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Camilla Carla Parenti: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft. **Andrea Binelli**: Conceptualization, Resources, Supervision, Writing - Review & Editing, Project administration, Funding acquisition. **Silvia Caccia**: Investigation, Methodology, Writing - Review & Editing. **Camilla Della Torre**: Methodology, Writing - Review & Editing. **Stefano Magni**: Investigation, Writing - Review & Editing. **Gabriele Pirovano**: Investigation. **Morena Casartelli**: Conceptualization, Project administration, Conceptualization, Resources, Supervision, Writing - Review & Editing.

Ingestion and effects of polystyrene nanoparticles in the silkworm *Bombyx mori*

Parenti[1], C.C., Binelli[1*], A., Caccia[2], S., Della Torre[1], C., Magni[1], S., Pirovano[1], G., Casartelli[1], M.

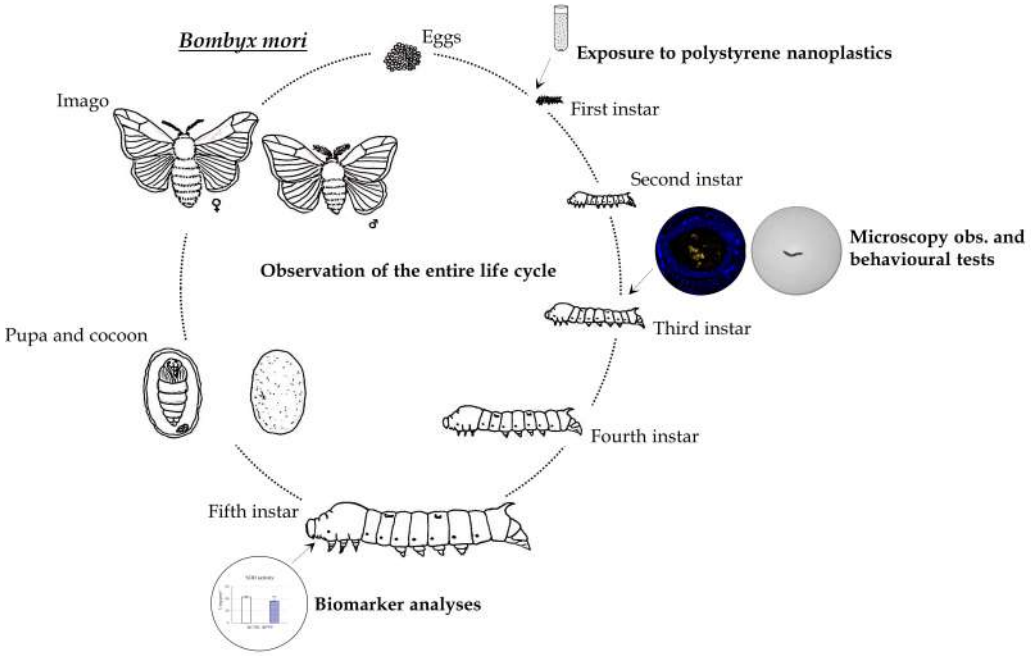
[1]Department of Biosciences, University of Milan, Via Celoria 26, 20133 Milan, Italy

[2]Department of Agricultural Sciences, University of Naples "Federico II", Via Università 100, 80055 Portici (Naples), Italy

*Corresponding author e-mail: andrea.binelli@unimi.it

ABSTRACT

Information on the occurrence and effects of nanoplastics in ecosystems worldwide currently represent one of the main challenges from the ecotoxicological point of view. This is particularly true for terrestrial environments, in which nanoplastics are released directly by human activities or derive from the fragmentation of larger plastic items incorrectly disposed. Since insects can represent a target for these emerging contaminants in land-based community, the aim of this study was the evaluation of ingestion of 0.5 µm polystyrene nanoplastics and their effects in silkworm (*Bombyx mori*) larvae, a useful and well-studied insect model. The ingestion of nanoplastics, the possible infiltration in the tissues and organ accumulation were checked by confocal microscopy, while we evaluated the effects due to the administered nanoplastics through a multi-tier approach based on insect development and behaviour assessment, as endpoints at organism level, and the measurements of some biochemical responses associated with the imbalance of the redox status (superoxide dismutase, catalase, glutathione s-transferase, reactive oxygen species evaluation, lipid peroxidation) to investigate the cellular and molecular effects. We observed the presence of microplastics in the intestinal lumen, but also inside the larvae, specifically into the midgut epithelium, the Malpighian tubules and in the haemocytes. The behavioural observations revealed a significant ($p<0.05$) increase of erratic movements and chemotaxis defects, potentially reflecting negative indirect effects on *B. mori* survival and fitness, while neither effect on insect development nor redox status imbalance were measured, with the exception of the significant ($p<0.05$) inhibition of superoxide dismutase activity.


Bombyx mori
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Exposure to polystyrene nanoplastics
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[1]Department of Biosciences, University of Milan, Via Celoria 26, 20133 Milan, Italy

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Information on the occurrence and effects of nanoplastics in ecosystems worldwide currently represent one of the main challenges from the ecotoxicological point of view. This is particularly true for terrestrial environments, in which nanoplastics are released directly by human activities or derive from the fragmentation of larger plastic items incorrectly disposed. Since insects can represent a target for these emerging contaminants in land-based community, the aim of this study was the evaluation of ingestion of 0.5 µm polystyrene nanoplastics and their effects in silkworm (*Bombyx mori*) larvae, a useful and well-studied insect model. The ingestion of nanoplastics, the possible infiltration in the tissues and organ accumulation were checked by confocal microscopy, while we evaluated the effects due to the administered nanoplastics through a multi-tier approach based on insect development and behaviour assessment, as endpoints at organism level, and the measurements of some biochemical responses associated with the imbalance of the redox status (superoxide dismutase, catalase, glutathione s-transferase, reactive oxygen species evaluation, lipid peroxidation) to investigate the cellular and molecular effects. We observed the presence of microplastics in the intestinal lumen, but also inside the larvae, specifically into the midgut epithelium, the Malpighian tubules and in the haemocytes. The behavioural observations revealed a significant ($p<0.05$) increase of erratic movements and chemotaxis defects, potentially reflecting negative indirect effects on *B. mori* survival and fitness, while neither effect on insect development

nor redox status imbalance were measured, with the exception of the significant ($p<0.05$) inhibition of superoxide dismutase activity.

