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Effects of zinc oxide nanoparticles (ZnO NPs) on the biology of *Galleria mellonella* L. (Lepidoptera: Pyralidae)

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Abstract

Background: Because of its ability to absorb UV radiation and possess catalytic, antibacterial, and semiconducting properties, zinc oxide nanoparticles (ZnO NPs) are increasingly being used in consumer goods. Because nanoparticles are used so often, accurate methods for determining any associated toxicity are crucial. The greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) is a suitable experimental model organism due to immune defense of the larvae is very strong. Also, larvae are a good model for carrying out toxicity studies. In this study, to determine the effects of zinc oxide NPs (ZnO NPs) on the biology (larval development time, the weight of the last stage larvae, the pupal development time, the pupal weight, the eclosion rate, the maturation period, the adult weights, the adult longevity, and the percentage of adults) of *G. mellonella*, zinc oxide NPs were added to the larvae diet (honeycomb) at different doses (100, 500, 1000, 3000, and 5000 ppm).

Results: Results showed that pupal weights significantly decreased at 1000, 3000, and 5000 ppm doses of zinc oxide NPs when compared with control. Adult weight increased at 100 ppm zinc oxide NPs and the adult longevity of *G. mellonella* in the group exposed to 5000 ppm zinc oxide NP was longer than the control group. Finally, total male longevity extended in 500, 1000, 3000, and 5000 ppm zinc oxide NPs groups when compared to the control group.

Conclusions: The findings of this study contribute to evidence that the negative effects of ZnO NPs on biological properties on *G. mellonella*. In addition, the study reveals the adverse effects of zinc oxide NPs on a model experimental organism and provides an idea for researchers working on this subject in terms of new studies that can be done in future.

Keywords: Biology, *Galleria mellonella*, Nanoparticle, Toxicity, Zinc oxide

Background

Nanoscience foundations of which are known to have been laid by the end of the 1950s with the well-known physicist Richard Feynman's statement "There is plenty of room at the bottom" is a science dealing with the values in 1 nm: 10^{-9} sized systems and the events occurring in these sizes (Köksal & Köseoğlu, 2014). With the works carried out after the emergence of the concept of nanoscience, scientific studies in the field of nanoscience gained momentum and it was aimed to maintain the control of the substances having sizes from 1 to100 nm and make sense of the events occurring in this size range (Zhang et al., 2008). Out of nanotechnological products, nano metals and nano metal oxides are used today in many consumption sectors for a variety of reasons. For instance, zinc oxide NP (ZnO NP) and titanium dioxide (TiO_2 NP) are used in sun protection and cosmetic products, silver NP (Ag NP) in detergent and antibacterial products, silver Np (Ag NP) and gold NP (Au NP) in

The data of the present study were obtained from Ph.D. theses of the first author

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printer ink, and silver NP (AgNP) in textile (Tourinho et al., 2012). They are also used in the fields of electronics, medicine, and chemistry and in fuel additives.

Heavy metals like zinc have a special physiological role in the regulation of development in cells and organs of organisms. Zinc is involved in more than three hundred types of enzymes and proteins in terms of physiological and biochemical functions (gene replication and transcription, protein synthesis, binding specifically to hormones and receptors) (Qiang et al., 2005). While it inhibits growing when found insufficiently in the body of the organism, high levels of zinc may be toxic (Kayhan et al., 2009). With the development of industrialization and urbanization, heavy metals as environmental pollutants have become highly toxic for living beings. Therefore, the overabsorption of zinc by the organism may inhibit normal body metabolism, affect physiological and genetic processes, and cause cancer. As a result of the generated nano pollution, the organisms living on the Earth may be directly or indirectly exposed to these man-made NPs. These substances that can float in the air can penetrate the plant and animal cells which have never been exposed to them (Singh et al., 2008). What's more, they can get in contact with proteins, cell membranes, DNA, and organelles at the cellular level (Nel et al., 2009).

The shapes of NPs have high importance in scientific studies on NPs since the shape of an NP affects the intracellular traffic of NPs in the living systems. For example, while the hexagonal NPs are absorbed into the cytoplasm, the rod-shaped NPs are inclined to move toward the cell nucleus through microtubules. While spheric NPs have been reported to penetrate the cell more quickly and in higher amounts than the rod- and disk-shaped ones through the process of endocytosis, there are also studies reporting that cells select and absorb rod-shaped and cylindric NPs (Hillaireau & Couvreur, 2009; Panariti et al., 2012; Xu et al., 2008).

The absorption of metals in the body and their physiological effects on the living systems can vary by species. The possible physiological effects depend on many factors such as sex, age, developmental stage, and seasonal differences. Insects have been reported to keep huge amounts of metals in their body (Hsu et al., 2006). That's why, insects are used as bioindicators for metal pollution (Nummelin et al., 2007; Kayıs & Emre, 2012). *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) is a holometabolous insect and is popularly known as the "greater wax moth" in colloquial speech". It causes great economic damage to the beekeeping sector (Özer, 1961). *G. mellonella* is also one of the best model organisms in ecotoxicology tests (Zorlu et al., 2018).

In research on the toxicity of NPs to insects, Kool et al. (2011) examined the chronic toxicity effects of non-nano zinc oxide, non-nano zinc chloride, and zinc oxide NP on *Folsomia candida* (Collembola). According to this study, the survival rates of the organism in the 6400 mg zinc/kg dose trial of the mentioned species were unaffected by these substances, but the insect's reproductive ability was reduced. The toxic effects could be caused by zinc ions released from NPs in the environment that contain nano and non-nano zinc oxide.

