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Alterations Induced by Nano-Polystyrene Administration in Biological Parameters of Host-Endoparasitoids (*Galleria mellonella* and *Pimpla turionellae*) and Host Hemocyte Counts

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Abstract: Plastic pollution is one of the biggest threats to the environment and human health. Micro and nanoplastics are encountered in many areas of our daily lives and may accumulate in organisms, causing reduced life span, genotoxicity, and altered metabolism. Plastic pollution around the environment may lead to reductions in insect biodiversity and populations. It may also lead to the collapse of food webs and ecosystems of organisms that feed on them in the food chain. Therefore, the effects of nano-polystyrene (PSs) on the life cycle, biological characteristics, total hemocyte count (THCs) of the host, and hemocyte types of the model organism *Galleria mellonella* and its endoparasitoid *Pimpla turionellae* were investigated. Nano-PSs were produced according to the single emulsion solvent evaporation method and larval feeds were prepared with solutions of different concentrations. These diets were given to the larvae until they developed. The developmental time of the host-larvae fed with nano-PS-containing diets and the parasitoids that emerged using the pupae of these larvae as hosts were shortened. While the host adult weight and size increased, the weight of the parasitoid decreased. Dose-dependent decreases in THCs were observed. Prohemocyte, plasmatocyte, oenocytoid, and spherulocyte counts decreased, while granulocyte counts increased. Furthermore, the changes in the biology of the host exposed to nano-PSs indirectly affected the endoparasitoids. In addition, this study emphasizes that nanoplastic toxicity in honey-bees is generally ignored and that the consumption of bee products may pose potential hazards to human health. This reveals the crucial role of taking necessary precautions in beekeeping.

Keywords: Cellular immunity, greater wax moth, life cycle, nano-plastic, parasitoid wasp.

Nano-Polistiren Uygulamasının Konak-Endoparazitoitlerin (*Galleria mellonella* ve *Pimpla turionellae*) Biyolojik Parametrelerinde ve Konak Hemosit Sayılarında Oluşturduğu Değişiklikler

Öz: Plastik kirliliği çevre ve insan sağlığı açısından en büyük tehditlerden biridir. Mikro ve nanoplastikler, günlük yaşamımızın birçok alanında karşımıza çıkmaktadır ve organizmalarda birikerek yaşam süresinin azalmasına, genotoksisiteye ve metabolizmanın değişmesine neden olabilmektedir. Çevredeki nanoplastik kontaminasyonu, böcek biyoçeşitliliğindeki ve popülasyonlarındaki azalmalara neden olabilir. Aynı zamanda besin zincirinde onlarla beslenen canlıların besin ağlarının ve ekosistemlerin çökmesine yol açabilir. Bu nedenle nano-polistiren (PS)'lerin model organizma *Galleria mellonella* ve endoparazitoiti *Pimpla turionellae*'nın yaşam döngüsüne, biyolojik özelliklerine, konağın toplam hemosit sayısına (THS) ve hemosit tiplerine etkileri incelendi. Nano-PS'ler tekli emülsiyon çözücü buharlaştırma yöntemine göre üretildi ve farklı konsantrasyonlarda solüsyonları ile larval besinler hazırlandı. Bu besinler, larvalara gelişinceye kadar verildi. Nano-PS içeren besinlerle beslenen konak larvaların ve bu larvaların pupalarını konak olarak kullanarak ortaya çıkan parazitoitlerin gelişim süreleri kısaldı. Konak ergin ağırlığı ve boyutları artarken, parazitoitin ağırlığı azaldı. THS'de doza bağlı azalmalar görüldü. Prohemosit, plazmatosit, önositoid ve sferülosit sayısının azaldığı, granülosit sayısının ise arttığı görüldü. Ayrıca nano-PS'lerle beslenen konağın biyolojisindeki değişiklikler, endoparazitoitleri dolaylı olarak etkiledi. Öte yandan bu çalışma ile bal arılarında nanoplastik toksisitesinin genellikle göz ardı edildiği ve arı ürünlerinin tüketilmesinin insan sağlığı için potansiyel tehlikeler yaratabileceği vurgulanmaktadır. Bu durum, arıcılıkta gerekli önlemlerin alınmasının önemini ortaya koymaktadır.

Anahtar kelimeler: Hücresel bağışıklık, büyük balmumu güvesi, yaşam döngüsü, nano-plastik, parazitoit yaban arısı.

1. Introduction

The high persistence of plastic wastes in ecosystems due to their high resistance to degradation and their interactions with living organisms has been a concern for the last century. Plastic materials accumulated in terrestrial and aquatic ecosystems have the potential to easily enter organisms by breaking down into micro and nanoplastics due to some factors such as sunlight, biodegradation, hydrolysis, photooxidation, mechanical abrasion, salinity, and temperature (Kögel et al., 2020; Murphy et al., 2016; Oliveira et al., 2019; Parenti et al., 2020). In comparison to large plastic wastes, micro and nanoplastics may create more risk as they are more likely to pass through biological boundaries, accumulate in tissues, or get into the food chain (Hu & Palić, 2020; Muhammad et al., 2021).

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Meanwhile, there is increasing concern about the pollution that microplastics may cause (Rothen-Rutishauser et al., 2021). The influences of plastic particles in terrestrial ecosystems have been almost underestimated compared to the aquatic ecosystems (Awet et al., 2018; El Kholy & Al Naggar, 2023; Muhammad et al., 2021; Wang et al., 2021). Insects both encompass a large part of the ecosystem and play an essential role in the ecosystem. Recently, it has been emphasized that there has been a significant decline in insect species due to micro/nanoplastic pollution (Shen et al., 2023). Plastic pollution in a terrestrial ecosystem may create serious problems owing to atmospheric cycling and transport to distant areas such as glaciers. Hence, it may adversely affect insects, plants and animals, food chains, ecosystems, and subsequently human-related activities that depend on these factors that may occur (Muhammad et al., 2021; Toussaint et al., 2019; Wang et al., 2021). Furthermore, the studies on the intake of plastic particles and their impacts on terrestrial organisms remain quite scarce and have only been considered in the last few years (Parenti et al., 2020; Wang et al., 2020). Among plastic particles, micro-sized particles have been particularly emphasized and their impacts on terrestrial ecosystems have recently been demonstrated (Deng et al., 2021; Dolar et al., 2022; Dolar et al., 2021; El Kholy & Al Naggar, 2023; Kalman et al., 2023; Kholy & Naggar, 2022; Zhang et al., 2023). For this reason, it is extremely critical to determine the effects of nanoplastics on living organisms, in particular insects.