1. INTRODUCTION

Almost 50 years have passed since plastic waste has been assessed as one of the emerging environmental problems (Kramm et al., 2018). Since then, the perceived impact of plastic has been linked to their widespread and growing use (Heidbreder et al., 2019) and their high persistence in ecosystems due to the extreme resistance to degradation (Horton et al., 2017).

Plastics reach the environment from several sources, mostly related to human activities, such as littering and inadequate disposal. Once in the environment, the larger plastic items are fragmented into microplastics (MPs) and nanoplastics (NPs) by sunlight, mechanical abrasion, salinity and temperature fluctuations, increasing their potential to be incorporated by organisms (Oliveira et al., 2019a). Although oceans have always been considered the major sink of plastics (Cózar et al., 2014), recent studies estimated that the contamination of terrestrial ecosystems might be up to 20-fold larger (Oliveira et al., 2019b). One of the major concerns related to plastic deposition in inland areas is the atmosphere circulation, which is responsible for the transport of plastic debris also to remote terrestrial areas, such as high-altitude glaciers (Zhang et al. 2019). Another source of plastics is the sewage sludge used as fertilizer in agricultural practises, since it represents the major fate of plastic debris removed from wastewaters (Nizzetto et al., 2016).

Nevertheless, terrestrial environments have been less studied than aquatic ecosystems with limited and variable monitoring data on plastic particle abundance, as demonstrated by the recent study by Wang et al. (2020) that reported a very wide range in plastic distribution from 1 to 40,000 items/kg in soils. As well as for monitoring, also studies concerning the uptake and the effects of plastic particles on terrestrial organisms are scarce. Among them, earthworms are the predominant model species used to investigate the potential negative effects in soil biota (Wang et al., 2020). For instance, growth rate, survival and reproduction of *Lumbricus terrestris* exposed to polyethylene

MPs (<150 µm) were significantly reduced (Huerta Lwanga et al., 2016), while histopathological analyses on the gut of *Eisenia andrei* demonstrated that the ingestion of polyethylene microbeads (250-1000 µm)caused tissue damage and immune reactions (Rodriguez-Seijo et al., 2017).

Regarding terrestrial ecosystems, there is few evidences also for the impact of NPs, which are recently redefined as debris in the range of 1 to <1000 nm (Hartmann et al., 2019). The main reason is due to the extreme difficulty in their detection and characterization because of their smaller size, factor that may underestimate the existing concentrations both in aquatic and terrestrial environments. Another problem is related to the bioavailability of NPs, since their capability to enter organisms and induce negative effects have to be surely assessed yet (Stapleton, 2019), especially because the nano-scale particles may cross the intestinal barrier more easily than larger plastic debris (Shen et al., 2019). NP effects were studied only on a few species of soil invertebrates: polystyrene NPs (50-200 nm particle size) were shown to reduce body weight and changed the gut microbiome of the soil oligochaete *Enchytraeus crypticus*, while they affected the energy metabolism in the soil nematode *Caenorhabditis elegans*, causing decreased locomotion and reproduction (Zhu et al., 2018; Kim et al., 2019). On the contrary, no data are available about ingestion and effects of NPs on insects that are the most varied and adapted group of organisms on earth. This is surprising in the light of their distribution worldwide that, combined with the ubiquitous nature of plastic contamination, make the contact between these arthropods and plastic particles unavoidable (Oliveira et al., 2019b). Furthermore, an interesting study by Al-Jaibachi et al. (2018) focused the attention on those insect groups, such as mayflies, dragonflies, midges and mosquitos, who become an important pathway for plastic particles dispersal, being their life cycle both aquatic and terrestrial. In detail, they demonstrated that polystyrene microplastics (2 and 15 µm) ingested by mosquito larvae were found in adults, exposing terrestrial predators to the risk of plastic ingestion.

Among insects, Lepidoptera is the second largest order (Powell, 2009) and their fundamental ecological role is not only related to their biomass and biodiversity, but also to their role as

herbivores, pollinators and food for insectivores. In ecotoxicological studies, adult stage of Lepidoptera is often used to test the impact of insecticides on non-target organisms, which is a major reason for pollinators decline (Mulé et al., 2017). Among lepidopteran species, the domesticated silkworm (*Bombyx mori*) is considered a useful model system to evaluate toxicity and mode of action of various substances such as drugs, pesticides and metallic nanomaterials (Meng et al., 2017a). Therefore, it can be used in health safety and environmental pollution assessment (Abdelli et al., 2018) since *B. mori* larvae own considerable advantages in comparison to other invertebrate and vertebrate models. They are easy to handle and have a relatively short life cycle, they can be easily maintained under laboratory conditions and are ideal to screen fast the toxicity of many substances at low cost (Abdelli et al., 2018). Moreover, the genome of *B. mori* was completely sequenced in 2008 (International Silworm Genome Consortium, 2008), giving a major boost in molecular and functional genomic studies. The efficacy of *B. mori* larvae as model organism in toxicological studies has been evaluated in a number of studies: Hamamoto et al. (2004) used silkworm to test the efficacy of several antibiotics, demonstrating that 50% of the effective dose (ED_{50}) is consistent with those reported for mice, and different studies established that it can be used as infection model for the identification of bacterial virulence factors and as human disease model (Kaito, 2016; Ishii et al., 2015; Meng et al., 2017a). This species has also been applied for environmental monitoring, since it has been found to be highly sensitive to pesticides, heavy metals and other harmful chemicals (Meng et al., 2017a). Silkworms were also employed to assess nanomaterial hazard, such as Ag nanoparticles, which can affect growth and survival in a dose dependent manner, causing a variation in gene expression pattern of the fat body (Meng et al., 2017b).

