E**). Enterocyte (*e*), nucleus (*n*), mitochondria (*m*), microvilli (*mv*), cisterns of the rough endoplasmic reticulum (*rer*), autophagosome (*ap*), electron-dense granules (*double arrows*), endocytosis (*arrows*), midgut lumen (*ml*), visceral muscles (*vm*). Transmission electron microscopy, scale bar = 2.0 μm.

somatic cells from the ovarian sheath/follicular cells (Fig. 8I,J).

3.3.3. *Ultrastructure of the testis and the course of spermatogenesis*

Testes showed the proper organization and ultrastructural details in all experimental groups (Fig. 9C-H) fed with PS particles and in the control group (Fig. 9A,B). As a rule, numerous syncytial cysts were located and developing along the long axis of the testis. Such cysts were in the consecutive stages of spermatogenesis. Packages of male germ cells developing within each cyst and being in the successive stages, such as spermatogonia, spermatocytes (not shown), and spermatids (Fig. 9), were usually observed. Spermatids undergo synchronous differentiation. Numerous changes occurred at a subcellular level, such as remodeling existing organelles (changing the shape of the cell nucleus from spherical to elongated and condensation of chromatin, localization of mitochondria below the cell nucleus, in the region of the future middle piece of the sperm), formation of new structures characteristic for sperm cells (i.e. acrosomes, flagellum with axonemes, and middle piece), and polarization of elongating spermatids and plasma membrane addition (Fig. 9). At the end of spermatid morphogenesis, cytoplasmic components not needed in mature sperm were stripped away (excess cytoplasm in the form of residual bodies were observed) (Fig. 9), and individualization of sperm occurred.

The ultrastructure of analyzed fruit fly testes indicated the occurrence of an intensive process of spermatogenesis in all experimental groups and the control group, and no changes or disturbances were observed.

3.4. *Cellular stress markers*

Fig. 10 illustrates markers of oxidative stress: the generation of reactive oxygen species (%ROS+ cells), antioxidant concentration, and GSH concentration in adult female and male *D. melanogaster*, while Fig. 11 shows the same parameters in *D. melanogaster* larvae. Additionally, a comparison between the levels of the studied parameters in the midgut of females and males was made, as presented in Table 2.

The oxidative stress markers in adult individuals showed tissue and sex dependence and also differed according to the size of the microplastic used (Fig. 10). The results obtained from samples of insects in the larval stages were analogous to those of adults, but their levels were significantly lower. In the larval stages, the highest level of cellular changes was confirmed after application of NPred microplastic. In the MPyellow group, a significantly lower level of %ROS+ cells and GSH concentration was demonstrated compared to the control group and other experimental groups (Fig. 11).

The level of each marker tested in adults was significantly higher in the midguts than in the sex organs. The most significant differences concerning the control group were observed for the MPpink and NPred groups. In the ovaries, the generation of reactive oxygen species was

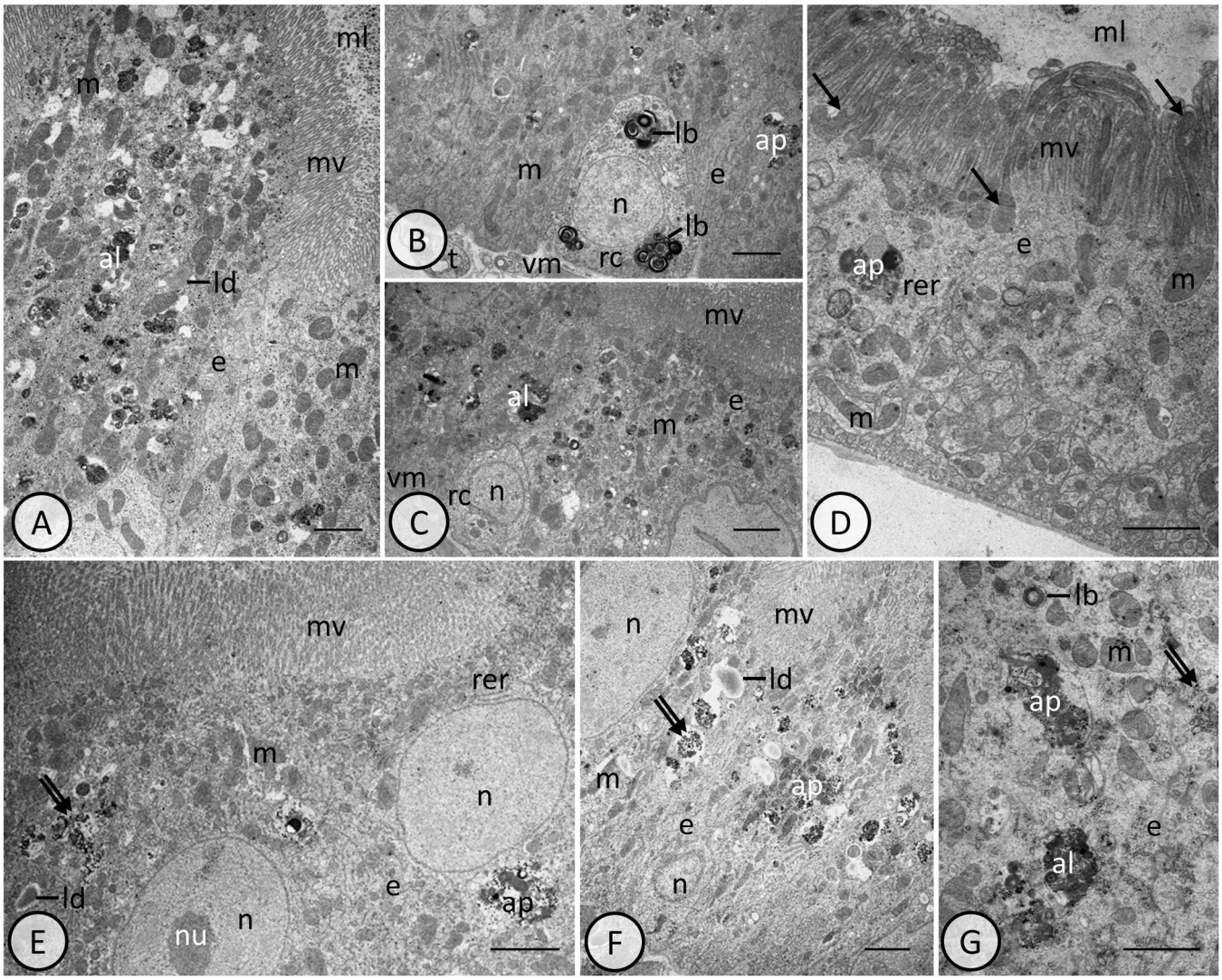

Fig. 7. Ultrastructure of the midgut in the male MPpink (**A-D**) and NPred experimental groups (**E-G**). Nucleus (*n*), nucleolus (*nu*), mitochondria (*m*), microvilli (*mv*), cisterns of the rough endoplasmic reticulum (*rer*), lipid droplets (*ld*), midgut lumen (*ml*), visceral muscles (*vm*), trachea (*t*). Note: numerous autophagosomes (*ap*) and autolysosomes (*al*) in enterocytes (*e*) and lamellar bodies (*lb*) in regenerative cells (*rc*), single and accumulated electron-dense granules (*double arrows*), and mitochondria localized in the enterocyte apical cytoplasm which entered the microvilli (*arrows*). Transmission electron microscopy, scale bar = 2.0 μm.