Mekiwi et al. (2012), in a study on *G. mellonella* in which they tested zinc oxide NP, reported that all doses (1×10^{-6}, 2×10^{-6}, 3×10^{-6} ve 4×10^{-6}) affected larval mortality drastically in a dose- and time-dependent manner, intermediate forms between larva and pupa forms (LP) were seen, and deformed adults disabled and having short wings were seen in the experiments as a result of the zinc oxide application.

In a study on *Spodeoptera litura* (Lepidoptera: Noctuidae) with copper oxide NPs and zinc oxide NPs, Abd El-Wahab and Anwar (2014) found that an amount of 0.01 g increased mortality (100% in zinc oxide NP and 33.3% in copper oxide nanoparticle), caused disability, and the disabled larvae then died by turning a dark gray color. The amount of apoptotic cells in hemocytes increased within 24 h, and they also noted that the activity of the enzymes ascorbate peroxidase and superoxide dismutase were decreased in comparison to the control group (at a dose of 1000 mg zinc oxide NP per kg).

Khooshe-Bast et al. (2016) tested hexagonal wurtzite zinc oxide NPs on *Trialeurodes vaporariorum* (Westwood, 1856) (Hemiptera: Aleyrodidae). Adult insects (3, 5, 10, 15, 20 mg l^{-1}) were exposed to zinc oxide NP doses in the study. The LC_{50} value was determined to be 7.35 mg l^{-1}, and the mortality rate was calculated to be 91.6% after exposure to the highest dose.

In a study published in 2018, Zorlu et al. (2018) investigated the developmental physiology, hemolymph total protein content, antioxidant enzyme (SOD, CAT, and GST) activities, and hemolymph MDA level of *G. mellonella* fed with diet containing TiO_2 NP at various doses (100, 500, 1000, 3000, and 5000 ppm) starting at the second instar stage. Due to exposure to TiO_2 NP, they discovered that male longevity, pupal and adult weights, pupal and adult development periods, and the percentage of morphological defects in adults all increased in dosage groups when compared to the control group. Additionally, it was found in the same study that the MDA level increased at dosages of 100, 500, and 1000 ppm while the total protein amount increased only at the 1000 ppm dose and SOD, CAT, and GST activities varied in a dose-dependent manner.

In another study in which Fe_3O_4 NP concentrations (0.4, 2, 10, 50, 250 µg/10 µl) of 18–38 nm-sized spherical nanopowders were applied to the sixth instar (180 ± 20 mg) *G. mellonella* larvae by force-feeding method, it was reported that the weight of the pupae evolving from the larvae exposed to 250 µg/10 µl Fe_3O_4 NPs and the adult developmental time increased significantly (Eskin et al., 2021a).

The effect of Zinc oxide NPs on the larval total hemocyte count of *G. mellonella* (Eskin et al., 2019), the effects of copper oxide NPs on viable, mitotic, apoptotic, necrotic, and micronucleated hemocyte indices in the larval hemocytes of *G. mellonella* (Eskin & Bozdogan 2021), and the effects of copper nanoflowers on hemocytes in the larval hemolymph of *G. mellonella* and on the responses of acetylcholine esterase (AChE), superoxide dismutase (SOD), and catalase (CAT) enzymes (Eskin et al., 2021b) have been studied so far.

However, no studies are found on the toxic effects of zinc oxide NPs on the biological parameters of the greater wax moth, *G. mellonella*. In this study, to determine the effects of zinc oxide NPs (ZnO NPs) on the biology of *G. mellonella*, they were added to the larvae diet at different doses (100, 500, 1000, 3000, and 5000 ppm). Thus, their effects on the larval development time, which is the duration needed to reach the last stage larva, the weight of the last stage larvae, the pupal development time, the pupal weight, the eclosion rate, the maturation period, the adult weights, the adult longevity, and the percentage of adults with morphological disorders were determined.

Methods

Insects

G. mellonella culture was reared in the Animal Physiology Laboratory of the Faculty of Arts and Sciences at Marmara University. The continuation of the greater wax moth *G. mellonella* culture was achieved by growing newly hatched larvae in non-aseptic conditions in a naturally darkened honeycomb medium. The culture was grown in dark conditions at 27 ± 1 °C temperature with 60 ± 5% relative humidity. For the continuation of the culture, *G. mellonella* females and males were kept in a beaker inside of which was covered with a white tissue paper for a few days and the healthy eggs were separated by cutting the paper. Eggs were placed into 1 L glass jars with honeycomb inside. The jars in which the diet and eggs were placed were covered with American cloth and perforated metal lids. The larvae that reached the final stage (weighing 0.18 ± 0.02 g) out of the eggs laid in the diet were transferred to beakers covered with folded white tissue paper inside to become pupas. It was determined that the larvae evolved into pupae in the cocoon within 5–7 days after being taken into the beaker and the pupae were taken into a separate beaker and they matured there. The emerging adult individuals were taken back into a beaker covered with white tissue paper. During the experiments, these procedures were carried out daily.

Preparation of wax honeycombs

The honeycombs used in the experiments were ground to better absorb the aqueous solution containing zinc oxide NP. A blender was used for the grinding process. To examine the physiological effects of NPs on *G. mellonella*, the larvae of the experimental and control groups were fed on a 1 g ground honeycomb.

Chemicals

Zinc oxide NP (Alfa Aesar NanoShield®, 70 nm, ZN-3008C, 50% in water, pH: 6.9 cationic dispersant colloidal dispersion) was used as a chemical in the study.