To the best of our knowledge, *B. mori* has never been used as a model species to unravel the effects of NPs in insects. This study reports, for the first time, the fate of spherical polystyrene nanoplastics (PNPs) administered by ingestion on *B. mori* larvae and their effects by a multi-tier approach based on development and behaviour assessment, as endpoints at organism level, and a biomarker suite

105 associated with the oxidative balance to measure the potential effects at molecular and cellular
106 levels. In detail, we measured the reactive oxygen species (ROS) and the detoxifying enzyme of
107 Phase II glutathione s-transferase (GST), as well as the activity of the main three anti-oxidant
108 enzymes, namely superoxide dismutase (SOD) and catalase (CAT), while the lipid peroxidation
109 (LPO) was measured as a typical endpoint of oxidative damage. Moreover, we evaluated the
110 capability of *B. mori* larvae to ingest PNPs through the observations of cryo-sections at confocal
111 microscopy, as well as the possible crossing of PNPs through the intestinal barrier, the transport by
112 the circulatory system and the potential accumulation in the internal organs.
113
114 ## 2. MATERIALS AND METHODS
115 ### 2.1 *Characteristics of PNPs*
116 We used fluorescent-labelled PNPs (Flash Red, excitation wavelength 660 nm, emission
117 wavelength 690 nm) with a mean diameter of 0.513 µm (Bangs Laboratories, Inc., Fishers, IN,
118 USA) for the microscopy observations, while non fluorescent PNPs with comparable size (diameter
119 range=0.4-0.6 µm; Spherotech, Inc., Lake Forest, IL, USA) for the exposure assays. Before the
120 exposures, size distribution and zeta potential of PNPs were certified by a Malvern Zetasizer Nano
121 ZS instrument (Malvern Panalytical Ltd, Malvern, UK; see Supplementary Materials). The
122 nanoparticles' characterization showed some differences in terms of size (Table S1), which were
123 not relevant to the purposes of our study (both PNPs did not exceed the nanosize range).
124
125 ### 2.2 *Insect rearing and bioassays with PNPs*
126 Eggs of *Bombyx mori* and the artificial diet for larvae rearing (Cappellozza et al., 2005) were
127 provided by the Research Centre for Agriculture and Environment (CREA-AA, Padova, Italy).
128 Larvae were reared on artificial diet based on mulberry leaves, under controlled conditions
129 (25±1°C, 65-70% relative humidity, 12:12 h light/dark period).

PNP exposures were performed by feeding larvae on artificial diet in multi-well plastic trays (Bio-Rt-32, Frontier Agricultural Sciences) covered with perforated plastic lids (Bio-Cv-4, Frontier Agricultural Sciences), maintaining the same rearing conditions reported above. We fed larvae *ad libitum* with artificial diet overlaid with an aqueous suspension of PNPs (pieces of diet of 0.5 g were overlaid with 50 µl of an aqueous suspension of PNPs at different concentration depending on the experiment). Control larvae were fed with diet overlaid with distilled water (50 µl/0.5 g diet) containing a proper dilution of the preservative of the PNP stock solution (Tween 20 or sodium azide). The diet was replaced every day.

Two different feeding bioassays on *B. mori* were carried out, depending on the analysis to be performed. To assess the presence of PNPs within tissues and organs by confocal microscopy, *B. mori* larvae were exposed to fluorescent PNPs (0.25 mg/0.5 g diet) for 10 days (from hatching to the second day of the 3rd instar). Subsequently, to evaluate the effect of PNP ingestion on larval behaviour, development and biochemical parameters, three independent experiments were performed exposing the larvae to 0.25 µg of PNPs per 0.5 g of diet. In detail, the exposure lasted for 10 days for behaviour analysis (from hatching to the second day of the third instar), for 21 days for biochemical analyses (from hatching to the third day of the last fifth instar) or from hatching until the end of the last instar to monitor insect development.

2.3 *Sample preparation for microscopy and image analysis*

Larvae were first anaesthetized with CO_2 and dissected immediately at the end of exposures. Then, the anterior and posterior ends of the larvae were discarded by cutting between the first and the second pair of legs and the fourth and fifth pseudolegs. The central portions of the body were individually fixed for 3 hours at 4 °C in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4). Samples were washed 3 times in PBS (rinses of 30 min each) and placed in 15% and 30% sucrose solutions (w/v in PBS) for 30 min and overnight, respectively, prior to inclusion in the cryostat-embedding

156 medium (Bio Optica, Milan, Italy). Samples were then frozen in liquid nitrogen and stored at -80
157 °C. A CM1850 cryostat (Leica, Wetzlar, Germany) was used to cut transversal sections of 15 µm at
158 -20 °C. Sections were mounted on glass microscope slides using the ProLong™ Gold mounting
159 medium containing 0.2 µg ml^{-1} DAPI (4',6-diamidin-2- phenylindole) (Invitrogen, Carlesbad, CA).
160 Controls were processed similarly.
161 To observe the PNP internalization in haemocytes, third instar larvae were anaesthetized with CO_2
162 immediately after the bioassays and haemolymph was collected from a cut of an abdominal
163 pseudoleg. After isolation, a 40 µl aliquot of haemolymph was placed on a glass microscope slide to
164 allow the haemocytes to settle and attach to the glass. After 15 minutes, the plasma was carefully
165 removed and adhered haemocytes rinsed three times with PBS, fixed for 10 min in 4% (w/v)
166 paraformaldehyde in PBS and rinsed again. Haemocytes were thus stained with Hoechst 33342
167 solution (10 µg ml^{-1}), rinsed three times with PBS and mounted for confocal microscopy with
168 PBS:glycerol 2:1 (v:v).
169 For the *in vitro* exposure to fluorescent PNPs, haemocytes were isolated from untreated fifth instar
170 larvae as described above and incubated for 1 h to a 10 mg L^{-1} solution of fluorescent PNPs in 5
171 mM Tris-HCl, 280 mM sucrose, 4.8 mM $MgSO_4$ and 1 mM $CaCl_2$. Fixation and mounting were
172 carried out as described above.
173 Sections and haemocytes samples (prepared from at least three larvae) were observed by confocal
174 microscopy (Laser Scanning Confocal Microscope Nikon A1, NITAL S.P.A., Moncalieri, Italy) and
175 acquisition details of different channels are included in Supplementary Materials (Table S2).
176 Imaging analyses were performed using the dedicated software (NIS-Elements) to exclude any
177 uncertainty on particle localization. Images were acquired merging different channels (bright field
178 and fluorescence), assuring that the fluorescent signal of beads was unequivocally distinguished
179 from autofluorescence.
180
181 2.4 *Growth and development analysis*

To monitor insect growth and development different parameters were recorded: larval mortality (reported as percentage of the initial number of larvae), length of the larval stage (from hatching to the occurrence of wandering behaviour), maximum larval weight (recorded just before the wandering phase), weight of the pupae and cocoon (registered on the sixth day of the pupal stage) and adult emergence (reported as percentage of the total number of pupae). Developmental stages of *B. mori* were defined according to Franzetti et al. (2012). Three groups of 25 larvae were treated with PNPs and the same number of larvae was kept as control.