1.87 times higher in the MPpink group than in the control group and 1.79 times higher in the NPred group (Fig. 10 I). A similar trend was observed in the testis (Fig. 10 II). In the midgut, the highest generation of reactive oxygen species was observed in the NPred groups, where % ROS + cells were 1.65 times higher than the control group for both females and males. However, the level of this parameter in males was significantly higher than in females (Table 2).

The concentration of antioxidants in all experimental groups differed significantly from the control group. In the ovaries, the level of antioxidants was about 1.5 times higher in all groups with applied microplastic than in the control group (Fig. 10 III). On the other hand, in the testis, the level was significantly higher only in the NPred group (Fig. 10 IV). The greatest differences were confirmed in the midgut with antioxidant levels in males being almost 2 times higher than in females in each experimental group (Table 2). In males, the concentration of antioxidants in the MPyellow group was significantly lower than in the control group, while it was significantly higher in both sexes in the MPpink and NPred groups.

Like the previous parameters, GSH concentrations in the ovaries and testes were the highest in the NPred group, but in the MPpink group they were also significantly higher than in the control group (Fig. 10 V, VI). The microplastic content of the diet also generated high GSH levels in the midgut of females and males (Fig. 10 V, VI). At the same time, the concentration of total glutathione in males was significantly higher than in females (Table 2). The largest increase in GSH levels was observed in the midgut of females in the NPred group, with levels 2.6 times higher than in the control group.

4. Discussion

Microplastic pollution is believed to be one of the most widespread and long-lasting changes on a global scale. Scientific attention on this topic has tremendously increased in recent years, mainly in marine and freshwater environments. In recent years, our understanding has been that microplastics have a significant impact on terrestrial systems, and it is a global change stressor. Scientific interest in this topic continues to grow. As the nature of microplastics is very complex and depends on such parameters as their type, particle size, modifications, additives used, and degradation processes, conducting research is often a challenge (de de de Souza Machado et al., 2018). At the same time, voices are being raised about the necessity and importance of research in different fields and at different biological degrees of sophistication, such

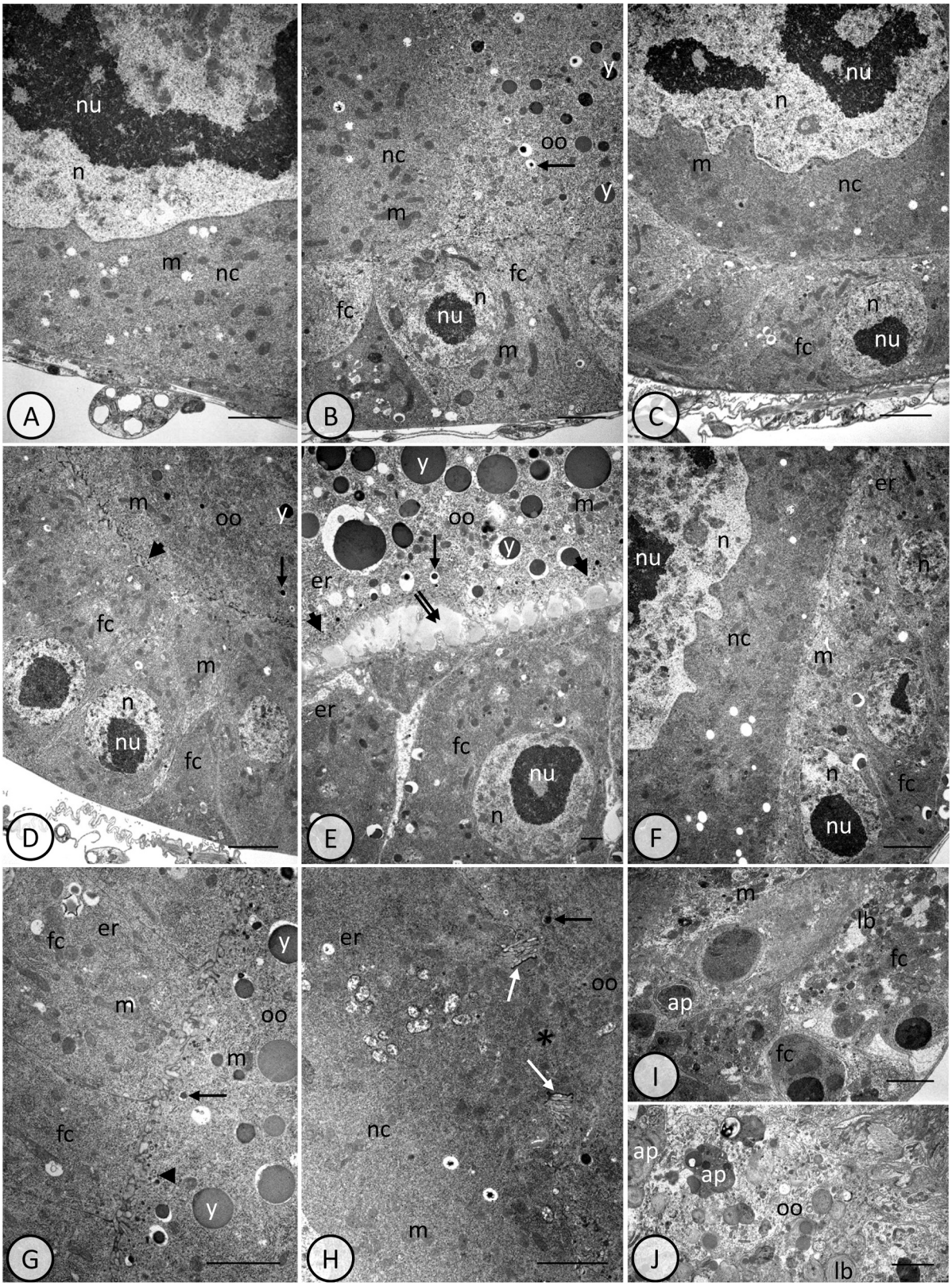

Fig. 8. Ultrastructure of ovaries of the control group (**A-B**) and experimental groups: MPyellow (**C-D**), MPpink (**E-F**), and NPred (**G-J**). Note growing oocyte (*oo*), nurse cell (*nc*), and somatic follicular cells (*fc*). Ring canals connecting the neighboring cells are also visible (*asterisk*), and they possess an electron-dense inner rim (*white arrows*). Nuclei (*n*), nucleolus (*nu*), mitochondria (*m*), cisterns of endoplasmic reticulum (*er*), small micropinocytotic vesicles (*arrowheads*), numerous small electron-dense granules that are the precursor of storage material (*dark arrows*), and electron-dense storage material – yolk (*y*). Continuous, smooth vitelline envelope on the oocyte surface (double arrows). Note in the NPred experimental group (**I, J**) the lamellar bodies (*lb*) and autophagosomes (*ap*) with electron-dense remnants in the oocyte and in follicular cells. Transmission electron microscopy, scale bar = 3.0 μm.