Characterization of ZnO NPs

The size, morphology, and crystal structure of zinc oxide NP were analyzed by the Scientific and Technological Research Council of Turkey (TUBITAK) using high-resolution transmission electron microscopy (HRTEM, JEOL 2100), scanning electron microscopy (JEOL/JSM-6510LV-INCA/EDS), and X-ray diffractometry (XRD). The zeta potential values of the NPs were calculated by a zeta (ζ) potential analyzer (ZetaSizer Nno ZS, Malvern Instruments Inc., UK) in Yeditepe University Genetics and Biotechnology Laboratory. Zeta potentials were measured by diluting the prepared nanoparticle mixture in deionized water. For this aim, the NP solution was diluted at a rate of 1/200. After the solution was vortexed for 15 s, it was placed into the Zetasizer device and measurements were made. To prevent the NPs from clustering into microparticles, reveal their special characteristics, and ensure their stabilization, they were sonicated for 2 min at an amplitude of 60% through an ultrasonic homogenizer (Bandelin Sonoplus, HD3200, Berlin, Germany). In all experimental processes, 1 mL of zinc oxide NP was drawn from different ppm doses (100, 500, 1000, 3000, and 5000 ppm) prepared in double-distilled water with an automatic pipette and added to each point of 1 g ground honeycomb. On the other hand, only 1 mL of double-distilled water was added to the honeycomb in the control group. To dry the liquid solution inside the combs, they were kept in laboratory conditions for one day.

Effects of zinc oxide NPs on the biology of *G. mellonella*

To determine the LD_{50} (lethal dose) of zinc oxide NP, many dose trials were carried out moving up to high doses. As no larval death of up to 50–90% was observed at NP doses below 50,000 ppm and since the nanoparticle formulation used in the study did not allow doses above

50,000 ppm to be studied, we determined 100, 500, 1000, 3000, and 5000 ppm ZnO NPs doses as experimental doses. To determine the effects of NPs on the biology of *G. mellonella*, 1 g of naturally darkened ground honeycomb was placed in 60 × 15 mm plastic petri dishes. The suspensions of zinc oxide NPs (100, 500, 1000, 3000, and 5000 ppm) were added to the diet that would be 1 ml in each petri dish. Only double-deionized water was added to the diet of the control groups. One 2nd instar *G. mellonella* larva was placed in each petri dish with a brush. For the control and experimental groups, 20 larvae in each group were studied in four replicates at different times. By following the developmental processes of the larvae; larval development time, which is the duration to reach the last instar larva, the weights of the last instar larvae, the pupal development time and pupal weights, the pupal eclosion rate, the maturation period, the adult weights, the adult longevity, the percentages of the adults with morphological disorders were determined. Dead larvae, pupae and adults of the replications were not included in the statistical analysis.

Statistical analysis

The means of the data obtained from the experimental and control groups were compared using the SPSS program (Version 20.0, SPSS Science, Chicago, IL). First of all, whether these obtained data showed a normal distribution or not was tested. After the variance analyses of the normally distributed group means were compared using the ANOVA parametric test, the differences between the means were compared with Tukey HSD if the variance was homogeneous. After the nonparametric Kruskal Wallis test was conducted to determine whether there was a difference between the groups in the parameters that did not show normal distribution, the differences between the groups were determined with the Mann Whitney *U* tests. In the tables of all statistical results found in this study, the control group was defined as "0." The effects of zinc oxide NP applications obtained from the experimental and control groups on the pupal eclosion rates of *G. mellonella* were subjected to statistical analysis after normalizing by taking arcsine square roots before the analysis. Results were presented in percentages. A confidence limit of 0.05 was taken as a basis in all statistical tests.

Results

Characterization of ZnO NPs

According to the obtained images shown in Fig. 1 and the analysis performed using the Shimadzu XRD-6000 device and a Cu X-Ray tube (λ = 1.5405 Angstrom), it was understood that the zinc oxide NP consisted of nanorods and particles with a polymorphic structure (See Fig. 1: A B, C, and D).

The zeta potential value of the zinc oxide NPs was calculated to be + 23.0 mV as a result of the measurements (Fig. 2).

Effect of Zinc oxide nanoparticles on the biology of *G. mellonella*

The results of the developmental times of *G. mellonella* larvae fed until the last stage in combs containing different doses of zinc oxide NP are given in Table 1. According to the statistical results, when the larval development times obtained from *G. mellonella* larvae fed with zinc oxide NP were examined, it was seen that there was no significant difference among the groups ($x^2 = 8.424$, $df = 5$, $P = 0.134$) (Table 1).

In our study, both the control group larvae and the experimental group *G. mellonella* larvae which were fed with five different doses of zinc oxide NP and reached the last stage were removed from the growth culture and placed between white tissue papers to become pupae. The pupation time during which each larva in each group passed from the last instar stage to the pupa stage was determined (Table 2). After the pupation times of the control and experimental groups were determined, the weight of each pupa was measured on a precision scale. The weights of the pupae belonging to the control and experimental groups are shown in Table 2.

As a result of the applications with zinc oxide NP, the eclosion rates of *G. mellonella* pupae were calculated from the data obtained. Data on the pupal eclosion rates are given in Table 3.

The maturation time (days) was determined for the larvae of the control group and the experimental groups fed with different doses of zinc oxide NP (Table 4). As a result of the statistical analysis, no significant difference between the groups in the zinc oxide NP was recorded ($x^2 = 8.818$, $df = 5$, $P = 0.117$) (Table 4). When the data in Table 4 were examined, it was understood that zinc oxide NP caused an increase in the maturation time resulting in prolongation but this increase was statistically insignificant. Data on the effects of zinc oxide NP on adult weights of *G. mellonella* are presented in Table 4. As a result of the statistical analysis, the increased mean value obtained in the 100-ppm zinc oxide NP test was found to be significant compared to the mean value of the control group ($x^2 = 14.980$, $df = 5$, $P = 0.010$) (Table 4).