2.5 *Biomarkers of oxidative stress*

We exposed 9 *B. mori* larvae to PNPs for 21 days and an equal number were reared as controls. Larvae were then dissected for homogenate preparation. Briefly, the anterior and the posterior ends of the larvae were discarded by cutting between the first and the second pair of legs and the fourth and the fifth pseudolegs. The central part of the body was opened lengthwise and midgut along with Malpighian tubules isolated and deprived of the midgut content. The carcass was scraped to recover internal tissues (fat body with adhered haemocytes, tracheae, nerves, muscles). Midguts, Malpighian tubules and tissues recovered from the carcass of three larvae were pooled and homogenized in 100 mM phosphate buffer (pH 7.4), with 100 mM KCl, 1 mM ethylenediamine tetra-acetic acid (EDTA), 100 mM dithiothreitol (DTT), and protease inhibitors (Roche, 1:100 v/v in deionized water). The homogenates were centrifuged at 15,000 × *g* for 15 min at 4 °C and supernatants were recovered and stored at -80 °C.

The measurement of GST, SOD and CAT, as well as the ROS production followed the methods described by Parenti et al. (2019), with minor modifications. The day of the analyses, homogenates were thawed, and protein concentration was measured by the Coomassie Brilliant Blue G-250 (Pierce) protein assay, with bovine serum albumin as standard.

The levels of enzymatic activity were assessed using the 6715 UV/Vis spectrophotometer (Jenway, USA). GST activity, expressed in mmol min^{-1} mg $protein^{-1}$, was measured at 340 nm. The activity

was quantified adding the reduced glutathione (20 mM) in 100 mM phosphate buffer (pH 7.4) and the substrate 1-Chloro-2,4-dinitrobenzene (CDNB) (20 mM). SOD activity, expressed in SOD units mg protein^{-1} (1 SOD unit = 50% inhibition of the xanthine oxidase reaction), was measured at 550 nm. The activity was quantified measuring the degree of inhibition of cytochrome C (0.3 mM) in 50 mM phosphate buffer + EDTA (0.1 mM), generated by the reaction mix of 1.5 mM hypoxanthine and xanthine oxidase 56.1 mU mL^{-1}. CAT activity, expressed in µmol min^{-1} mg protein^{-1}, was analysed at 240 nm. The activity was determined by measuring the consumption of H_2O_2 (150 mM) in 100 mM phosphate buffer (pH 7). ROS level was assessed using the dichlorofluorescein-diacetate (DCFH-DA) method. Larval homogenates were added in triplicate to a 96-well plate and incubated for 5 min at room temperature. In each well, 100 µL of PBS and 8.3 µL of DCFH-DA (10 mg mL^{-1} in DMSO) were also added and the plate was then incubated for 30 min at 37 °C. ROS concentration, expressed in fluorescence units (FU), was measured using the EnSight™ multimode plate reader (PerkinElmer) at λ_{ex} 485 nm and λ_{em} 530 nm. Enzymatic activities and ROS production were normalized on the total protein content of each sample.

The oxidative damage was evaluated measuring the LPO, following the method of Ohkawa et al. (1979). In brief, 100 µl of each homogenate were placed in glass tubes with 500 µl of 12% (v/v) trichloroacetic acid (TCA) in deionized water, 400 µl of 0.6 M Tris-HCl and 500 µl of 0.37% (v/v) thiobarbituric acid (TBA) in deionized water, and incubated 1 h in boiling water. The suspension was then refreshed in ice and centrifuged at 15,000 × *g* for 10 min at 20 °C. The level of TBA-reactive substances, expressed in nmol g^{-1} w.w.$^{-1}$ (wet weight), was estimated in the supernatants, reading the absorbance at 535 nm.

2.6 *Behavioural tests*

To evaluate the potential effects of ingested PNPs on behaviour, *B. mori* larvae were exposed to PNPs for 10 days and an equal number of larvae were reared as controls.

Control and PNP-exposed larvae were subjected to an alternating dark/light test (8 min) and a chemotaxis test (5 min) and filmed by an infrared camera (sample rate of 6 frames/second) using the DanioVision™ observation chamber (Noldus Inc., Wageningen, The Netherlands). The evaluation of chemotaxis defects was performed by a single odour assay by using 20 µL of mulberry leaves extract as olfactory stimulus that was produced by mixing the artificial diet powder with distilled water (4 ml of water each gr of powder) followed by centrifugation at 10,000 × *g* for 5 min and supernatant recovery. The motion of single larvae exposed to the odour source was recorded during the entire duration of the trial (5 min with light on). The EthoVision XT® software (Noldus Inc., Wageningen, The Netherlands) was used to elaborate several parameters for behavioural quantification (total mobility, time course of distance to odour source). Measurements have been performed on groups of at least 6 larvae.

2.7 *Statistics*

The effect of PNP exposure on larval growth was investigated by means of a generalized linear model (GLM) with mean weight as depended variable and day and treatment (control and PNPs) as response variables. Since mean weight variable distribution was right-skewed, we assumed a Poisson distribution of the variable. In a former model, we included the two-way interaction between treatment and day to assess the potential effect of the treatment, considering the day. However, since the two-way interaction did not significantly affect the mean weight ($p>0.9$), we removed this interaction in the final model. Only the variable day had a significant and positive effect on larval mean weight (estimate Standard Error: 0.33(0.05), $z=6.15$, $p<0.001$, $n=96$), while no effects of treatment have been observed ($p>0.8$). A generalized linear model (GLM) was performed using R v.3.6.1. To identify any significant effects of PNPs on larval stress and behaviour, we analysed data using the STATISTICA 7.0 package. After having certified the normality of the data and homoscedasticity using the Shapiro-Wilk and Levene tests, the significant differences between

the two independent groups (control and PNP) were analysed by one-way ANOVA followed by the Fisher LSD *post-hoc* test, taking $p<0.05$ as significant cut-off.