Among plastic materials, polystyrene (PS) polymers are recognized as one of the most globally used plastics in the production of disposable cutlery, egg cups, spectacle frames, food packaging, and building insulation (Deng et al., 2021; El Kholy & Al Naggar, 2023; Long et al., 2017). When the wide range of usage areas is considered, it is unlikely to be unaffected by chemical, mechanical, and direct/indirect toxic potential impacts (Kim & An, 2019; Kögel et al., 2020; Muhammad et al., 2021). In recent years, it has been stated that some terrestrial organisms, notably *Bombyx mori* (Muhammad et al., 2024; Muhammad et al., 2021; Parenti et al., 2020; Wang et al., 2023), *Drosophila melanogaster* (Aloisi et al., 2024; El Kholy & Al Naggar, 2023; Kholy & Naggar, 2022; Tu et al., 2023; Urbisz et al., 2024), *Galleria mellonella* (Demirtürk et al., 2024), *Eisenia fetida* (Wang et al., 2019), *Aphylla williamsoni* (Guimarães et al., 2021), *Apis mellifera,* and *Apis cerana* (Deng et al., 2021; Wang et al., 2021), *Chironomus riparius* (Martin-Folgar et al., 2024), *Chironomus kiinensis* (Zhang et al., 2023), and *Tenebrio molitor* (Peng et al., 2024) may uptake micro and nano PSs from the environment; accumulate in the intestine, adipose tissue, ovary, and hemolymph; cause substantial changes in weight, development, and life span; cause necrosis and apoptosis; suppress the immune system; display neurotoxic effects; increase susceptibility to viral infection; and cause DNA damage. However, studies on the biological development of PS NPs in terrestrial insects are deficient and information on the aggregation effect of PS NPs in the food chain is limited (Wang et al., 2023).

Honey-bees are highly valued species that provide pollination services for the production of a wide variety of agricultural crops (Calderone, 2012). The greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae) is the world's most severe pest of honey-bees worldwide. They infest stored wax combs and bee colonies, causing extensive damage. Additionally, when the apiary is queenless or exposed to pesticides, the hive suddenly weakens and problems arise (Gounari et al., 2024). Apart from these, potential adverse biological and cellular effects can be seen on the host *G. mellonella* directly exposed to nanoplastics, especially PS NPs. On the other hand, these host species may interact indirectly with honey-bees by parasitism and their endoparasitoids *Pimpla turionellae,* and even by invading combs and may have adverse effects on their biological processes such as survival and lifespan. Also, host hemocytes, which are critical in the insect immune system and are involved in the defense against parasitoid invasion, may weaken the immune system by affecting its vitality and function with the effect of nanoplastics. Conversely, nanoplastic parasitoids may also have the ability to successfully parasitize the host. Nanoplastics released into the environment may carry potential risks for non-target organisms, including useful insects such as honey-bees. For instance, PS and three other different plastic microparticles were detected in bee species from fields in six different provinces of China (Deng et al., 2021). Insight into the specific effects on *G. mellonella* and *P. turionellae* helps to assess broader ecological consequences, informing guidelines for the use/management of nanoplastics in pest management and other applications. Hence, in the present study, nano-PSs were manufactured and administered to the larvae of *G. mellonella*, a model greater wax moth host, mixed with their diet. Subsequently, changes in the life cycle, weight, and length of *G. mellonella* and its endoparasitoid *P. turionellae* were analyzed. Differences in total and differential hemocyte counts in *G. mellonella* larval hemolymph were determined. In addition, multivariate correlation and principal component analysis were performed to determine the correlation between biological features. The same analyses were also performed for the types of hemocytes of *G. mellonella.*

2. Material and Method

2.1. Host and endoparasitoid

The greater wax moth *G. mellonella* was reared in the laboratory at 25 ± 2°C, 60 ± 2% relative humidity, and 24 h in the dark. Host larvae were fed a synthetic diet containing honeycomb, honey, glycerin, bran, and distilled water (Bronskill, 1961; Sak et al., 2006). The host endoparasite *P. turionellae* was cultured at 25 ± 5°C, 60 ± 5% relative humidity, and 12 h light/12 h dark. Endoparasitoid species were fed with sterile cotton wool soaked in honey solution (30%, V: V) diluted with distilled water and pupal hemolymph of *G. mellonella* (two pupae for five females) three times a week.

2.2. Preparation of polystyrene nanoparticles

Demirtürk et al. (2024) optimized the single emulsion solvent evaporation (w/o) method to prepare PS NPs. In brief; 20 mg of weighed PS pellets (Sigma Aldrich, MW: 35,000 g/L, density: 1.06 g/mL at 25°C) were dissolved in 2 mL of dichloromethane and 4 mL of 0.75% poly(vinyl alcohol) (PVA) (Sigma Aldrich, Mowiol® 4-88, MW: 31,000 g/L) was added to form an oil-in-water emulsion (o/w). The emulsion was sonicated (Bandelin Sonopuls HD 2070.2) for 4 min and 0.75% PVA was added. The organic solvent was removed by stirring for 3 h. The particles were

then centrifuged at 15,000 × g (Gyrozen 1580R, Seoul, Korea) and washed twice with distilled water. The properties of the NPs formed are given in the previous study (Demirtürk et al., 2024). Various doses of solutions (50, 100, 500, and 1000 ppm) of the obtained NPs were prepared. The solutions were prepared by adding the amount of water specified in the diet of the larvae. The larvae were fed with NP-containing food until they developed from early instars to final instars (~25 days).