Data on the effects of zinc oxide NP on the adult longevity of *G. mellonella* are given in Table 5. As a result of the statistical analysis, the difference between the group exposed to 5000 ppm zinc oxide NP and the control group was found to be significant ($x^2 = 18.804$, $F = 2.471$, $df = 5$, $P = 0.033$) (Table 5).

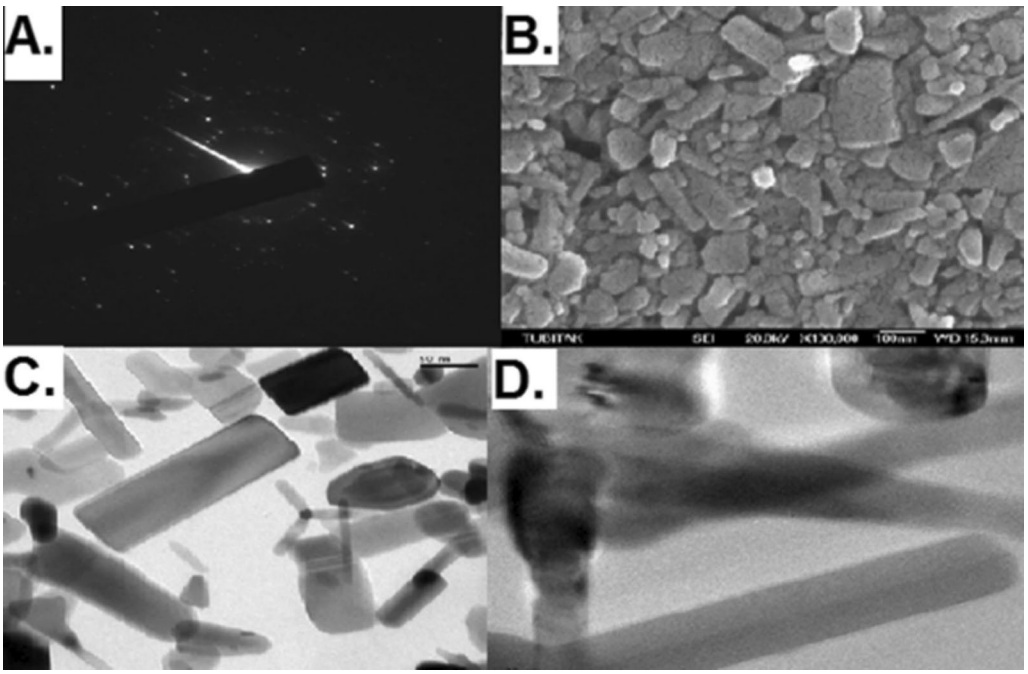

Fig. 1 Electron diffraction pattern image of zinc oxide nanorods (**A**), SEM image of zinc oxide NP at a 100.000 magnification (the bar indicates a length of 100 nm) (**B**), TEM image of zinc oxide NP (the bar indicates a length of 100 nm) (**C**), TEM image of zinc oxide NP (the bar indicates a length of 20 nm) (**D**)