3. RESULTS AND DISCUSSION

3.1 *General considerations*

One of the most critical aspects in the study of NP toxicity is to clearly demonstrate their internalization in the organism by different intake routes (e.g. gills, gut and skin), as well as the infiltration of plastic particles in the tissues and/or internalization in the cells and the eventual clearance. To do this, it is crucial the selection of reliable methods which make conclusive evidence of plastics intake. Indeed, if the use of fluorescent-labelled particles solves many interpretation problems by the elimination of some interferences, the lack of adequate controls can give inconclusive evidence of plastic accumulation in the tissues of the selected biological model. Catarino et al. (2019) showed that commercial fluorescent NPs can leach the fluorophores that accumulate in the same internal tissues of zebrafish larvae where NP accumulation was observed. Thus, we performed a double-check method based on the use of labelled-PNPs coupled with the identification of single nanospheres by the observation of cryo-sections, able to preserve the sample avoiding too much aggressive treatments. This guaranteed, together with the orthogonal projections (see Fig. $1B_2$), a conclusive evidence about their accumulation in the larvae tissues and the lack of accidental transport of the administered PNPs during the cryo-section preparation. The need to check the capability of plastic particles to enter the organisms forced the use of PNP concentrations much higher than environmental ones, decreasing the ecological realism. On the other hand, this is the only possible approach in a preliminary study whose purpose was the evaluation of the uptake, transport and accumulation of PNPs and their ecotoxicological potential, without any claim to reach an environmental risk assessment (ERA).

3.2 *Detection of PNPs in B. mori larvae*

The observation of sections of third instar *B. mori* larvae by confocal microscopy showed the presence of fluorescent PNPs inside the intestinal lumen (Fig. $1B_2$), demonstrating the ingestion of the food along with the plastic particles.

Furthermore, PNPs were observed not only inside the intestinal lumen, but also into Malpighian tubules (Mts; Fig. $1B_2$), which are excretory and osmoregulatory organs of insects (Xia et al., 2007). Mts are considered analogous to the nephridia in annelids or kidneys in vertebrates (Wigglesworth, 2003), but they are also involved in other essential functions, such as the immunological defence, regulation and secretion of calcium and also in chemical detoxification processes (Beyenbach et al., 2010). Mts transport solutes and water from haemolymph forming the primary urine which is collected in Mt lumen and poured into the intestine between the midgut and the hindgut. In the latter the primary urine is modified and discarded with the feces (Nocelli et al., 2016). The observed presence of PNPs in these organs can be due to the possible movement of PNPs from the gut to the Mt lumen, but it might also suggest the involvement of an unidentified process that leads to the movement of PNPs from the haemolymph to the Mt lumen in the attempt to eliminate these potential hazardous materials. Our results are also similar to those revealed in a recent study that showed the presence of 2 µm MPs in the Mts of adult *Culex* mosquitoes (Al-Jaibachi et al., 2018). The authors fed aquatic larvae with MPs and detected their presence in the abdomen and Mts of adults, indicating the transfer of ingested microparticles from larval to the adult stage, although no effects on body weight and mortality were recorded (Al-Jaibachi et al., 2019). This accumulation of plastics in terrestrial organisms at low trophic level may support further transfer and accumulation along food chains, posing a threat to insect-eating species.

Microscopy observations of *B. mori* sections showed also the presence of PNPs into the midgut epithelium (Fig. $1C_2$), pointing out the capability of these physical contaminants to pass through the gut barrier and infiltrate in the near larval tissues.

The most intriguing result obtained by microscopy was probably the observation of PNPs in the cytoplasm of haemocytes sampled from larvae after the treatments (Fig 2A and B). Given that this

evidence is crucial to demonstrate the transport of these physical pollutants by the circulatory system and their potential accumulation in all the organism tissues, we carried out *in vitro* experiments by incubating haemocytes isolated from untreated larvae with PNPs which confirmed the capability of haemocytes to internalize them (Fig. 2C). The presence of plastic particles in the haemolymph was already demonstrated in different bivalves (Brown et al., 2008; Ribeiro et al., 2017; Magni et al., 2018), indicating that plastic debris may be retained in the organisms and potentially transported in all the tissues.

3.3 *Effects of PNPs on insect development*

After demonstrating the presence of PNPs in many larval tissues, we investigated their possible negative effects at different level of biological organization. Moving to results obtained at organism level, PNP exposure caused neither larval mortality nor alteration in the duration of the larval cycle (Table 1). Moreover, no significant difference in body weight was observed in respect to controls over the entire larval stage (Fig. 3, Table 1). To clarify whether the exposure may affect metamorphosis, the pupal and cocoon weight, as well as adult emergence, were also recorded, showing no detrimental effects (Table 1). Emerged adults were monitored and no differences in mortality was observed between exposed and untreated larvae, as all the adults survived and females oviposited (data not shown). The overall data indicated that PNP exposure did not alter the individual and population fitness of *B. mori*, at least in our experimental conditions.

3.4 *Behavioural tests*

To further assess the potential effects of PNPs at organism level, we performed also several assays based on larval behaviour, whose endpoints link molecular and physiological effects with ecological processes, providing information at various levels of biological organization (Scott and Sloman, 2004). Indeed, any behavioural variation caused by the exposure to environmental contaminants may compromise directly or indirectly the organism survival and fitness. Behavioural

effects caused by MPs and NPs, in terms of changes in locomotor activity and feeding behaviour, were already observed in several aquatic organisms, such as *Daphnia magna* (De Felice et al., 2019), *Carcinus maenas* (Watts et al., 2014) and *Danio rerio* embryos and adults (Parenti et al., 2019; Qiang and Cheng, 2019; Mak et al., 2019).