2.3. Host and endoparasitoid biology

We recorded the time required to complete the larval, pupal, and adult stages of *G. mellonella*, as well as the adult weights and lengths. Pupae were parasitized by reproductively mature *P. turionellae* females. Afterwards, the emergence time, longevity, weight, and size data of *P. turionellae* adult parasitoids were regularly observed and recorded (Uçkan et al., 2011).

2.4. Hemolymph collection

The last instars of host larvae (0.21 ± 0.01 g) were randomly selected from the experimental groups to determine total and differential hemocyte counts. The larvae were anesthetized on ice for five minutes and sterilized with 70% ethanol. Under sterile conditions, the hind leg of the larvae was punctured and hemolymph samples were taken with a micropipette (Eppendorf, St. Louis, MO) (Altuntaş et al., 2012).

2.5. Total and Differential hemocyte counts

To detect the effects of NPs on circulating total hemocyte counts (THCs), the protocol recommended by Altuntaş et al. (2012) was applied. In short, 3 µL hemolymph was obtained and transferred to microcentrifuge tubes containing 27 µL anticoagulant solution. From the obtained cell suspension, 10 µL of hemolymph was removed and transferred to a Neubauer slide. Hemocytes were counted under 60 × magnification under phase-contrast microscopy (Nikon Eclipse Ti-U phase contrast microscopy). Results were expressed as THCs × 10^6 cells/mL hemolymph (Altuntaş et al., 2012).

The Giemsa staining protocol was used for differential hemocyte counts (DHCs) (Uçkan & Sak, 2010). In summary, 5 µl of hemolymph was spread on a sterile slide, dried, and fixed in methanol:acetic acid (3:1) for five min. The slides were stained in Giemsa stain solution (3 mL Giemsa, 57 mL PBS) for 10 min. The slides were then washed with distilled water and PBS and dried. DHCs in the prepared slides were examined under phase-contrast microscopy. For DHCs, 500 cells from a single larva were counted on each slide and the results are given as cells/500 prohemocytes, granulocytes, plasmatocytes, oenocytoids, and spherulocytes. Hemocyte types were defined by using the morphological characters described by Uçkan and Sak (2010).

2.6. Multivariate correlation and principal component analysis

Multivariate correlation analysis (MCA) was applied to detect the correlation between the nine biological features of *G. mellonella* and *P. turionellae* obtained. Principal component analysis (PCA) was used to analyze the proximity between different factors on five important biological features related to development. MCA and PCA were also applied to determine the correlation between the five types of hemocytes of *G. mellonella* and larval development.

2.7. Statistical Analysis

SPSS analysis software program (IBM SPSS Statistics, Version 27.0. IBM Corp.) was used for statistical analyses. Normal distribution analysis between groups was performed using Levene's test. For data sets that did not show normal distribution, the data were first normalized by taking the square root of the data. All data obtained from the experiments and the determined standard errors were expressed as mean and the results were shown as mean ± standard error. ANOVA (One-Way Analysis of Variance) was used when the means were homogeneous and Tukey's HSD (Tukey's Honestly Significant Difference) test was used to determine significant differences. The Kruskall Wallis test was used for nonparametric data. Dunn's post hoc test was used if the mean data were not homogenous. In all statistical analyses, the p-value was taken as 0.05 ($p < 0.05$). OriginLab OriginPro 2024 SR1 v.10.1.0.178 software was used in MCA and PCA analyses. In MCA analyses, the symbols ** and * indicate extreme significance at $p < 0.01$ and statistical significance at $p < 0.05$, respectively.

3. Results

3.1. Biological Features of host-endoparasitoid

All doses of PS NP reduced the larval development time of *G. mellonella* in a dose-dependent manner (degree of freedom 1 (df1), df2 = 4.70, F = 33.92, sig = 0.001, Table 1). Pupal development time showed the first effect at a 100 ppm PS NP dose and this time decreased with increasing dose (df1, df2 = 4.70, F = 74.82, sig = 0.001, Table 1). The most effective dose of PS NP that shortened adult longevity was 50 ppm; however, adult longevity was also shortened at other doses (df1, df2 = 4.70, F = 29.88, sig = 0.001, Table 1). The most statistically significant increase in *G. mellonella* adult weights was observed at the 500 ppm PS NP dose (df1, df2 = 4.70, F = 4.90, sig = 0.002, Table 1). Moreover, statistically significant increases in *G. mellonella* adult size were detected in the 500 and 1000 ppm groups (df1, df2 = 4.70, F = 20.34, sig = 0.001, Table 1).

Adult emergence time of *P. turionellae* at 50 and 100 ppm were similar to each other, while adults grown at doses of 500 and 1000 ppm were also similar to each other. In general, adult emergence time decreased at all doses (df1, df2 = 4.70, F = 35.74, sig = 0.001, Table 2). Interestingly, while the 1000 ppm dose had no change in adult parasitoid longevity compared to the control, the other NP doses decreased adult longevity. Specifically, the 50 ppm NP dose was the most effective group (df1, df2 = 4.70, F = 27.98, sig = 0.001, Table 2). In contrast, similar decreases in *P. turionellae* adult weights were detected in all PS NP groups but no significant differences in size were noticed (df1, df2 = 4.70, F = 5.59, sig = 0.001; df1, df2 = 4.70, F = 1.55, sig = 0.198, Table 2).