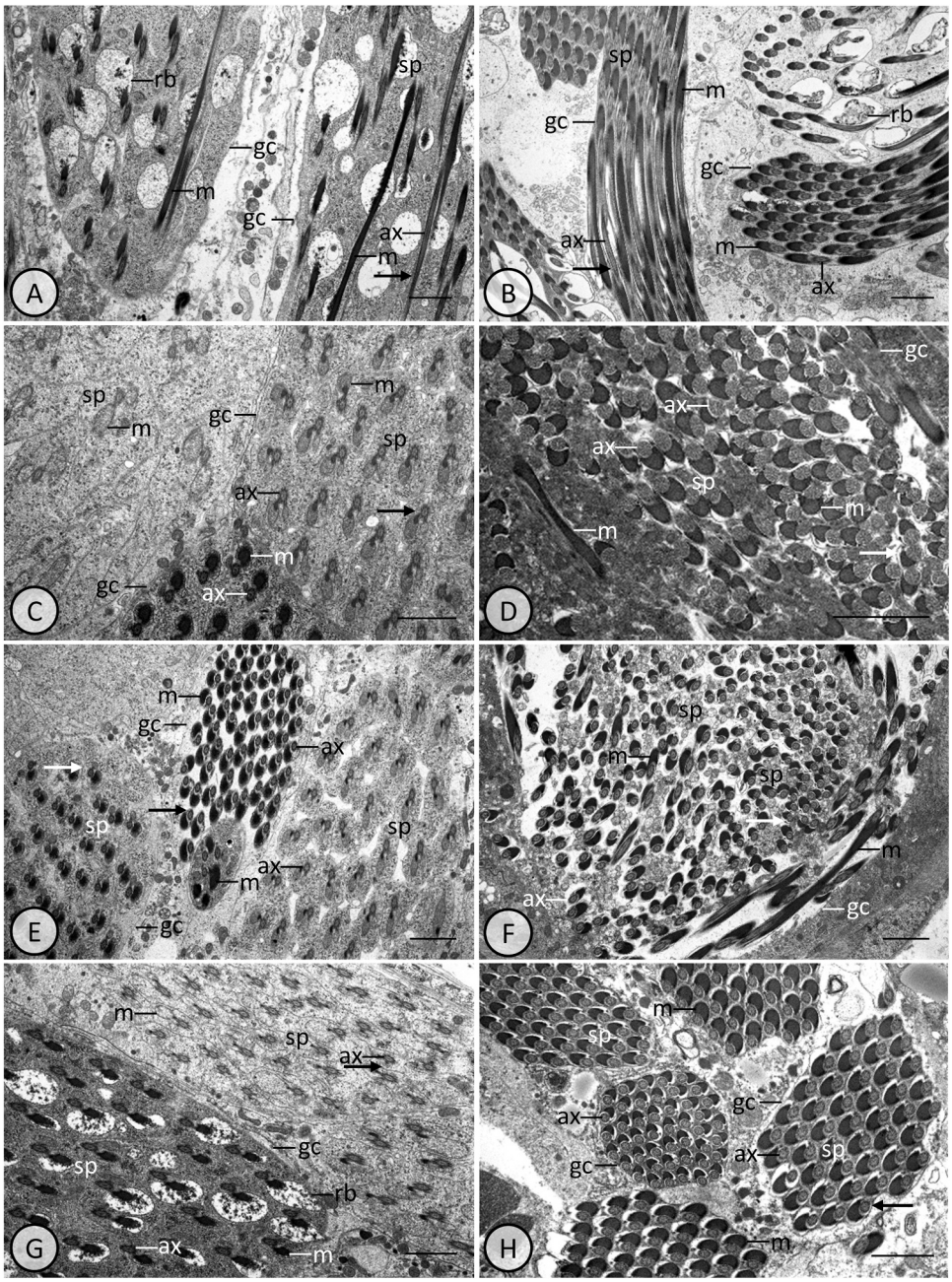

Fig. 9. Ultrastructure of testes of the control group (**A-B**) and experimental groups: MPyellow (**C-D**), MPpink (**E-F**), and NPred (**G-H**). Germ-line elongated cysts (*gc*) containing synchronically developing spermatids (*sp*) are visible. Cross and oblique sections of spermatids at different levels across their long axes show individual structures characteristic of elongated cells; note the axonemal sheath marked by *arrows*, axonemes (*ax*) from the forming flagellum, and mitochondrial derivatives (*m*) from the forming middle piece. The excess cytoplasm in the form of residual bodies (*rb*) is also marked. Transmission electron microscopy, scale bar = 2.0 μm.

as ecological, ecotoxicological, environmental, in vitro cell and tissue laboratory studies, and in vivo laboratory studies. Studies that can assess plastic particles' toxicity and environmental risks are critical and should not be overlooked. Each such study provides new data and expands our awareness of the risks to the environment, to humans and animals, and makes us aware of the scale of the challenge we face (de Souza Machado et al., 2018). Here we focused on particles of one commonly used and widespread plastic – PS – and their micro- and nano-size-dependent impact on living organisms.

Invertebrates, especially insects, are an excellent target for such studies due to their abundance and importance in terrestrial ecosystems. In the present study, we focused on a terrestrial organism commonly used as a model in biological studies, the fruit fly *Drosophila melanogaster*. *D. melanogaster* has been a model in research fields such as developmental biology and genetics, while its utility in toxicological studies has only recently become widely recognized (Barik and Mishra, 2019; Buszczak and Cooley, 2000; Chintapalli et al., 2007; Hudson and Cooley, 2014). There are many reports devoted to certain environmental contaminants and toxic substances studied on *D. melanogaster*, including mercury, manganese, arsenic, lead, ethanol, pesticides, solvents, and nanoparticles (Barik and Mishra, 2019; Rand et al., 2014). The significance of the fruit fly model for understanding the human response under stress conditions has been broadly accepted, as we have been able to understand the wealth of highly conserved genes and pathways controlling development, the stress response, and metabolism in these two species. *D. melanogaster* is commonly used not only for comparative