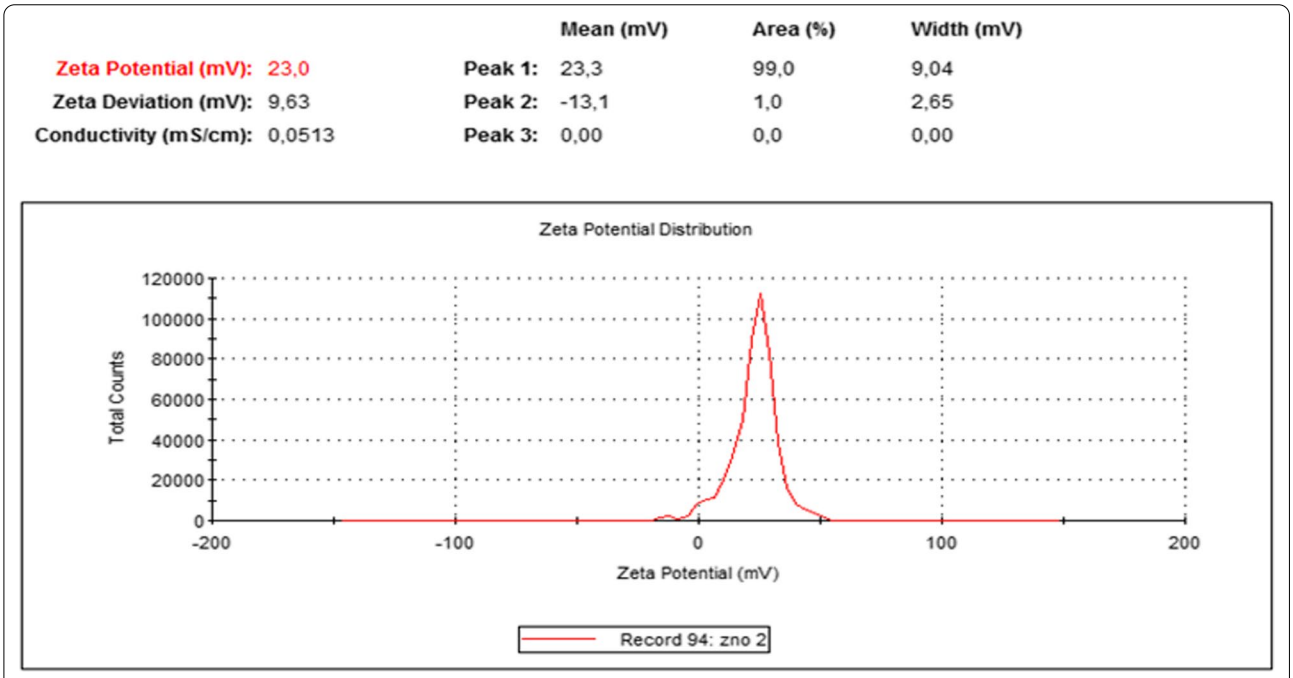

Fig. 2 Zeta potential value of zinc oxide NP

The period from the 2nd stage larval stage to the day of death of the adult was determined as "total longevity." The effects of zinc oxide NP doses on total longevity were examined separately in both female and male individuals; and thus, sex-related effects were revealed (Table 6). In the zinc oxide NP application,

Table 1 Effect of zinc oxide NP on the larval development time and larval weight of *G. mellonella*

Dose (ppm)	Min.–Max	Larval Development Time (Day) (Mean ± SE)*	Min.–Max	Larval Weight (Gram) (Mean ± SE)*
0	17–53	32.91 ± 1.52^{a}	0.12–0.36	0.2007 ± 0.0082^{a}
100	17–57	28.42 ± 1.47^{a}	0.14–0.37	0.2129 ± 0.0087^{a}
500	17–52	30.81 ± 1.35^{a}	0.15–0.31	0.2165 ± 0.0080^{a}
1000	17–72	32.81 ± 2.08^{a}	0.13–0.35	0.2015 ± 0.0093^{a}
3000	17–50	29.00 ± 1.60^{a}	0.14–0.33	0.1921 ± 0.0065^{a}
5000	17–72	31.01 ± 1.61^{a}	0.12–0.36	0.1943 ± 0.0076^{a}

*Kruskal Wallis, $P > 0.05$

The difference between the groups with the same letter in the same column next to the mean values is not statistically significant

Table 2 The effect of zinc oxide NP on *G. mellonella*'s pupal development time and pupal weight

Dose (ppm)	Min.–Max	Pupal Development Time (Day) (Mean ± SE)*	Min.–Max	Pupal weight (Gram) (Mean ± SE)**
0	4–41	13.44 ± 1.31^{a}	0.06–0.23	0.11 ± 0.0052^{ab}
100	5–47	17.06 ± 1.61^{a}	0.04–0.25	0.12 ± 0.0667^{a}
500	5–63	19.82 ± 2.22^{a}	0.04–0.28	0.12 ± 0.0846^{a}
1000	5–63	16.11 ± 1.97^{a}	0.06–0.17	0.10 ± 0.0503^{bc}
3000	4–66	18.29 ± 2.01^{a}	0.03–0.16	0.10 ± 0.0423^{bc}
5000	3–39	15.61 ± 1.37^{a}	0.03–0.18	0.09 ± 0.0534^{c}

*Kruskal Wallis, $P > 0.05$

**Mann Whitney U, $P < 0.05$

The difference between the groups with the same letter in the same column next to the mean values is not statistically significant

Table 3 Effect of zinc oxide NP on the pupal eclosion rates of *G. mellonella*

Dose (ppm)	Pupal Eclosion rate (%)*
0	85.45^{a}
100	86.00^{a}
500	74.46^{a}
1000	88.88^{a}
3000	85.41^{a}
5000	92.30^{a}

*Tukey HSD, $P > 0.05$

The difference between the groups with the same letter in the same column next to the mean values is not statistically significant

there was a statistically significant difference in the total male longevity between 500, 1000, 3000, and 5000 ppm zinc oxide NP applied groups and the control group and the total male longevity extended in these experimental groups compared to the control group ($x^2 = 11.438$, $df = 5$, $P = 0.043$) (Table 6). There was a statistically significant difference between the experimental groups and the control group in the effect of zinc oxide NP on the total female longevity of *G. mellonella* ($x^2 = 19.436$, $df = 5$, $P = 0.950$) (Table 6).

The data of the study in which the morphological abnormalities were pursued in the adults of the larvae fed with the honeycomb containing different doses of zinc oxide NP are presented in Table 7. When the percentage data of the morphological abnormalities of the adults obtained from *G. mellonella* pupae were examined, it was observed that there was a very low level of abnormality in the groups (2–11%) (Table 7). Although morphological abnormalities were detected in some individuals, they were not statistically significant ($F = 0.623$, $P = 0.684$).

Discussion

As a result of the application of different doses of NP, there was no statistically significant result in mean larval weights when compared with the mean weight of the control group ($x^2 = 8.006$, $df = 5$, $P = 0.156$) (Table 1). In the study by Kılıç et al. (2015), one of the studies in which larval development was not affected and fed in an environment containing low-dose antibiotics, the effect of the anthelmintic triclabendazole on the survival and development of *G. mellonella* larvae fed with artificial diet was investigated. In the study, it was determined

Table 4 Effect of zinc oxide NP on the maturation time and adult weights of *G. mellonella*

Dose (ppm)	Min.–Max	Maturation time (Day) (Mean ± SE)*	Min.–Max	Adult Weight (Gram) (Mean ± SE)**
0	4–30	12.10 ± 1.00^{a}	0.02–0.15	0.0578 ± 0.0031^{a}
100	6–31	13.33 ± 0.89^{a}	0.03–0.17	0.0764 ± 0.0049^{b}
500	5–29	12.03 ± 1.12^{a}	0.04–0.13	0.0644 ± 0.0037^{ab}
1000	5–33	13.58 ± 1.12^{a}	0.02–0.15	0.0588 ± 0.0044^{a}
3000	4–51	15.42 ± 1.39^{a}	0.03–0.10	0.0538 ± 0.0022^{a}
5000	4–34	14.22 ± 1.08^{a}	0.02–0.15	0.0621 ± 0.0041^{ab}