3.2. Multivariate correlation and principal component analysis for biological parameters

Pearson's r correlation coefficient was applied to inspect the relation between the detected data. The results in Figure 1 showed that there were significant positive

Table 1. Polystyrene nanoparticles (PS NPs)-associated changes in larval, pupal development, adult longevity (day), adult weight (mg), and size (mm) of *Galleria mellonella*

Groups	Larval Development (day)*	Pupal Development (day)*	Adult Longevity (day)*	Adult Weight (mg)*	Adult Size (mm)*
Control	28.0 ± 0.25 ^{a}	16.0 ± 0.22 ^{a}	16.0 ± 0.33 ^{a}	79.2 ± 0.29 ^{a}	12.6 ± 0.18 ^{a}
50 ppm	26.5 ± 0.23 ^{b}	15.1 ± 0.27 ^{a}	12.2 ± 0.22 ^{c}	79.6 ± 0.25 ^{a}	12.2 ± 0.11 ^{a}
100 ppm	25.7 ± 0.20 ^{b}	14.0 ± 0.21 ^{b}	13.1 ± 0.27 ^{bc}	80.1 ± 0.27 ^{ab}	12.5 ± 0.16 ^{a}
500 ppm	24.7 ± 0.30 ^{c}	12.0 ± 0.23 ^{c}	14.0 ± 0.22 ^{b}	80.0 ± 0.35 ^{b}	14.0 ± 0.18 ^{b}
1000 ppm	24.0 ± 0.25 ^{c}	11.1 ± 0.23 ^{c}	13.8 ± 0.20 ^{b}	80.3 ± 0.31 ^{ab}	13.4 ± 0.16 ^{b}

*All data for each group are represented as "Means ± Standard Errors". In each group, the mean of 15 individuals was given and 3 replicates were analyzed. The means in each experimental group followed by the same letter are not significantly dissimilar but different letters (a-c) are statistically significant (Tukey's HSD, $p < 0.05$).

Table 2. Polystyrene nanoparticles (PS NPs)-associated changes in adult emergence time, longevity (day), adult weight (mg), and size (mm) of *Pimpla turionellae*

Groups	Adult Emergence Time (day)*	Adult Longevity Time (day)*	Adult weight (mg)*	Adult size (mm)*
Control	21.6 ± 0.19 ^{a}	23.7 ± 0.11 ^{a}	17.7 ± 0.11 ^{a}	10.7 ± 0.11 ^{a}
50 ppm	20.6 ± 0.15 ^{b}	21.9 ± 0.18 ^{c}	17.4 ± 0.13 ^{ab}	10.5 ± 0.13 ^{a}
100 ppm	20.4 ± 0.13 ^{b}	22.3 ± 0.12 ^{cb}	16.9 ± 0.15 ^{b}	10.4 ± 0.13 ^{a}
500 ppm	19.3 ± 0.12 ^{c}	22.6 ± 0.12 ^{b}	17.0 ± 0.16 ^{b}	10.6 ± 0.13 ^{a}
1000 ppm	19.6 ± 0.12 ^{c}	23.2 ± 0.11 ^{a}	17.0 ± 0.16 ^{b}	10.3 ± 0.12 ^{a}

*All data for each group are represented as "Means ± Standard Errors". In each group, the mean of 15 individuals was given and 3 replicates were analyzed. The means in each experimental group followed by the same letter are not significantly dissimilar but different letters (a-c) are statistically significant (Tukey's HSD, $p < 0.05$).

correlations between Host Larval Development (HLD), Host Pupal Development (HPD), Host Adult Longevity (HAL), Host Adult Weight (HAW), Host Adult Size (HAS), Endoparasitoid Adult Emerge Time (EAET), Endoparasitoid Adult Longevity (EAL), Endoparasitoid Adult Weight (EAW), and Endoparasitoid Adult Size (EAS) at 0.05 and 0.01 levels. The color and size of the circles shown in Figure 1 indicate the correlation coefficient between the two parameters. HLD was positively correlated with HPD, EAET, EAW, and EAS 89.9%, 74.9%, 31.9%, and 31.5%, respectively, whereas it was negatively correlated with HAW and HAS 78.0% and 66.7%, respectively ($p < 0.01$). It was also found that HLD was not correlated with HAL and EAL. HPD was strongly positively correlated with EAET and EAW 75.5% and 40.7%, ($p < 0.01$), weakly positively correlated with EAS 26.6% ($p < 0.05$), and strongly negatively correlated with HAW and HAS 82.2% and 66.9% ($p < 0.01$), respectively. On the other hand, HPD was not correlated with HAL and EAL. HAL showed a strong positive correlation with HAS and EAL with 50.4% and 61.2% ($p < 0.01$), respectively, a weak positive correlation with EAW with 23.0% ($p < 0.05$), and no correlation with HAW and EAS. HAW exhibited a 73.4% strong positive correlation with HAS, whereas it exhibited a 67.8% and 49.5% strong negative correlation with EAET and EAW, respectively ($p < 0.01$). At the same time, EAL and EAS were not correlated with EAW. HAS was strongly negatively correlated ($p < 0.01$) with EAET (61.6%) and EAW (30.9%) but not with EAL and EAS. EAET was positively correlated with 44.3% EAW ($p < 0.01$) but not with EAL and EAS. EAL was positively correlated with EAW by 45.1% ($p < 0.01$) but not with EAS. On the other hand, no correlation was observed between EAW and EAS (Fig. 1).

Varimax rotated PCA revealed that the first two principal components (PC1 and PC2) greater than 1 explained 84.61% of the data variability (Kaiser-Meyer-Olkin test value = 0.66, Fig. 2A). PC1 accounted for 52.50% and PC2 accounted for 32.12% (Fig. 2B). HLD 95.02%, HPD 95.17%, and EAET 89.02% were positively correlated with PC 1, while HAL 89.60% and EAL 89.78% were positively correlated with PC 2 (Fig. 2B). Two factors can explain 90.4% of the variance of the HLD variable, 90.6% of the variance of the HPD variable, 80.4% of the variance of the HAL variable, 80.4% of the variance of the EAET variable, and 81.4% of the variance of the EAL variable. As shown in Figure 2B, three development-related variables (HLD, HPD, and EAET) loaded the highest on the first component and two variables (HAL and EAL) loaded the highest on the second component.