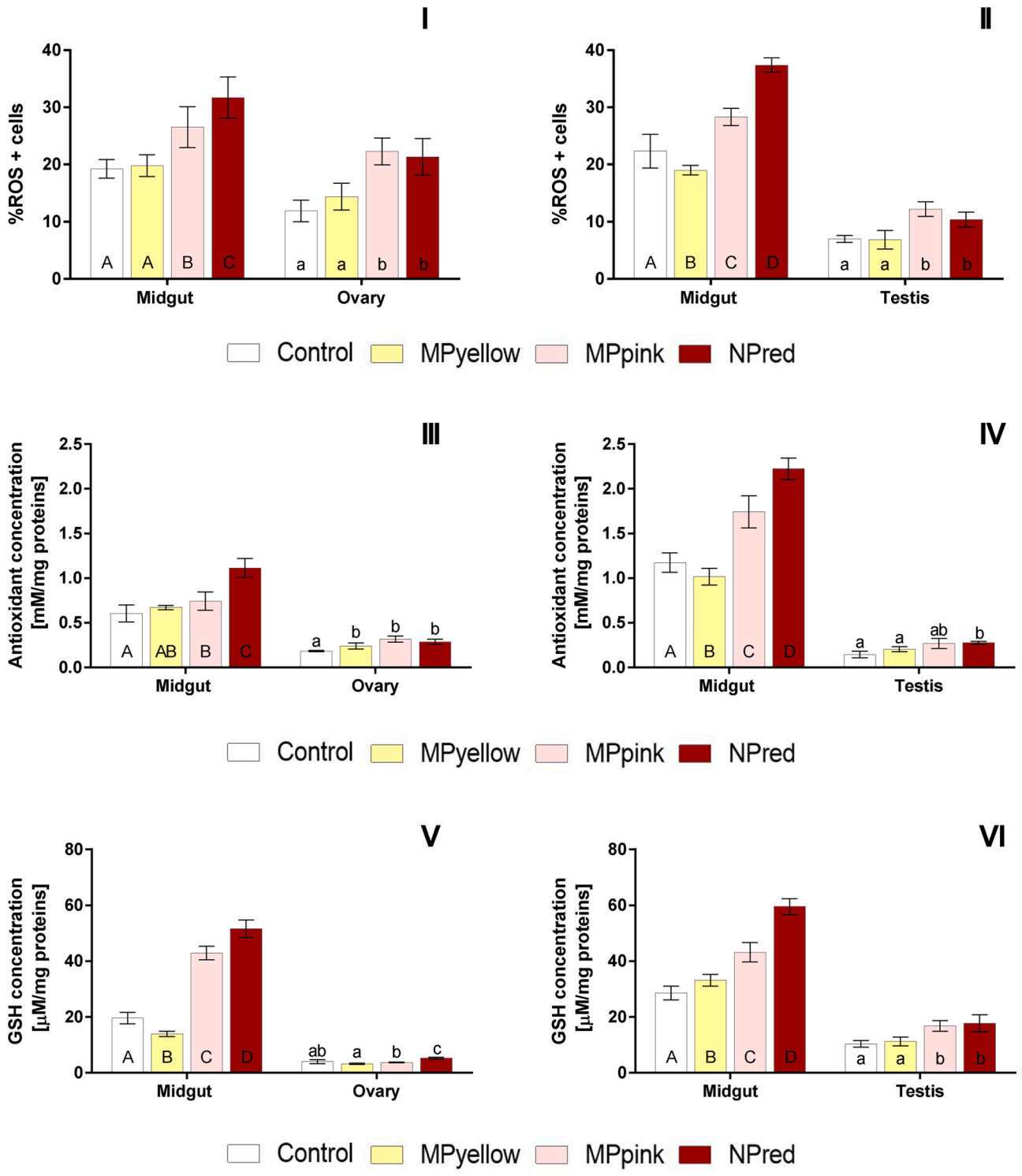

Fig. 10. Level of oxidative stress markers [mean ± SD] by calorimetric methods: antioxidant concentration, total glutathione concentration (GSH), and flow cytometer: reactive oxygen species (%ROS + cells) in *Drosophila melanogaster* females (**I, III, V**) and males (**II, IV, VI**). Different letters indicate statistically significant differences: **A-D** differences between experimental groups in the midguts, **a-d** differences between experimental groups in ovaries and testes (Tukey's multiple comparisons test, $p \leq 0.05$, $n = 6$).

purposes of ongoing processes in humans but also due to the fact that it is a representative of common and globally distributed terrestrial invertebrates (Barik and Mishra, 2019; Buszczak and Cooley, 2000; Chintapalli et al., 2007; Hudson and Cooley, 2014). That is why the observation and analysis of such parameters as survival, reproduction, changes in the midgut, and tissue and cell responses in exposed insects, as we have performed here, can be an indication of possible toxic effects of the test substances that can be expected in other taxa in terrestrial ecosystem. In the present study, we investigated the impact of PS micro- and nanoparticles on midgut epithelium – which is the first place of direct contact and the site of most significant exposure to the supply of plastic from food in adult *D. melanogaster* individuals. Twenty-eight days of exposure to plastic, in the case of the fruit fly, represents most of its life span, making it possible to determine the long-term effects of plastic

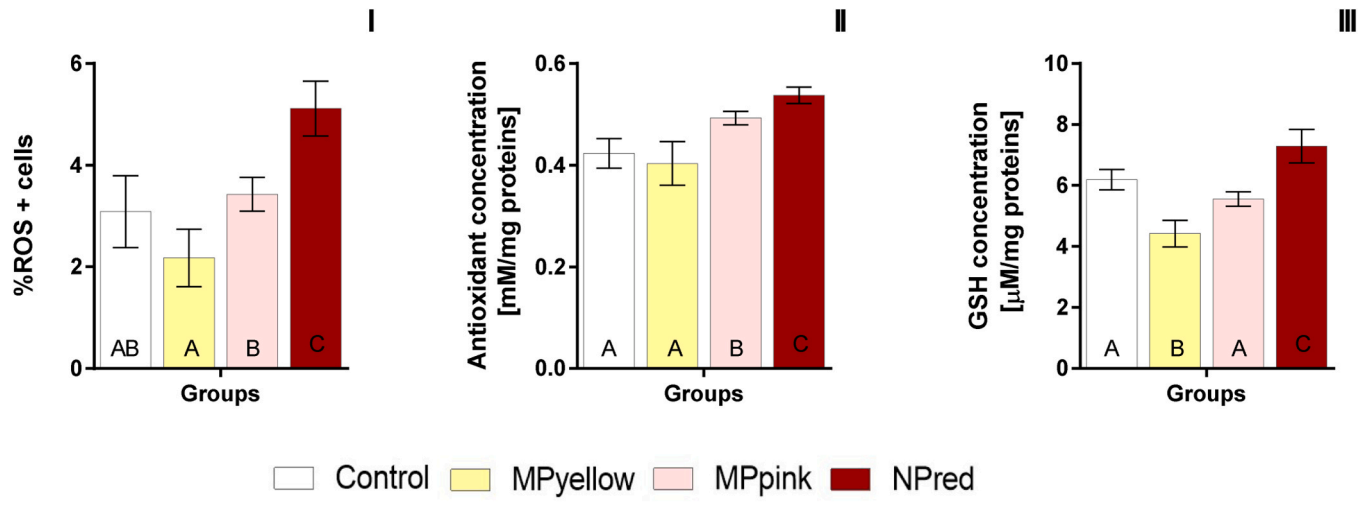

Fig. 11. Level of oxidative stress markers [mean ± SD] in *Drosophila melanogaster* larvae, by flow cytometry: reactive oxygen species (ROS + cells) (**I**) and calorimetric methods: antioxidant concentration (**II**), total glutathione concentration (GSH) (**III**). Different letters indicate statistically significant differences between experimental groups (Tukey's multiple comparisons test, $p \leq 0.05$, $n = 6$).