*Kruskal Wallis, $P > 0.05$

**Mann Whitney *U*, $P < 0.05$

The difference between the groups with the same letter in the same column next to the mean values is not statistically significant

Table 5 Effect of zinc oxide NP on adult longevity of *G. mellonella*

Dose (ppm)	Min.–Max	Adult Longevity (Day)* (Mean ± SE)[a]
0	3–36	16.30 ± 1.01^{a}
100	6–41	20.92 ± 1.52^{a}
500	7–44	20.63 ± 1.37^{a}
1000	3–40	20.67 ± 1.60^{a}
3000	7–35	21.51 ± 1.16^{a}
5000	4–53	21.89 ± 1.51^{b}

*Tukey, $P < 0.05$

The difference between the groups with the same letter in the same column next to the mean values is not statistically significant

that low doses of anthelmintic substances (0.001 and 0.01 g/100 g diet were not significantly effective on the time required for the development of *G. mellonella* larvae up to the seventh stage. The reason why no significant difference was observed in larval development times in this study conducted with zinc oxide NP may be that the larvae can regulate the possible toxic effect on the larval development time of the insect during the long period of time until the last stage. There are similar studies on the effects of various nano and non-nano heavy metals on the weights of last instar *G. mellonella* larvae. For instance, Yılmaz (2013), in her study examining the effects of aluminum chloride ($AlCl_3$) at 100,000, 200,000, and 300,000 ppm doses on the biology and hemocyte count of *G. mellonella*, diagnosed no effect of the mentioned microparticles on the larval weight and explained this situation with the fact that *G. mellonella* larvae could regulate $AlCl_3$. In our study, the reason why statistically significant effects were not observed in some biological parameters of *G. mellonella* on which we tested the toxic effects of zinc oxide NP may be that the insect regulated the tested doses. As an example of the regulation mechanism, some non-essential metals (Cd) can be accumulated in lepidopteran larvae, while others (Pb) are hardly assimilated. While the concentration of essential metals such as zinc (Zn) and nickel (Ni) in insect larvae may vary, metals such as copper (Cu) are generally controlled

Table 6 Effect of zinc oxide NP on total male and total female longevity of *G. mellonella*

Dose (ppm)	Min.–Max	Total Male Longevity (Day) (Mean ± SE)*	Min.–Max	Total Female Longevity (Day) (Mean ± SE)**
0	37–107	66.71 ± 4.72^{a}	44–140	83.00 ± 4.67^{a}
100	44–123	80.45 ± 5.42^{ab}	45–127	81.37 ± 4.56^{a}
500	44–114	82.66 ± 5.90^{b}	44–108	78.66 ± 4.53^{a}
1000	44–119	84.90 ± 4.71^{b}	62–118	86.41 ± 4.49^{a}
3000	51–118	85.05 ± 4.37^{bc}	49–122	81.61 ± 3.92^{a}
5000	50–119	85.00 ± 3.68^{b}	43–126	83.65 ± 5.60^{a}

*Mann Whitney *U*, $P < 0.05$

**Tukey HSD, $P > 0.05$

The difference between the groups with the same letter in the same column next to the mean values is not statistically significant

Table 7 Effect of zinc oxide NP on the emergence of morphological abnormalities in the adults of *G. mellonella*

Dose (ppm)	Emergence of Abnormalities (%)*
0	2.12[a]
100	11.62[a]
500	5.71[a]
1000	5.55[a]
3000	2.12[a]
5000	8.33[a]

*Tukey, $P > 0.05$

The difference between the groups with the same letter in the same column next to the mean values is not statistically significant

by their regulation mechanisms. Regulation mechanism occurs with the lysis in the digestive system of intestinal cells located in the places where metals accumulate in the fecula for excretion. Or, metals are excreted by passing from the hemolymph to the Malpighi tubes and from there to the hindgut of the insect (Hopkin, 1989; Noret et al., 2007). The same researchers reported that the larvae of *Issoria lathonia* (Lepidoptera: Nymphalidae) feeding on leaves containing high doses of zinc regulate their internal zinc concentrations by excretion of fecula containing a large amount of metal concentrate. In a study, Kharbouche et al. (2007) investigated the effects of codeine, a psychoactive alkaloid obtained from the poppy (*Papaver somniferum*), on the development of the greenbottle fly *Lucilia sericata* (Diptera: Calliphoridae) and they found that codeine caused no significant difference in the mean larval weights of the insect during the pupal stage. The results of this study are in agreement with the above results and the literature. The reason why the weights of the larvae exposed to zinc oxide NP and the larvae of the control group did not differ statistically may be due to the fact that the insect regulated the nanoparticles with the activation of the regulation mechanisms described in detail above (Table 1).