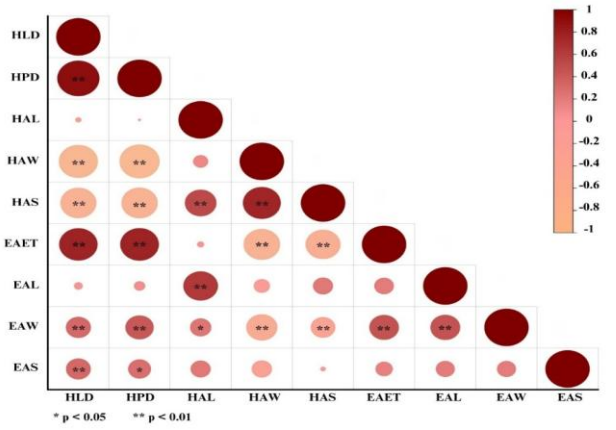

Figure 1. Correlation coefficient plot between nine biological parameters in host *Galleria mellonella* and endoparasitoid *Pimpla turionellae*. Host Larval Development (day) = HLD, Host Pupal Development (day) = HPD, Host Adult Longevitiy (day) = HAL, Host Adult Weight (mg) = HAW, Host Adult Size (mm) = HAS, Endoparasitoid Adult Emergence Time (day) = EAET, Endoparasitoid Adult Longevitiy (day) = EAL, Endoparasitoid Adult Weight (mg) = EAW, Endoparasitoid Adult Size (mm) = EAS

3.3. Total and Differential Hemocyte Counts

THCs in *G. mellonella* larvae statistically decreased at all PS NP doses (df1, df2 = 4.70, F = 127.5, sig = 0.001, Fig. 3). In the control group, THCs were determined as 40 ± 0.33 × 10^{6} cells/mL. At the lowest concentrations of 50 and 100

ppm NP, THCs reduced to 32.4 ± 0.64 and 29.6 ± 0.69 × 10^6 cells/mL, respectively. In the 500 and 1000 ppm NP groups, which constituted the highest concentrations, THCs declined by 26.6 ± 0.44 and 24.8 ± 0.42 × 10^6 cells/mL, respectively (Fig. 3).

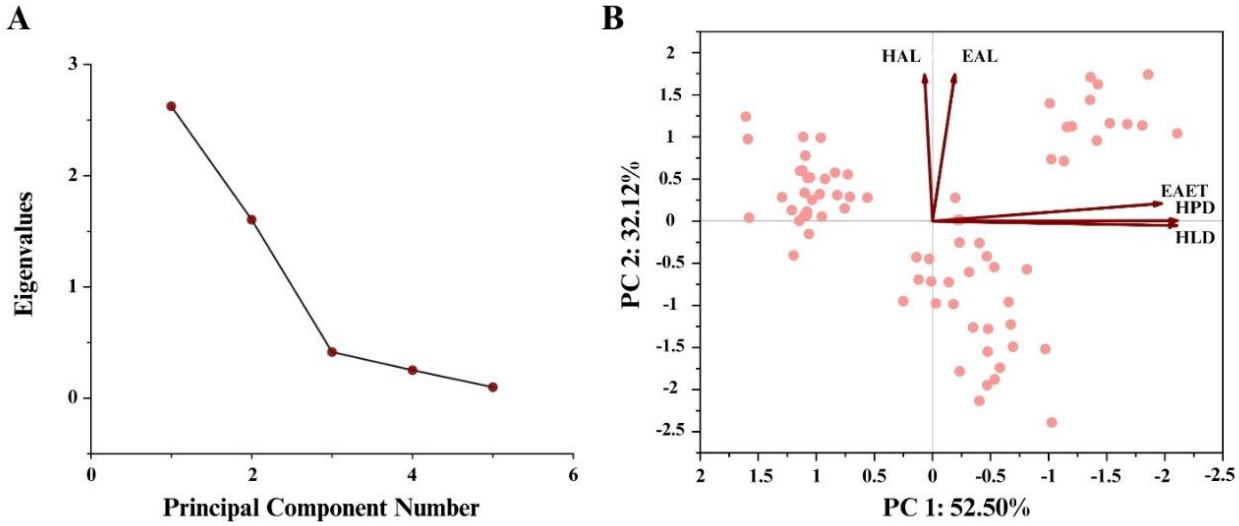

Figure 2. Principal Component Analysis (PCA) on all developmental variables measured in *Galleria mellonella* larvae exposed to Nano-PS, A) Scree plot of all variables, B) Proximity of developmental parameters to components. Host Larval Development (day) = HLD, Host Pupal Development (day) = HPD, Host Adult Longevity (day) = HAL, Endoparasitoid Adult Emerge Time (day) = EAET, Endoparasitoid Adult Longevity (day) = EAL

Changes in DHCs (cells/500) of *G. mellonella* larvae associated with PS NPs are given in Figure 4. Significant increases in granulocyte counts were observed in all groups of PS NP concentrations (df1, df2 = 4.70, F = 192.05, sig = 0.00 < 0.001). Granulocyte counts were 27.66 ± 191 cells/500 in the control group. The mean values of granulocyte count from low to high concentrations were 36.77 ± 1.00, 48.69 ± 0.99, 55.57 ± 1.03, and 58.45 ± 0.64 cells/500, respectively. On the contrary, plasmatocyte counts were significantly diminished at all concentrations compared to the control (df1, df2 = 4.70, F = 89.82, sig = 0.00 < 0.001). Significant changes were observed in each experimental group, while 55.88 ± 1.22 cells/500 in the control group. Plasmatocyte cells constituted 52.84 ± 0.98, 42.42 ± 1.06, 38.09 ± 0.91, and 33.81 ± 0.76 cells/500 of the total hemocyte population at all concentrations (50, 100, 500, and 1000 ppm) of *G. mellonella* larvae, respectively. Similarly, significant decreases in prohemocyte, oenocytoid, and spherulocyte counts were observed at all concentrations (df1, df2 = 4.70, F = 46.63, sig = 0.00 < 0.001; df1, df2 = 4.70, F = 53.05, sig = 0.00 < 0.001; df1, df2 = 4.70, F = 35.96, sig = 0.00 < 0.001, respectively).

3.4. Multivariate correlation and principal component analysis for hemocytes

Pearson's r correlation coefficient was applied to inspect the relation between the detected data. The data in Table 3 and Figure 4 showed highly significant positive correlations between prohemocytes, granulocytes, plasmatocytes, oenocytoids, and spherulocytes at the 0.01 level. The darker the color and the larger the size of the circles shown in Figure 4, the correspondingly bigger correlation coefficient and the greater correlation. A negative correlation was observed between granulocytes, other hemocytes, and larval development.