Table 2
Analysis of variance (ANOVA) in oxidative stress markers tested between females and males.

	Control F-Control M	MPyellow F-MPyellow M	MPpink F-MPpink M	NPred F-NPred M
%ROS + cells	0.1212	> 0.9999	0.7961	0.0007
Antioxidant concentration	< 0.0001	< 0.0001	< 0.0001	< 0.0001
GSH concentration	< 0.0001	< 0.0001	> 0.9999	< 0.0001

Data marked in red are statistically significant p-values ($p \leq 0.05$).

supply on the studied individuals (Barik and Mishra, 2019). Moreover, as different sexes may show different tolerance and response to a given factor in the environment, examination and comparison of experiments between males and females, as performed here, also carries additional cognitive value. To assess the impact of size-dependent PS particles on reproductive organs – testes in males and ovaries in females – and the impact on gamete formation was also interesting in the context of the impact on future generations and the known defense mechanisms in the germ line. Finally, we investigated the larval stage and possible changes in cellular response levels. Testing fruit fly embryos or larvae is widespread and various analyses have been conducted, focusing on different parameters and testing agents (Barik and Mishra, 2019; Rand et al., 2014).

4.1. *Effects at the organism level*

Plastic particles given with food did not interfere with food intake in adult fruit flies. It is known from the literature that plastic particles of different sizes can cause various food intake problems in animals, especially aquatic ones, and this is associated with accumulation or blockage of the digestive tract, particularly by larger particles. Generally speaking, plastic particles of different sizes can be ingested or inhaled, causing pseudosatiation and blockage of the digestive tract, or abrasion and irritation of the mucosa (de Souza Machado et al., 2018). Micro- and nanoparticles of PS given in the food had no negative effect on the lifespan of the studied fruit flies. The culture was in good condition, the individuals were active, and the food supply was not reduced or restricted over time. A change in the mortality (survival probability) of individuals was noted, but this was due to the length of their lives and the length of time they were kept breeding rather than a direct effect of the plastic. Our observation agrees with the general trend noted in the literature and the lower survival probability of both sexes observed in the experimental group fed the largest microplastic (MPyellow in our case). However, we did not observe a similar relationship in the smaller particles (MPpink and NPred). The lower survival in the MPyellow group was also reflected in fewer eggs laid in this group than in the groups fed with smaller PS particles and the control group. This observation is similar to other studies, as in marine zooplankton – copepods – in which PS micro- and nanoplastics had negative effects, including decreased survival and retarded development, especially diminution of reproduction (Lee et al., 2013). Also, Pacific mole crab, experimentally exposed to plastic (polypropylene), had higher mortality than the control group. The authors suggested that larger particles might induce a decrease in fecundity which could be attributed to insufficient nutrition or the inhibition of digestion due to the large number of microplastics ingested. What is more, limitation of food quantity led to low egg production in studied copepods and a decrease in the number of days that crabs held viable eggs (Horn et al., 2020; Lee et al., 2013).

4.2. *Impact of PS particles in midgut*

In terrestrial invertebrates, such as insects, toxic substances and nanoparticles can enter the body through the trachea and the digestive system. The midgut is an essential part of this system in which the intense processes constitute a barrier against the effects of these agents on the body. Changes in the midgut epithelium after exposure to stressors from the external environment have been observed in numerous studies on invertebrates (Ostróżka et al., 2022). The changes in the midgut epithelium may involve degeneration processes, necrotic changes, and cell death processes, such as autophagy and organelle alteration. Mainly the mitochondria are the first organelles to react to any changes and manifest this, among other things, by changes in their ultrastructure (Ostróżka et al., 2022). Therefore a thorough analysis of fruit fly midgut ultrastructure under TEM is essential to show any changes caused by the studied different-size PS particles. Our recent study focused on using the fruit fly midgut to assess the ability of nanoparticles to enter the body and confirmed the intake and passing of the cell boundaries of the midgut epithelium in fruit fly by such particles as [70]fullerene nanomaterial (Dreszer et al., 2023). In the case of such promising nanomaterials as fullerenes, with the potential for extensive application, it is clear that the first step for their use is to characterize their interaction with cells, especially in terms of toxicity and internalization pathways. In the case of micro- and nanoplastic, their possible

intake or internalization is undesirable and can cause unwanted effects at the cell and tissue levels. Here we found that the PS particles did not significantly disrupt midgut function because the ultrastructure of most epithelial cells demonstrated proper ultrastructure. However, besides these proper cells, we observed degenerative changes in the midgut cells, which were more numerous the smaller were the particles supplied to the fruit flies, and also more numerous in males than females of a given experimental group. Most alterations occurred in the formation in the cytoplasm of enterocytes, lamellar bodies, autophagosomes/residual bodies, and cell degeneration (necrosis). Some changes, such as autophagosomes, also occur in regenerative cells and muscle cells. These observations indicate that PS micro- and nanoparticles studied here can impact the midgut and induce a strong cell response.

According to the literature, cell death can be activated by many stressors coming from the external environment, and this process plays a role in eliminating damaged or unwanted cells from tissues and organs. Among numerous types of cell death reported in animals, three are best known: apoptosis, autophagy, and necrosis. They are common processes that occur in the intestinal epithelia of invertebrates, and these processes can be interlinked (Klionsky et al., 2016; Rost-Roszkowska et al., 2018; Teixeira et al., 2013). It is believed that autophagy initially plays the role of a survival factor but eventually causes cell death. When the cytoplasm becomes rich in autophagic structures, apoptosis and/or necrosis can be activated (Klionsky et al., 2016; Levine and Yuan, 2005; Rost-Roszkowska et al., 2016).