When the pupal development times of *G. mellonella* fed with zinc oxide NP-added honeycombs were examined, it was seen that the difference among the groups was not statistically significant ($x^2 = 6.226$, $df = 5$, $P = 0.285$) (Table 2). However, although there was no statistically significant difference, it was observed that there was an increase in the mean pupal development times compared to the control group. The reason for this increase can be explained by the reasons mentioned in the study by Uçkan et al. (2014). These researchers investigated the effects of indole acetic acid (IAA) on the biochemical parameters of *Achoria grisella* Fabr. (Lepidoptera: Pyralidae) hemolymph and *Apanteles galleriae* Wilkinson (Hymenoptera: Bracomidae) larvae. They reported that IAA induced changes in the biochemical parameters of both insect species, and the reason for this was that IAA could cause hormonal changes effective in the development and metabolism of the insect. Similarly, the changes in the hormonal balance and metabolism of *G. mellonella* may have caused an increase in the mean pupation time in our study. When the pupal weights obtained from *G. mellonella* pupae fed with zinc oxide NP-containing diet were examined, it was seen that there was a significant difference between the groups ($x^2 = 17.344$, $df = 5$, $P = 0.004$) (Table 2). The pupal weights seen in the 5000 ppm-dose experimental group were recorded less than the pupal weights in the control group, and the decrease in the pupal weights was found to be statistically significant compared to the related groups (Table 2). In a study investigating the effect of cypermethrin on the pupation and death rates of *G. mellonella*, Sak and Uckan (2009) explained that the decrease in the pupal weight may be due to the toxic effects of the insecticide. In the same study, the toxic effect was reported to have occurred as follows: First of all, after the insecticide is taken by the insect with diet, stress occurs in the living being. Overcoming stress conditions requires a high amount of energy. Various repair mechanisms also cause energy consumption in this process, resulting in a decrease in the energy stores (glycogen storage and adipose tissues) of *G. mellonella*. The reason for the decrease in the pupal weights in some doses of the experimental groups in our study may be similar to the situation described in the study of Sak and Uckan (2009) (Table 2). Zinc oxide NPs may have caused a decrease in the insect's energy stores by creating stress on the insect after being taken into the insect's body with diet. From other similar studies on the effects of heavy metals on insects, in a study in which Huang et al. (2012) investigated the effects of copper ions, one of the heavy metals, on the growth, development, and population dynamics of *S. litura*, the pupal weights of individuals exposed to all copper doses in the experimental groups (25, 50, 100, and 200 mg/kg) decreased significantly compared to the control group similar to the study performed with zinc oxide NP. It has been reported that zinc and copper bind to cytosol metallothionein in the midgut in many organisms and can be toxic at high concentrations (Baghban et al., 2014; Cheruiyot et al., 2013).

As a result of the statistical analysis, it was seen that there was no significant difference among the groups in terms of the pupal eclosion rate ($F = 1061$, $P = 0.414$) (Table 3). There are not enough studies on the effects of zinc oxide NP on the pupal eclosion rates of *G. mellonella.* Zorlu (2016) investigated the effects of titanium dioxide NP at the doses included in our study on the

pupal eclosion rates of *G. mellonella*. In the study, it was found that the mentioned NP did not affect the eclosion rate. The results in the study are consistent with the results obtained in our study. To give examples from other studies with non-nano and heavy metals, Abu Elela and Elsayed (2015) investigated the effects of lead and cadmium on *S. litura*'s eclosion rate and found that both heavy metals did not affect the eclosion rate of this species. Yılmaz (2013) reported that the eclosion rate of *G. mellonella* was not adversely affected by aluminum chloride at high doses (100,000, 200,000, 300,000 ppm). Safaee et al. (2014) investigated the effects of lead on the development of *D. melanogaster and* found that this heavy metal had no effects on the eclosion rate at low doses. The researchers observed no discernible negative effects on pupae. They chalked this up to the fact that metallothioneins, which are cysteine-rich proteins found in insects (these proteins bind to heavy metals and neutralize their toxic effects), are capable of detoxifying low doses of lead. The presence of copper-binding proteins in the gastrointestinal tract of *G. mellonella* was reported by Polek et al. (1993). The reason why no significant toxic effect was observed on the eclosion rate of the experimental groups compared to the control group in our study may be the fact that the aforementioned detoxification system works in *G. mellonella* (Table 3).

In studies on the effects of heavy metals on insects, Nurullahoğlu et al. (2014) explained that there is a selective physiological mechanism in insects against heavy metals, depending on the insect species. Baghban et al. (2014), in a study in which they investigated the effects of three different heavy metals (Cd, Cu, and Zn) on the nutrition of *H. armigera*, reported that heavy metals do not always have negative effects on organisms. In our study, when the data on the maturation time are examined, it is understood that the insect can regulate the nanoparticle stress at this stage. Detailed studies on the weight gain in adult insects as a result of heavy metal stress and its causes are currently insufficient. In this study conducted with zinc oxide NP, the reason for the increase in adult weight observed at 100 ppm dose compared to the control group can be explained by another mechanism (Table 4). Hormesis is a phenomenon put forward to explain the ability to survive at low concentrations of toxic substances and the adaptive features developed against these substances. This phenomenon actually tells us that toxic molecules and environmental conditions have a biphasic effect that is both supportive and destructive for life. The mechanism is activated by low doses of toxic agents and adds to the living beings an adaptive response. Protective proteins, antioxidant enzymes, and chaperone proteins take part in the adaptive response (Hoffman et al., 1986, Salem, 2000; Calabrase and Blain, 2005; Kısım & Uzunoglu, 2012). This phenomenon was tried to be explained by the effects of sublethal doses of heavy metals, insect growth regulators, and pesticides on juvenile hormones, fertility, and other biological parameters (Altuntas et al., 2012). In our study, the increase in weight at low doses, the activation of hormesis phenomenon in the insect, and the insect response to heavy metal stress may have increased the protective proteins, antioxidant enzymes, and chaperone proteins in *G. mellonella* (Table 4). One of the studies that recorded an increase in body weight was a study testing an antibiotic on *Nezara viridula* (L.) (Heteroptera: Pentatomidae). It was reported that low concentrations of the mentioned antibiotic accelerated the development of the insect, increased the survival rate, adult longevity, and body weight (Hirose et al., 2006). In a study by Zorlu (2016) which investigated the effects of titanium dioxide NP on the biology of *G. mellonella*, adult weights increased at 3000 and 5000 ppm doses compared to the control group, similar to our study. The results of our study are compatible with the results of the studies in the literature.