In this study, DanioVision™ was used to record the mobility state of larvae in two different tests, measuring the percentage of the movement time and the larval response to a chemical stimulus, respectively. In the first assay, we measured a mobility parameter able to detect the movement independently by the spatial displacement of the organism. This allowed to evaluate the whole movement of the larva, even in the absence of a real movement within the test arena. The mobility test showed a significant hyperactivity of treated larvae (Fig. 4A), both during the light phase ($p<0.05$) and especially the dark phase ($p<0.001$), suggesting that the stress condition represented by darkness may further increase the effect. The higher mobility of larvae exposed to PNPs was not related to an increase in the distance moved, but rather to erratic movements that can have crucial ecological consequences because lepidopteran larvae may be, for example, particularly vulnerable to detection by predators. An excitatory effect of NPs on locomotor behaviour was also observed in *Caenorhabditis elegans* (Lei et al., 2018), explained as a potential interaction of NPs with neurons.

Olfaction is one of the most important ways through which insects interact with their surroundings (Gadenne et al., 2016). Since, among its many functions, insects rely on chemoreception to locate food sources, we performed another behavioural test using an olfactive stimulus to investigate if the PNP exposure could alter silkworm feeding behaviour. Very interestingly, collected data reported a significant increase of mobility during the first 2 minutes of the test for exposed larvae (Fig. 4B, $p<0.05$), while the cumulative duration of time spent in the area surrounding the olfactive stimulus was not significantly different (Fig. 4C). Thus, the PNP effect seemed to interfere mainly on the feeding initiation (meal-start), which is driven by a complex co-action of physical and chemical factors (Audsley and Weaver, 2009).

362 3.5 *Biomarkers of oxidative stress*

363 We applied a biomarker suite for the evaluation of possible impacts due to PNPs at cellular and
364 molecular level, focusing mainly on their possible role in the imbalance of redox homeostasis
365 through the antioxidant response. Oxidative stress is included in the fourth main category
366 (physiological stress) of effects reported for plastic particles <10 µm on aquatic and shoreline biota
367 (Kögel et al., 2020). A recent review by Prokić et al. (2019) reported a huge and sometimes
368 controversial variety of oxidative stress responses for organisms exposed to plastic beads (ranging
369 from 0.05 to 100 µm), depending on several factors, such as tissue chosen for the analysis or
370 composition, size, shape and concentration of plastics. For instance, the effect of the particle size
371 was highlighted by Jeong et al. (2016; 2017) who observed that lower-sized polystyrene particles
372 (50 nm) were able to induce a higher oxidative stress in the rotifer *Brachionus koreanus* and in the
373 copepod *Paracyclopina nana* while, in contrast, Lu et al. (2016) showed as the greatest antioxidant
374 response was observed in *Danio rerio* as a response to larger plastic particles (5 µm). The same
375 study found that the induction of two antioxidant enzymes (SOD and CAT) increased with higher
376 concentration of MPs. Furthermore, biomarkers of oxidative stress often showed a tissue-specific
377 response to MPs (20 µm), as observed in gills and digestive gland of the clam *Scrobicularia plana*
378 (Ribeiro et al., 2017). In our study, the exposure to PNPs did not induce any variation in ROS level
379 (Fig. 5A) or CAT (Fig. 5B) and GST (Fig. 5D) activities. On the contrary, a significant ($p<0.01$)
380 inhibition of SOD (Fig. 5C) emerged after the exposure to PNPs. A study by Liu et al. (2019) of NP
381 effects on *Daphnia pulex* showed that NPs (75 nm) lead to changes in the relative expression of
382 some oxidative stress-mediated genes (*SOD*, *GST*, *GPx* and *CAT*), which increased or decreased
383 depending on the concentration of the particles (from 0.1 to 2 mg L^{-1}), showing a downward trend
384 with the higher concentrations. In the same way, the SOD inhibition observed in treated larvae
385 might suggest the onset of oxidative stress imbalance caused by the high PNP concentration used in
386 the study. Nevertheless, no oxidative damage was observed, concerning the level of lipid
387 peroxidation (Fig. 5E), which is considered an indicator of the adaptive response to oxidative stress.

This result is consistent with the level of ROS (Fig. 5A), which did not present any difference between controls and exposed groups.

4. CONCLUSIONS

This study represents the first investigation on the uptake and effects of PNP exposure in the Lepidoptera model organism *B. mori*. The overall dataset demonstrated the capability of silkworm larvae to ingest PNPs, which were then able to pass the intestinal barrier and reach the internal tissues and organs and the circulatory system, as clearly shown by the presence of PNPs in the haemocytes. Another crucial result of this study was the application of a multi-tier approach to investigate the potential toxicity of these physical contaminants. The non-exhaustive results obtained by the measured endpoints at different levels of the biological organization confirmed the need to use several approaches that allow the comparison and integration of the entire dataset. Indeed, neither biochemical analyses nor endpoints of development revealed significant impact at cellular and molecular levels or an alteration of larval life cycle, while the most relevant outcome emerged from the behaviour observations, since PNPs affected locomotor activity, with potential negative indirect effects on *B. mori* survival and fitness from the ecological point of view.

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FIGURE CAPTIONS

Fig. 1 Confocal microscopy observations of 15 µm transversal cryo-sections of third instar *B. mori* larvae. Fluorescent beads are shown in red, while yellow fluorescence resulted from the merge of PNPs and administered diet. Cell nuclei were stained with DAPI. (A_1)(A_2) Transversal section of a control larva [L: intestinal lumen; scale bar 50 µm] (B_1)(B_2) Transversal section of a larva exposed to PNPs with orthogonal projections of Z-stacks evidencing the nanobeads at cellular level [L: intestinal lumen, E: gut epithelium, Mt: Malpighian tubule; scale bar 50 µm] (C_1)(C_2) Detail of a transversal section of a larva exposed to PNPs [E: gut epithelium, T: trachea; scale bar 10 µm].

Fig. 2 Haemocytes collected from control (A_1) and treated larvae (A_2 and A_3) [scale bar 10 µm]. Isolated haemocytes of *B. mori* larva incubated *in vitro* for 60 min: (B_1) control and (B_2) exposed to PNPs [scale bar 10 µm]. White arrows indicate the nanobeads, nuclei are stained with Hoechst 33342.