The ultrastructure analysis in the fruit fly midgut epithelium suggests that the negative effects of micro- and nanoparticles of PS correlate with the plastic size. In the case of microplastic particles (0.4–1.9 μm – MPyellow and NPpink groups), their impact is not acute and involves activation of the process of cell rescue – autophagy, leading to cell death. Larger microplastic particles (MPyellow) caused the cell changes only in the main midgut epithelial cells – enterocytes. At the same time, the smaller ones (MPpink) also activated autophagy in regenerative cells and muscles. The most significant changes were observed after exposure to the smallest particles. These consisted of both autophagy and degeneration – necrosis of the midgut epithelial cells. Thus, in the examined fruit flies exposed to the nanoplastic, the initial autophagy activated necrosis and led to the degeneration of midgut cells. It agrees with the literature suggesting that autophagy is a pro-survival process enabling organisms to maintain homeostasis, while necrosis can be its consequence when the accumulated alterations are too many and when the cell is no longer able to save itself (Ostróżka et al., 2022; Rost-Roszkowska et al., 2018; Tettamanti et al., 2019). A similar phenomenon was observed in the midgut epithelium in such invertebrates as tardigrades, and the stressor, in that case, was the infection of microsporidia (Rost-Roszkowska et al., 2018, 2013). There are also some reports of abnormal ultrastructure of intestinal epithelial cells in brine shrimp cultured with 10 μm PS microparticles. In this case, alteration involved fewer disordered microvilli, an increased number of mitochondria, and the formation of autophagosomes. According to the authors, such larger particles could affect nutrient absorption and energy metabolism (Wang et al., 2019).

It is worth paying attention to one specific ultrastructural observation we made in the midgut of a fruit fly after exposure to micro- (NPpink) and nanoparticles (NPred). In the cytoplasm of some enterocytes, the mitochondria became filamentous, moved toward the apical part of the cell, and entered the microvilli. Generally, mitochondria are known to be dynamic organelles. They are among the first organelles that react to changing conditions and stressors within cells and tissues. Two opposing processes – mitochondrial fusion and fission – have been demonstrated as such cellular responses. During mitochondrial fusion, individual organelles are merged into more extensive mitochondrial tubules or networks, while fission leads to the networks splitting into individual mitochondria. The balance between these processes has implications for mitochondrial morphology and their distribution throughout the cell, which in turn is directly linked to mitochondrial function (Chan, 2006; Hoitzing et al., 2015; Ramalho-Santos et al., 2009; Scott and Youle, 2010; Westermann, 2012). Mitochondrial fusion has been described in different cell types. Also the molecular mechanisms of these events are well known from model yeast and mammalian tissue cell cultures (Benard and Karbowski, 2014, 2009; Cherok et al., 2017; Dimmer and Scorrano, 2006; Mishra and Chan, 2016; Yang et al., 2016). Additionally, changes in mitochondrial ultrastructure can be associated with activation of cell death in different tissues and organs (Mannella, 2008; Orrenius, 2004; Willems et al., 2015). For example, recent data have shown that a mitochondrial pathway and changes in mitochondrial morphology are major mechanisms for cell death activation in the fruit fly mid-oogenesis. In egg chambers, mitochondria form extensive tubular networks, while the lack of nutrients causes the degeneration of egg chambers and the formation of clusters of mitochondria (Tanner et al., 2011; Tanner and McCall, 2011). Similarly, in *C. elegans*, mitochondrial morphology changes during the process of germ cell differentiation from globular (predominantly in the distal gonad arm) to more elongated organelles (in the proximal gonad arm) (Charmpilas and Tavernarakis, 2019). It is also stated that mitochondrial fusion allows more effective ATP production and better distribution in cell compartments (Glancy et al., 2020; Twig et al., 2008), but a secondary aim is to keep the mitochondria in good condition and protect them from the effects of mitochondrial damage or mutations (Otera and Mihara, 2011; Shutt and McBride, 2013; Zemirli et al., 2018). In particular, mitochondrial fusion may compensate for minor damage, whereas severe damage or mutations lead instead to mitochondrial fission, splitting of defective mitochondria, and finally their elimination, e.g. by autophagy (Benard and Karbowski, 2009; Chan, 2006; Garbern and Lee, 2021; Makino et al., 2010; Picard et al., 2013; Shutt and McBride, 2013; Twig et al., 2008; Youle and Bliek, 2012). Considering the above findings, our observations of long filamentous mitochondria localizing in the microvilli of the midgut epithelium of flies from the MPpink and NPred experimental groups may be an expression of mitochondrial fusion. They may represent a protective mechanism in cells exposed to the stressor studied. On the other hand, the mitochondria in the microvilli may facilitate the production and supply of energy that is not necessary for nutrient absorption. A similar phenomenon was described in older papers devoted to Malpighian tubules (Bradley, 1984; Bradley and Satir, 1979). To our knowledge, this is the only example known from the literature of mitochondria filling the microvilli. According to the authors, localization of mitochondria within the cell microvilli was connected with transport activity (of some ions) and/or developmental changes in Malpighian tubules in insects such as mosquito, skipper butterfly, and *Rhodnius* assassin bugs (Bradley, 1984; Bradley and Satir, 1979). Nothing is known, however, about other possible functions of such mitochondria within the microvilli.

Although there are growing numbers of examples of the negative effects of nano- and microplastics, such as PS, the mechanisms of their toxic effects are still largely unclear and need further study. It is generally speculated that smaller particles would be more toxic than larger particles due to the increase in specific surface area (Decuzzi et al., 2010; Lu et al., 2016). Like other nanoparticles which can easily cross the cell boundaries and enter cells (Barik and Mishra, 2019), PS micro- and nanoparticles can cause changes in membrane properties that affect the activity of membrane proteins (Rossi et al., 2014). Research from cell cultures has revealed that PS particles can impact cell homeostasis and interact with cell organelles and genetic material, causing various reactions such as alterations in gene expression, biochemical responses, ROS production, and even carcinogenesis (Forte et al., 2016; Kato et al., 2003). The real impact of micro- and nanoparticles is hard to estimate due to the great variety of specific properties of tested particles, including particle size and shape, surface area, electrical charge, various surface modifications as well as hydrophobic properties (Ruenraroengsak and Tetley, 2015). For instance, the PS particles with modified surfaces (amine- and carboxyl-surface) and unmodified ones showed different effects on the cultured cells of the

human alveolar cells. Amine-surface particles caused oxidative damage and apoptotic cell death, while carboxyl-surface and unmodified particles showed low cytotoxicity via oxidative stress. The authors also noted the variety in bioreactivity of different-sized particles depending on the type of cells (Ruenraroengsak and Tetley, 2015). Similarly, in the phagocytic cell line, exposure to amine-modified PS nanoparticles led to apoptotic cell death, while BEAS2-B cells exhibited necrotic cell death (Xia et al., 2006). It is worth mentioning, however, that oxidative stress and high ROS level are not always linked to cell death, because ROS can induce different oxidative stress processes in different cell types. As mentioned in the study of human alveolar cells, nanoparticles can induce oxidative stress or can result in cell death accompanied by ROS production (Ruenraroengsak and Tetley, 2015).