Table 3. Pearson's r correlation coefficient matrix among the detected differential hemocyte counts and larval development

Correlations	Prohemocytes	Granulocytes	Plasmatocytes	Oenocytoids	Spherulocytes	Larval Development
Prohemocytes	1					
Granulocytes	-0.790**	1				
Plasmatocytes	0.660**	-0.967**	1			
Oenocytoids	0.825**	-0.832**	0.700**	1		
Spherulocytes	0.816**	-0.781**	0.602**	0.853**	1	
Larval Development	0.796**	-0.882**	0.828**	0.795**	0.729**	1

**Correlation between differential hemocyte counts and larval development are significant at the 0.01 level.

PCA for differential hemocyte counts revealed that only one principal component (PC) explained 82.71% of the data variability (Kaiser-Meyer-Olkin test value = 0.49). Prohemocytes correlated with 90.0%, granulocytes with 96.1%, plasmatocytes with 86.4%, oenocytoids with 92.7%, and spherulocytes with 89.2%. A single factor (Component 1 = 'related to hemocyte types') can explain 81.0% of the variance of prohemocytes, 92.4% of the variance of granulocytes, 74.7% of the variance of plasmatocytes, 86.0% of the variance of oenocytoids, and 79.5% of the variance of spherulocytes. We therefore extracted a principal component comprising the five types of hemocytes identified in our analysis.

4. Discussion

Insects are an extremely valuable part of ecosystems and have a strong influence on the survival of the entire biosphere (Outhwaite et al., 2022). Hitherto, a remarkable

decline in insect species and populations globally has been documented owing to microplastic-induced toxicity (Shen et al., 2023) rather than insecticides (Shafiq ur, 2009). Microplastics may adversely affect insect survival, reproduction, development, and gut microbiota (Shen et al., 2023). Besides, nanoplastic-induced toxicity findings, which have a smaller structure, constitute a very new research area. As a result of the idea that nano-sized plastics can be taken into the body and penetrate tissues and cells more easily, we planned our study on the model *G. mellonella*. Our research was carried out based on the hypothesis that the counts of hemocytes involved in the development, longevity, growth, and immune system in insects may change/impair their function. *P. turionellae*, the endoparasitoid of *G. mellonella*, which is harmful to honeycombs and creates a major challenge in beekeeping by affecting honeybee colonies, is responsible for maintaining the ecological balance. Thus, here we first manufactured nano PSs, then determined the biological effects of PS NPs with an approximate size of 275 nm on these host-parasitoid species and on host hemocytes.