Fig. 3 Growth curve of *B. mori* larvae exposed to PNPs and controls. The weight was recorded from 6^{th} day to 24^{th} day (mean body weight of larvae from each experimental group), when larvae started to enter the wandering phase.

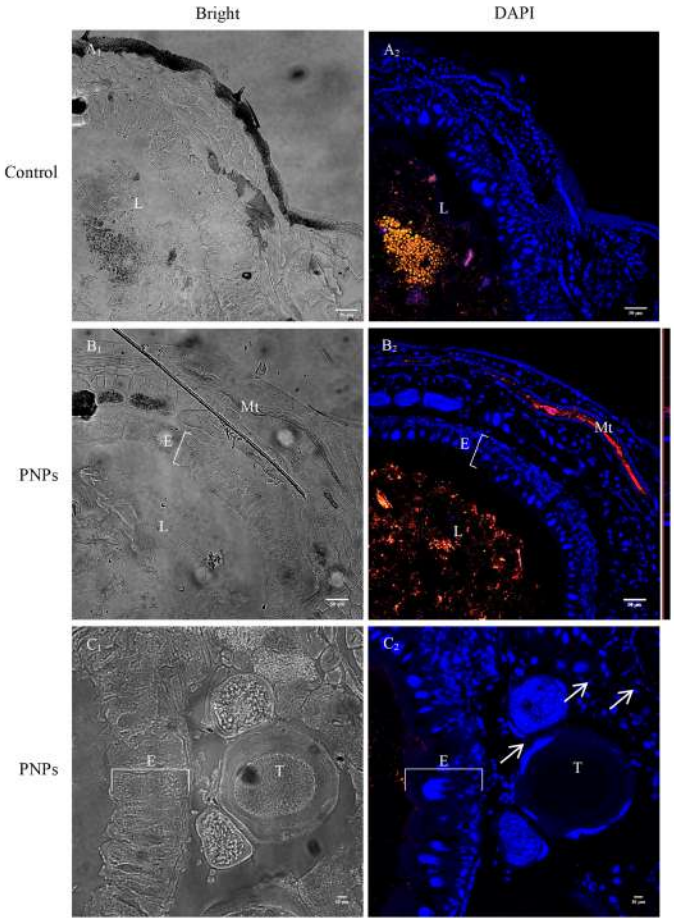
Fig. 4 Behavioural effects of PNP ingestion on third instar larvae, respect to controls. (A) Mean percentage of maximum mobility during the 8 min of the alternating light-dark test. (A1) Percentage of maximum mobility during the alternating light-dark test, calculated every 30 s. (B) Mean percentage of maximum mobility during the first 2 min of the chemotaxis test. (B1) Percentage of maximum mobility during the first 2 min of the chemotaxis test, calculated every 30 s. (C) Cumulative duration of the stay in the stimulus zone during the 5 min of the chemotaxis test. (C1) Cumulative duration of the stay in the stimulus zone during the 5 min of the chemotaxis test, calculated every 30 s. Data are from three experiments and are presented as mean ± standard error. (one-way ANOVA followed by Fisher LSD post-hoc test, *** = $p<0.001$, * = $p<0.05$).

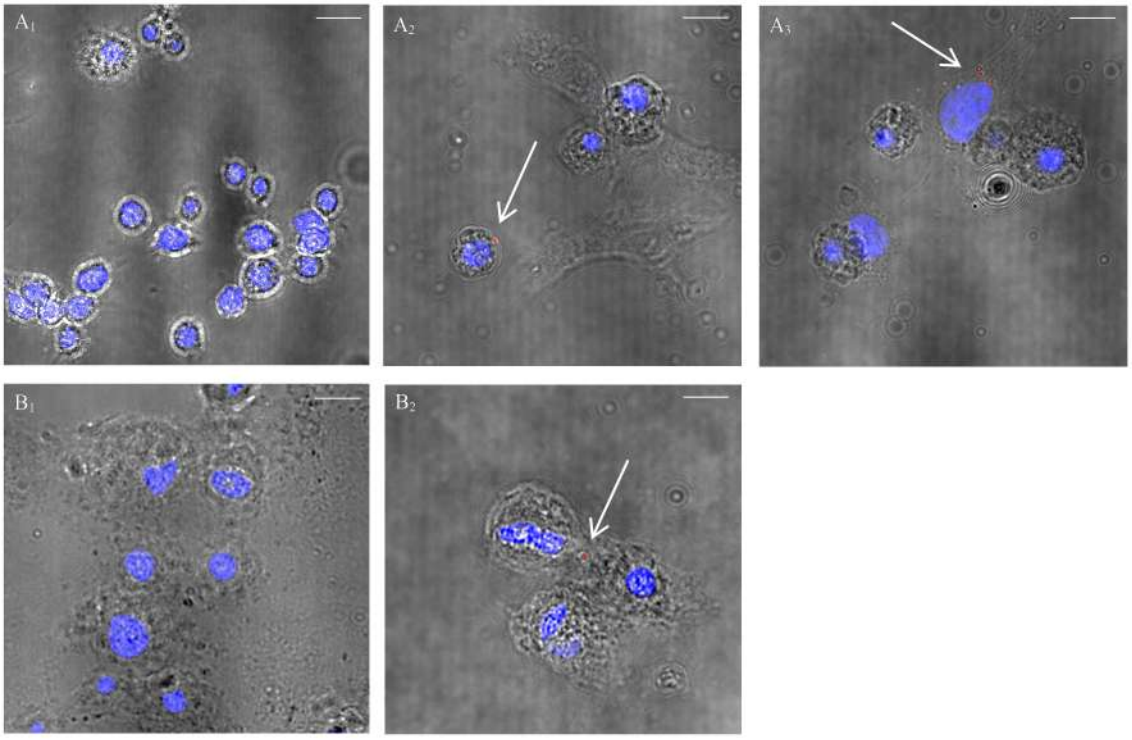
Fig. 5 Sub-individual effects of PNP ingestion on fifth instar larvae, respect to controls. (A) Quantity of ROS. (B) Activity of CAT. (C) Activity of SOD. (D) Activity of GST. (E) Level of LPO. Data are from three experiments and are presented as mean ± standard error. (one-way ANOVA followed by Fisher LSD post-hoc test, ** = $p<0.01$).