Data from the study conducted in vivo on living organisms are unfortunately limited so far and show mainly the accumulation and potential impact of PS particles of different sizes (from micro, i.e., 20 μm and 5 μm, to nano, i.e., 70 nm or 24 nm) on different organs of organisms inhabiting marine and freshwater ecosystems. These reports point to various disorders at the level of cells, tissues, and whole organs, for example, inflammation (including vacuolation, infiltration, and necrosis) and lipid accumulation after exposure to 70 nm particles, and moreover, increased oxidative stress markers disturbed the lipid and energy metabolism in zebrafish liver exposed to different size PS micro- and nanoplastic (Lu et al., 2016), and also disturbance of the lipid metabolism in crucian carp exposed to 24 nm PS nanoparticles (Cedervall et al., 2012). Other reports described immunomodulation, increasing ROS and NO production, and induction of apoptosis in marine bivalve hemocytes exposed to cationic PS nanoparticles (Canesi et al., 2015), and the impact on apoptosis and embryotoxicity in Mediterranean sea urchin embryos exposed to amine PS nanoparticles (Della Torre et al., 2014). Our analysis of the cell and tissue response in the midgut of fruit flies corresponds to our ultrastructural observations and is also in line with other in vivo studies described above. Generally speaking, the smaller the PS particles were, the more substantial was the effect on midgut tissue and cells they caused. The highest level of cellular changes was detected after applying PS NPred. All of the studied parameters, i.e., the generation of ROS (whose overproduction generates oxidative stress), total glutathione concentration (involved in the defensive role against ROS acting in distinct pathways), and total antioxidant concentration (an effective oxidative defense system) (Hellou et al., 2012) were the highest in the midgut cells after exposure to the smallest nanoparticles; by contrast, the lowest level of these parameters was shown by midgut cells exposed to the largest microparticles tested. Our analysis clearly shows the negative impact of PS particles on the fruit fly midgut, at both the micro- and nanoscale, which causes oxidative stress and activates processes of antioxidative protection. In referring to changes in ultrastructure observed in epithelial cells, it suggests that oxidative stress triggers autophagy and/or, ultimately, necrosis. In conclusion, even unmodified PS particles without additives, albeit with a sufficiently small size, mainly at the nanoscale, can induce negative changes and disorders. These changes are more severe the smaller the particles are.

As mentioned above, our analysis showed sex-related differences in the effects of micro- and nanoplastics on the midgut, with male flies appearing to be more sensitive. Negative effects of exposure to PS MP and PS NP in males were more substantial and more prominent, even after contact with larger particles, compared to changes observed in females. It was demonstrated in ultrastructural changes in the midgut epithelial cells, showing the undergoing processes of autophagy and consequently necrosis, and also clearly visible in tested cell stress markers (Table 1). The level of markers tested in males was significantly higher than in females. Considering these differences between the sexes in an ecological context, it can be assumed that such differential effects may bring negative consequences, for example, in the form of disruption of the normal distribution of sexes and numbers of individuals in a given population in the environment, as in our study we also observed higher mortality in males.

In conclusion, the fruit fly midgut is the first place of contact with various agents and constitutes the barrier separating the inside of the body from external agents. Therefore, it should react strongly and be the place that protects the rest of the body's tissues and organs. The mechanisms of cell responses to maintain homeostasis in the midgut work quite well. Each marker tested in adult females and males was significantly higher in the midgut than in the reproductive organs. Moreover, the most significant reactions were observed again for the smaller micro- and nanoparticles (MPpink and NPred groups). It indicates that the midgut reacts strongly to ROS production. However, the fruit fly individuals are also forced to activate repair processes, such as oxidative stress protection, autophagy, and even necrosis, to nullify the negative effects of micro- and nanoparticles. As PS does not cause dramatic disorders in the midgut, this, however, does not change the fact that it disrupts the body's homeostasis to some extent. The intestine of a fruit fly seems to be an organ perfectly adapted to cope with such a factor of external origin.

4.3. Reproductive and transgenerational effects of PS MP and PS NP

Exposure to micro- and nanoplastic has been widely reported to alter gonadal morphology, the quality and number of gametes in both sexes, fecundity, and the number and quality of offspring in numerous aquatic and terrestrial species. Most data come from larger particles (about several micrometers in diameter) and/or from exposure in high doses. Therefore, it is not surprising that various types of plastic have acute negative effects on animal reproduction and fertility disorders in humans. Even considering only experiments with PS, it has been reported that such microparticles, for example: led to a decrease in sperm velocity in Pacific oysters (2 μm and 6 μm PS-MPs); delayed maturation of the gonads, decreased egg reproduction, and reduced the fertility of the marine medaka *Oryzias melastigma* (10 μm PS particles); caused reproduction toxicity, a lower percentage of pregnancy, and number of embryos in *Poecilia reticulata* (~23 nm PS-MPs); induced granulosa cell apoptosis and pyroptosis of the ovary, and ovary fibrosis in Wistar rats (0.5 μm PS-MPs); led to gametogenesis and gonad regression in the pearl oyster *Pinctada margaritifera* (6 μm and 10 μm PS-MPs); increased apoptosis of the testis in zebrafish (1 μm PS-MPs); decreased sperm motility and testosterone levels, induced abnormal sperm morphology, and increased sperm deformation, atrophy, and apoptosis in mice (0.5 μm, 4 μm, 5.0 μm, 10 μm PS-MPs) and Wistar rats (0.5 μm PS-MPs); for details and more examples, see (Jewett et al., 2022; Yuan et al., 2022).

One of the mechanisms responsible for these alterations is connected with the homeostasis of reactive oxygen species (ROS), as it is crucial for female and male reproductive functions. Oogenesis, quality of oocytes, and embryo development, as well as spermatogenesis, sperm functions, and fertilization-related processes, depend on the proper ROS level. It was found, for example, that exposure to plastic particles can lead to an increase in ROS levels in organisms and can induce oxidative and inflammatory damage in the reproductive systems (for details see Hou and Zhu, 2017; Wei et al., 2022; Yuan et al., 2022). However, there are also known reports of a lack of negative effects of microplastics on reproduction, such as in the blackworm, in which ingestion of polyethylene MPs increased ROS level and decreased aerobic energy production but did not alter reproduction (Silva et al., 2021). Similarly, in Java and Japanese medaka (Assas et al., 2020) and *Daphnia magna* (Kelpsiene et al., 2020) exposure to PS microparticles caused no reproductive defects, probably due to, according to the authors, too low doses to induce any defects in the reproductive function of animals.