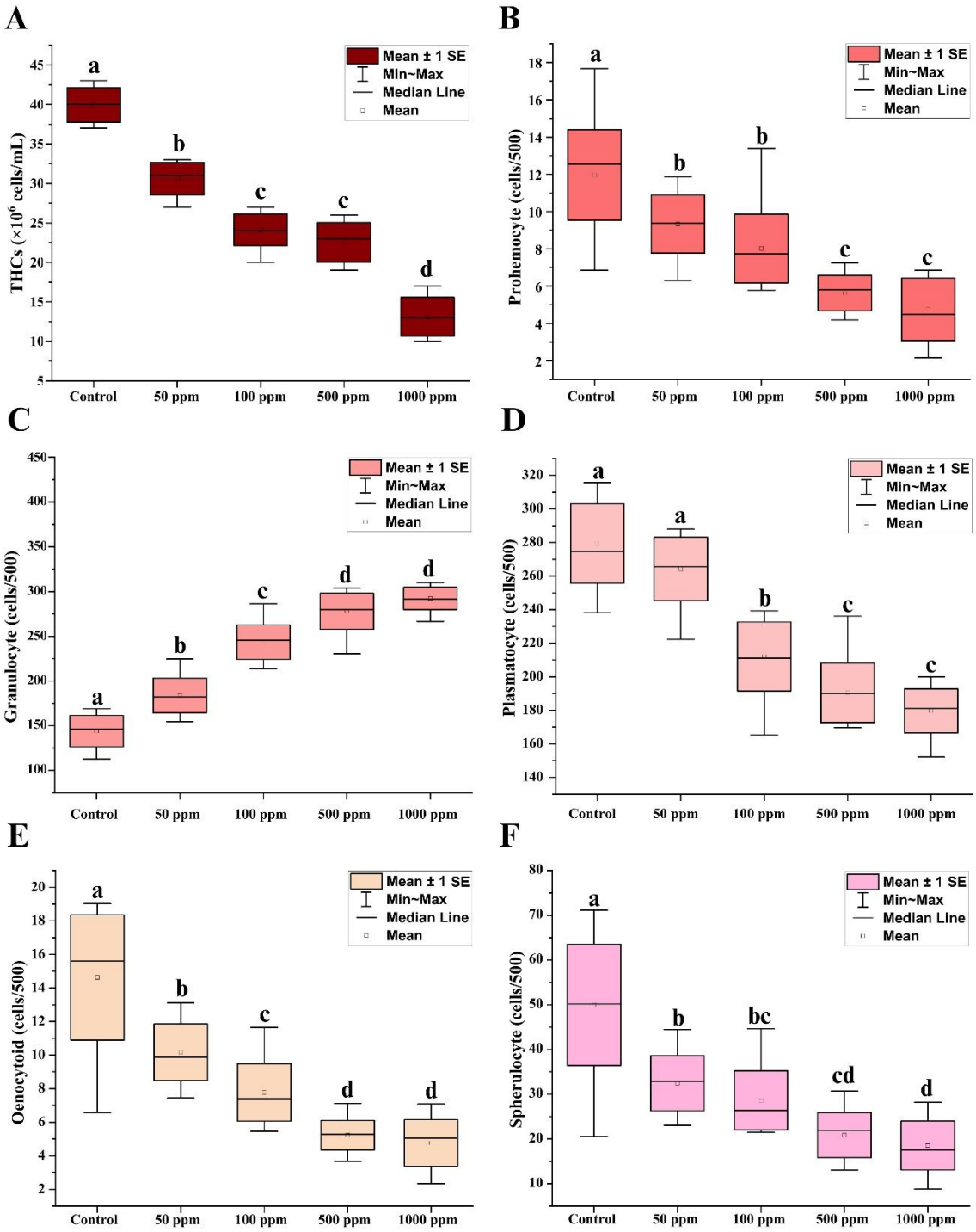

Figure 3. Influence of Polystyrene nanoparticles (PS NPs) on, A) total hemocyte counts (×10⁶ cells/mL), B) prohemocyte, C) granulocyte, D) plasmatocyte, E) oenocytoid, and F) spherulocyte counts (cells/500) in the hemolymph *Galleria mellonella* larvae. Dissimilar letters (a-d) in the control and experimental groups represent statistically significant differences (Tukey's HSD $p < 0.001$ for THCs, prohemocytes, granulocytes, plasmatocytes, and spherulocytes; Kruskall Wallis for oenocytoids). Data are indicated as "Means ± Standard Errors" of three replicates using five larvae in one replicate

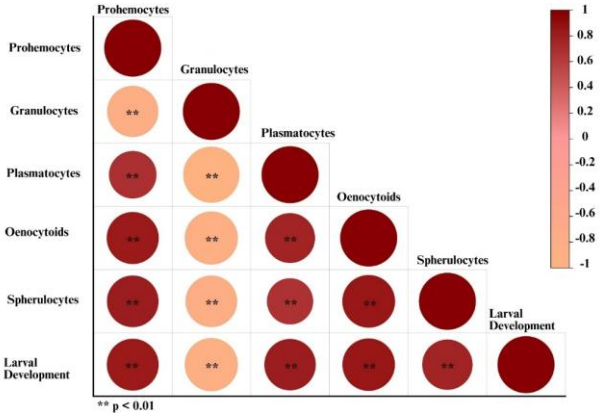

Figure 4. Correlation coefficient plot between differential hemocyte counts and larval development in *Galleria mellonella*

Terrestrial organisms such as *B. mori* (Muhammad et al., 2021; Parenti et al., 2020; Wang et al., 2023), *D. melanogaster* (Aloisi et al., 2024; El Kholy & Al Naggar, 2023; Tu et al., 2023), and *A. williamsoni* (Guimarães et al., 2021) may intake micro and nano PSs from the environment and accumulate them in the body cavity, intestine, intestinal lumen, midgut epithelium, Malpighian tubules, adipose tissue, and larval tissues such as ovary, head, digestive system, and hemolymph. Accordingly, it is concluded that plastic pollutants may easily enter and retain organisms and may be transported through the intestinal barrier to larval tissues. The data obtained in the literature on insect development are highly variable. Parenti et al. (2020) fed *Bombyx mori* larvae an artificial diet containing non-fluorescent 716 nm and fluorescent 533 nm PS NP (0.25 mg/0.5 g diet) and concluded that it did not cause changes in larval mortality, cycle time, adult emergence, and body weight. Under the researchers' experimental conditions, PS NPs were shown not to alter individual and population fitness of *B. mori* (Parenti et al., 2020). Likewise, PS NPs (50–100 nm, 2.22 × 10^{10}/mL, acute exposure) administered to *B. mori* had no significant effect on larval body mass or survival (Muhammad et al., 2021). Further, in *B. mori*, environmental concentrations of PS MNPs (0.25 to 1.0 µg/mL, 91.92 nm, 5.69 µm, 9.7 µm) increased body weight without affecting survival but did not cause significant changes in the development time and life span (Muhammad et al., 2024). In the present study, *G. mellonella* adult weights and adult sizes increased at high doses, while *P. turionellae* adult weights decreased similarly in all groups but no significant change in size was detected. The existence and accumulation of PS NPs in adipose tissue are probably due to the lipophilic properties of PS NPs. Accumulation of NPs in adipose tissue may enhance the weight and size of the organism as seen in *B. mori* (Muhammad et al., 2024) and *G. mellonella*. These findings suggest that chronic exposure to micro and nano PSs may affect host energy homeostasis and induce weight gain in organisms. In addition, PS NP treatment diminished the larval and pupal development time of *G. mellonella* and adult emergence time of *P. turionellae* in a dose-dependent manner. The concentration at which adult longevity reduced the most was the lowest concentration (50 ppm) in both *G. mellonella* and *P. turionellae*. The impacts of PS NP treatment on *G. mellonella* and *P. turionellae* exhibit interesting differences when compared to the impacts on *B. mori* in the literature. While Parenti et al. (2020) and Muhammad et al. (2021) did not reveal any significant effect of PS NPs in these species, our findings revealed meaningful reductions in developmental times. These results suggest that the effects of PS NPs may vary between species and that even low concentrations may be harmful. The idea that plastic pollution has a negligible but potential effect on the development of some insect species becomes relevant. At the same time, this evidence suggests that PS NPs have a unique effect on insect physiology, potentially by accelerating metabolic processes or through toxicological stress promoting faster development as a survival response.