When the data in Table 5 were examined, it was understood that the adult longevity of *G. mellonella* in the group exposed to 5000 ppm zinc oxide NP was longer than the control group. When the existing literature on these results is examined, Uckan and Ergin (2003) reported that both carbohydrates and water were responsible for the extending adult longevity of *Apanteles galleriae* Wilkinson (Hymenoptera: Braconidae). Uckan et al. (2011) also reported that lipid was decreased in the hemolymph of *G. mellonella* larvae exposed to gibberellic acid (GA_3) at all doses while carbohydrate was decreased at most of the doses. In their study examining the effects of niclosamide on some biological and physiological properties of *G. mellonella.* Buyukguzel and Kayaoglu (2014) studied the survival rate, development time, sex ratio, longevity, egg productivity of *G. mellonella* at different concentrations of niclosamide taken with diet during the larval period and determined the negative effects on the eclosion rate. The researchers reported that the reason for these negative effects may be the oxidative stress caused by the related antibiotic in the midgut. In addition, the effects of copper oxide and copper sulfate NPs on detoxification enzymes in the midgut and fat body of *G. mellonella* were determined by Tunçsoy and Özalp (2016).

It was understood that the means of total male and female adult longevities were very close to each other. From the data we obtained and showed in Tables 6, it was understood that male individuals exposed to zinc oxide NP were more sensitive to NP than females and

their longevity was extended as a result of the toxic effect. Zorlu (2016) found in his study that TiO_2 NP increased the total female longevity of *G. mellonella*, but this difference was insignificant in statistical comparison. However, there were fluctuations in total male longevity. The findings of our study were similar to the results obtained by Zorlu (2016) in terms of statistical differences. Cervera et al. (2004) reported that female individuals are more sensitive than male individuals and that sexual toxicity may develop (Cervera et al., 2004; Zorlu, 2016).

Yılmaz (2013) found that the percentage of morphological abnormalities seen in *G. mellonella* adults as a result of aluminum chloride application did not cause a statistical difference between the experimental groups and the control group. The researcher reported that aluminum chloride did not negatively affect the development of *G. mellonella* larvae or that possible adverse effects could be regulated by various physiological mechanisms. Zorlu (2016) tested titanium dioxide NP on *G. mellonella* at the same doses as in our study and achieved a statistically significant result compared to the control group at only 100 ppm dose. The insignificant difference found at other doses and the findings obtained by Yılmaz (2013) are compatible with the findings of our study. Al-Salim et al. (2011) found quantum dots in Malpighi tubes of winged adults formed as a result of metamorphosis of lepidopter larvae fed with quantum dots in the larval stage. The reason for the 11% (zinc oxide NP) morphological abnormality observed in our study may be the toxic effects of NPs, which cannot be detoxified by metamorphosis and other mechanisms, during adult formation, with some preservation in the insect (Table 7).

Conclusions

Nanotechnological products which started to be manufactured with the development of nanotechnology after the emergence of nanoscience as a branch of science are being used for various purposes in many consumer products today. NPs, which have gained popularity thanks to the practical advantages (self-cleaning glass, bacteria-proof beehives, antibacterial bandages, nanorobots, etc.) they offer, can threaten the living beings on earth and their biological systems when they are used in excessive amounts and unconsciously released into natural ecosystems The findings obtained in this study were tried to be interpreted by comparing them with the existing very few studies conducted, and in cases where the existing literature was insufficient, our results were interpreted by reviewing the data in the studies testing non-nano heavy metals and chemicals on living beings. In addition to our current study, further studies are needed for understanding the toxic effects of NPs on insects and the accumulation of NPs in the organs and tissues of insects and we recommend that they should focus on the detection of hemolymph stress proteins synthesized in the insect exposed to NPs, the examination of the gene-level profiles of metallothioneins synthesized as a result of heavy metal stress, hormonal changes in the larva of insects (such as juvenile hormone and ecdysone hormone), morphological changes in the hemolymph and intestinal epithelial cells in the cellular basis (detection of excessive vacuolization and membrane thickening), the examination of the parts with heavy metal accumulation by staining the histological sections of the intestine and Malpighi tubes, etc. In this study, the toxic effects of zinc oxide NPs on *G. mellonella*, a model experimental animal, were tested to determine the possible negative effects that may occur today and in future.

Abbreviations

$AlCl_3$: Aluminum chloride;, Ag NP: Silver nanoparticle; Au NP: Gold nanoparticle; CAT: Catalase; Fe_3O_4 NP: Iron oxide nanoparticle; g: Gram; GST: Glutathione S-Transferase; HRTEM: High-resolution transmission electron microscopy; L: Liter; MDA: Malondialdehyde; min: Minute; mL: Mililiter; nm: Nanometer; mV: Millivolt; NPs: Nanoparticles; SEM: Scanning electron microscopy; SOD: Superoxide dismutase; TiO_2 NP: Titanium dioxide nanoparticle; XRD: X-ray diffractometry; ZnO NPs: Zinc oxide nanoparticles.

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Authors' contributions

AE carried out the toxicity studies, read, wrote, and approved the final manuscript. ZUN managed and coordinated the toxicity studies. All authors read and approved the final manuscript.

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Availability of data and materials

All data and materials used and/or analyzed during the current study are available in this manuscript.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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