We intended to test the possible reproductive and transgenerational effects induced by PS at both low dose and small sizes at the micro- and nanoscale. Our study showed for the first time the gonads and the process of spermatogenesis and oogenesis at the ultrastructural level and correlated this with levels of oxidative stress markers in these gonads, as

well as in the next generation, that is, in larvae coming from exposed flies. We found that low doses of micro- and nano-sized PS particles did not alter the reproduction of female and male flies. Spermatogenesis and oogenesis proceeded normally. Only some minute alterations and ultrastructural changes were noted, such as autophagy in developing oocytes and the somatic ovarian sheath. Throughout the experiment, females laid eggs intensively, and larvae were hatching. A reduced number of eggs laid was observed in the case of the largest microparticles (MPyellow) tested. PS particles were noted inside the ovaries. Smaller microparticles (MPpink) and nanoparticles (NPred) accumulated in the cytoplasm of growing oocytes, nurse cells, and somatic follicular cells. Only the largest particles tested (MPyellow) did not pass into the ovaries and germ cells. It indicates that at least smaller particles (MPpink and NPred) can pass from the midgut to other tissues, accumulate in germ cells, and alter oogenesis. ROS and the cellular antioxidant response increased after exposure to PS nanoparticles (NPred) and smaller microparticles (MPpink), respectively. They did not change after exposure to the largest (MPyellow) particles. This clearly demonstrates the size-dependent negative effect of PS on oogenesis, even at a low plastic dose.

Additionally, the ultrastructure of analyzed testes indicated an intensive process of spermatogenesis, and no changes or disturbances were detected. Similarly to ovaries, testes seem not to be highly affected by PS. The process of sperm formation occurred intensively in each of the study groups, and the sperm that were formed showed proper ultrastructure. However, after analyzing the results from the cell stress response, it became clear that, similarly to females, in males, the supply of smaller microparticles (MPpink) and nanoparticles (NPred) also affected the testes and spermatogenesis, changing parameters such as the ROS levels and antioxidant response. Therefore in males and females, PS induced cellular stress and activated antioxidant protective mechanisms. Only the exposure of males to the largest particles tested (MPyellow) did not change the level of ROS and cellular oxidative responses. It indicated that the largest particles did not trigger sperm formation, and again, a size-dependent correlation was observed between PS particles and the negative impact on spermatogenesis. A low dose, however, did not lead to morphological changes in testes and did not trigger spermatogenesis, despite the changes in cellular response.

Our ultimate question was whether PS particles could negatively impact the next generation. A transgenerational effect was noted, for example, after the exposure of polyethylene particles in the soil, where it reduced the production of juveniles of the earthworm *Eisenia fetida* (Sobhani et al., 2022, 2021; Yuan et al., 2022). Also, high concentrations of PS particles (0.05 μm and 0.5 μm) affected survival and development of marine copepods in the F1 generation (Lee et al., 2013). In *C. elegans*, the exposure of microplastic particles in females caused a significant reduction in brood size, decreased locomotion, and increased the level of intestinal ROS in offspring (Jewett et al., 2022; Zhao et al., 2017). In turn, the exposure of fruit flies to polyvinyl chloride particles (23–500 μm) led to shorter life cycles and smaller sizes of their offspring (Jimenez-Guri et al., 2021). Interestingly, also in mammals, exposure to microplastics in mouse dams caused numerous alterations in the offspring, including tissue damage, disturbance of the immune response, fewer live births, changes in sex ratio, decrease in body weight and changes in lymphocyte composition within the spleen, thus revealing long-term intergenerational effects of microplastics (D'Angelo and Meccariello, 2021).

The accumulation of smaller microparticles (MPpink) and nanoparticles (NPred) in oocytes, as shown in our study, suggested that they could potentially affect the quality of the offspring and therefore have a transgenerational effect. Indeed, when we measured the levels of ROS and antioxidant response in larvae from exposed flies, parameters were higher in larvae of those groups in which the particles could accumulate in oocytes (MPpink and NPred). Such larvae were negatively affected even though they were reared under clean conditions and had no direct contact with PS particles. The differences were not dramatic, but they indicate that even a small dose of exposure to PS micro- and nanoparticles negatively affects larvae. Again, particle size-dependent differences are noticeable, with a tendency for smaller nanoparticles to have more significant effects and negative changes than larger microparticles. Only in the case of exposure to the largest microparticles (MPyellow), which did not pass into the gonad and did not accumulate in oocytes, were larvae not triggered. The ROS level and oxidative responses were even lower than the control group. Further research is required to identify the causes of these differences and to provide more data for the transgenerational impact of PS described here.

5. Conclusions

The current investigation of the impact of PS micro- and nanoparticles in low dosage indicated that:

- PS MP and NP given with food did not interfere with food intake in adult fruit flies,
- The ingested particles did not cause a negative effect on the lifespan and culture conditions of the fruit flies; however, the largest microplastic particles were associated with lower survival of individuals of both sexes, compared to smaller particles; probability of survival in males from all analyzed groups was always lower than in females,
- PS particles can induce size-dependent and sex-dependent ultrastructural alterations in midgut epithelium and cause cellular responses connected with increased levels of ROS and triggering the mechanism of oxidative cell response; ultrastructural changes were more severe, and ROS and oxidative stress parameters were higher the smaller were the ingested particles; male flies seem to be more sensitive to exposure to PS particles; the alteration in the midgut and tested parameters of cellular response in males were more substantial and more prominent when compared with females,
- PS particles did not alter spermatogenesis and oogenesis; however, despite egg and sperm production, a size-dependent negative effect of PS particles at a low dose was observed; the smaller MPs (MPpink) and NPs (NPred) could be transferred to the ovary and accumulated in the future gametes; this was reflected in the level of ROS and cellular antioxidant response, which increased in the case of MPpink and NPred exposure respectively, while they did not change in the case of the largest particles tested (MPyellow).
- A transgenerational negative effect was observed after exposure to a low dose of PS MPs and NPs; exposure to PS particles tested negatively affected larvae and demonstrated size-dependent alterations, where the smaller the particles were, the higher were the noted parameters of ROS and cellular oxidative response; as the largest MPs (MPyellow) did not pass into the gonad, only in this case were tested parameters in larvae not altered.
- Our study demonstrated that micro- and nanoscale PS particles, even in a low dosage, can induce numerous negative effects on the terrestrial invertebrate *D. melanogaster*.

CRediT authorship contribution statement

Chajec Łukasz: Data curation, Formal analysis, Investigation. **Małota Karol:** Data curation, Formal analysis, Investigation. **Sawadro Marta Katarzyna:** Data curation, Formal analysis, Investigation. **Urbisz Anna Zofia:** Conceptualization, Formal analysis, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing. A.Z.U. – study conception and design, prepared the material for the study, wrote the main manuscript text; A.Z.U., Ł. Ch., K.M., M.K.S. – performed the experiments, analyzed the data; M.K.S. – performed statistical analysis; A.Z.U., K.M., Ł. Ch., M.K.S – analyzed and interpreted the data; A.Z.U. – critical revision of the manuscript.

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.

Data Availability

Data will be made available on request.

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