Recent developmental studies in terrestrial organisms have indicated that, in parallel with the present findings, developmental time is more likely to be reduced in insects. For instance, adverse effects such as growth retardation and body weight loss have been described in *B. mori* fed with feed containing fluorescent PS NP (Wang et al., 2023). In *D. melanogaster* fed with food treated with PS-MPs (2 µm) at different concentrations (0.005, 0.05, 0.5 µg/mL), life span was shorter in males than females (Kholy & Naggar, 2022). The same researchers found that 7 days of acute treatment of adult D. melanogaster with almost all concentrations of PS MP significantly diminished survival of both male and female flies (El Kholy & Al Naggar, 2023). Micro (1.0-1.9 µm, 0.4-0.6 µm) and nano (0.04-0.06 µm) sized PS particles have been studied to reduce survival and alter the structure of the midgut, ovary, and testis in *D. melanogaster* (Urbisz et al., 2024). This impact was found to be sex-dependent, with male flies being more susceptible (Urbisz et al., 2024). In *D. melanogaster* fed with fluorescent PS NPs (100 µm) for prolonged periods, a reduction in larval-pupal development time and a reduction in the weight of male and female adults were determined (Aloisi et al., 2024). Also in another study, it was shown that the number of egg production and hatching rate of *D. melanogaster* were significantly reduced with delayed development after 5 days of exposure to PS NPs (100 nm; 1, 10, 50, and 100 mg/L) (Tu et al., 2023). Moreover, Martin-Folgar et al. (2024) have stated that PS NPs (30 nm, 24 h acute exposure) impeded the expression of genes involved in the endocrine system and development in *C. riparius* (Diptera) larvae. An almost 90% survival rate was found in larvae (Martin-Folgar et al., 2024). Results showed important declines in larval and pupal development of *G. mellonella* and *P. turionellae* in a dose-dependent manner. The adverse effects seen in Wang et al. (2023), El Kholy and Al Naggar (2023), and Kholy and Naggar (2022) studies further highlight the toxic effects of PS NPs in different organisms, while the sex-linked sensitivity described by Urbisz et al. (2024) also highlights the potential effects of PS NP exposure on ecosystem dynamics. Additionally, the effects of experimental conditions such as NP size and exposure times on the results are crucial to understanding the reasons for different findings. Thereby, examining the long-term effects of PS NPs on population fitness will also help to develop a more comprehensive understanding of this issue.

In insects, it is also very critical to obtain data on the immune system related to the effects of PS NPs and MPs such as accumulation in tissues, development, weight, growth, life span, and survival. For instance, it was emphasized that exposure to PS NPs in *B. mori* suppressed the immune response and rendered the host defenseless

(Muhammad et al., 2021). PS NPs have been demonstrated to diminish the activity of superoxide dismutase (Muhammad et al., 2021; Parenti et al., 2020) and glutathione transferase (Muhammad et al., 2021) which are crucial in antioxidant defense in the same organism. In general, it was concluded that PS NPs and MPs trigger cellular and oxidative stress and cause REDOX imbalance in *D. melanogaster*, *A. williamsoni*, *C. riparius* (Guimarães et al., 2021; Martin-Folgar et al., 2024; Urbisz et al., 2024). Besides, concentration-dependent impacts of PS on the cellular structure of midgut cells in *D. melanogaster*, indicative of cell necrosis and apoptosis, have been demonstrated (El Kholy & Al Naggar, 2023; Kholy & Naggar, 2022). In the study, we determined the responses of *G. mellonella* hemocytes, which are effective in cellular immune defense in insects, against PS NPs. Granulocytes increased dose-dependently, whereas other types of hemocytes and THCs decreased. Our data are similar in conjunction with our previous findings of cellular encapsulation responses (Demirtürk et al., 2024). Since encapsulation reactions against PS NPs entering the host as a foreign substance are realized with the help of host granulocytes. Granulocytes attach to the surface of PS NPs, disintegrate, and deposit their granules on the NPs in a thick layer. Plasmatocytes also mediate this defense mechanism. Even in this process, REDOX interactions may become dysregulated, and oxidative stress, apoptosis, and necrosis may occur. The decreases in THCs and DHCs also suggest that these NPs may weaken the insect's immune defenses, making them more susceptible to pathogens and parasites. This may have wider implications for insect health and survival in environments contaminated with nanoplastics. On the other hand, the changing developmental duration and immune responses of the host *G. mellonella* as well as the changing adult lifespan and weight of *P. turionellae* suggest a complex interaction in which the parasitoid may be indirectly affected by host exposure to PS NPs. Such a relation is critical for understanding the ecosystem dynamics and potential cascading effects in food webs.

Micro- and nanoplastics are an emerging threat to biodiversity and ecological conservation but little is known about their possible effects on pollinator species (Deng et al., 2021; Hu et al., 2019). Four types of MP, including polystyrene (PS), were identified in 66.7% of honey-bee samples (*Apis mellifera* and *Apis cerana*) collected from fields in six provinces in China. In a study conducted to measure how the application of PS MPs (0.5, 5, and 50 µm) for 21 days affected the proliferation of Israeli acute paralysis virus, it was revealed that PS was ingested, accumulated in the midgut, and increased the susceptibility of bees to viral infection. It has also been documented that PS, mainly the smallest sizes, damages the midgut tissue, which is then transferred to the hemolymph, Malpighian tubules, and trachea (Deng et al., 2021). Considering the ecological and economic significance of honey-bees, it is extremely important to expand similar studies on *Apis mellifera* to directly assess how PS NPs affect their development, immune responses, and overall health. However, bees and bee products as biological indicators of environmental pollution have been indicated as an economic alternative method for monitoring pollutants (Voget, 1989). Within this scope, bees and bee products may be considered to be effective in monitoring plastic pollution. Understanding the similarities and differences between the responses of *G. mellonella* and honey-bees to nanoplastics may help to predict the findings and develop specific guidelines for beekeeping. Further, how PS NPs affect pollination efficiency and hive health, bee behavior, foraging patterns, and the nutritional quality of pollen and nectar are also of interest.

With the results, it was determined that PS NPs caused shortening in the life cycle of *G. mellonella* and *P. turionellae*, and significant declines in the total and differential hemocyte counts of the host species. Data highlighted the possibility of transfer of nano PSs along the food chain in terrestrial ecosystems. Simultaneously, it demonstrates the potential of using the wax moth as a model of fully metamorphosed insects to investigate the biological impact of PS NPs on conspecific terrestrial animals and indirectly on the endoparasitoid *P. turionellae* to which NP toxicity can be transferred. The positive correlation of host larval and pupal development with endoparasitoid emergence time provides new information about the developmental toxicity of PS NPs due to host-parasitoid interaction. In addition, the data obtained will be useful for the assessment of environmental risks associated with PS NPs or potential adverse impacts on people and emphasizes the neglected nanoplastic toxicity in honey-bees affected by anthropogenic pollution and the potential hazards to human health from ingestion of bee products. The insights gained from studies of *G. mellonella* and *P. turionellae* may be translated into practical applications in beekeeping, providing knowledge about the health and sustainability of bee populations and their critical role in pollination and agriculture. Consequently, the study contributes to being able to fill the knowledge gaps on insect development at different doses of PS NPs.

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