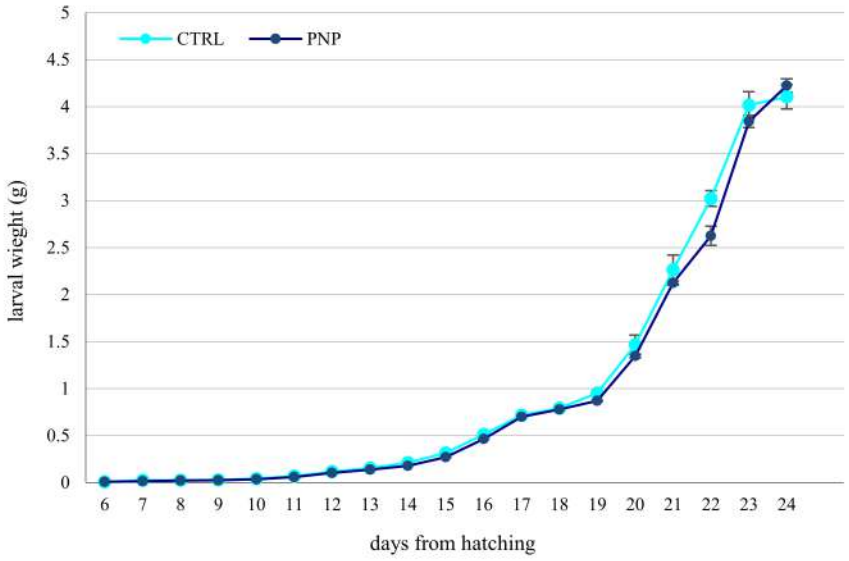
Table 1 Growth parameters of *B. mori* larvae exposed to PNPs. The maximum larval weight refers to the maximal weight reached by last instar larvae. The experiment was performed in triplicate with groups of 25 larvae. The values are reported as mean ± standard error.

Table 1. Growth parameters of *B. mori* larvae exposed to PNPs. The maximum larval weight refers to the maximal weight reached by last instar larvae. The experiment was performed in triplicate with groups of 25 larvae. The values are reported as mean ± standard error.

Treatment	**Mortality**	**Larval cycle (days)**	**Maximum larval weight (g)**	**Pupal weight (g)**	**Cocoon weight (g)**	**Adult emergence (%)**
Control	0	24.58 ± 0.92	4.48 ± 0.26	1.92 ± 0.47	0.28 ± 0.01	100
PNP	0	24.78 ± 0.94	4.50 ± 0.07	1.61 ± 0.08	0.28 ± 0.04	100



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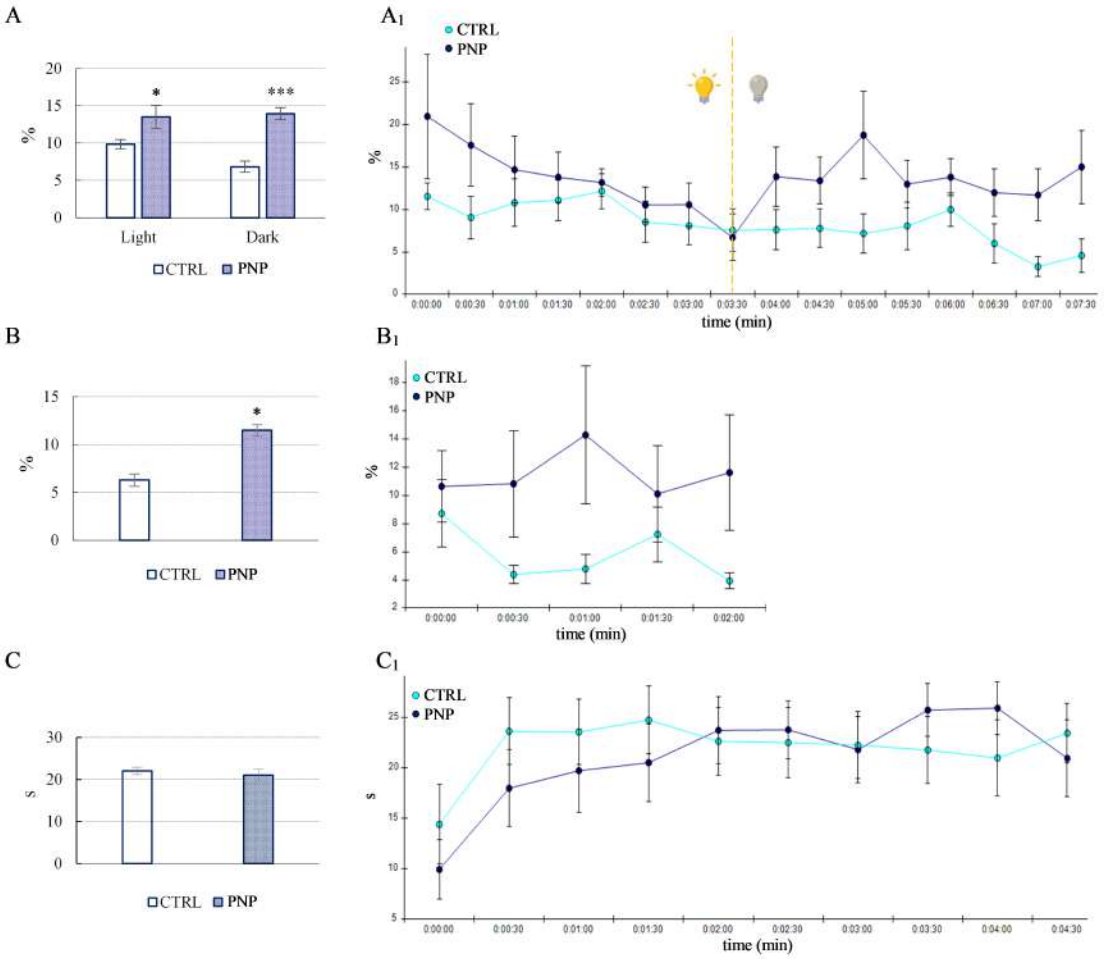
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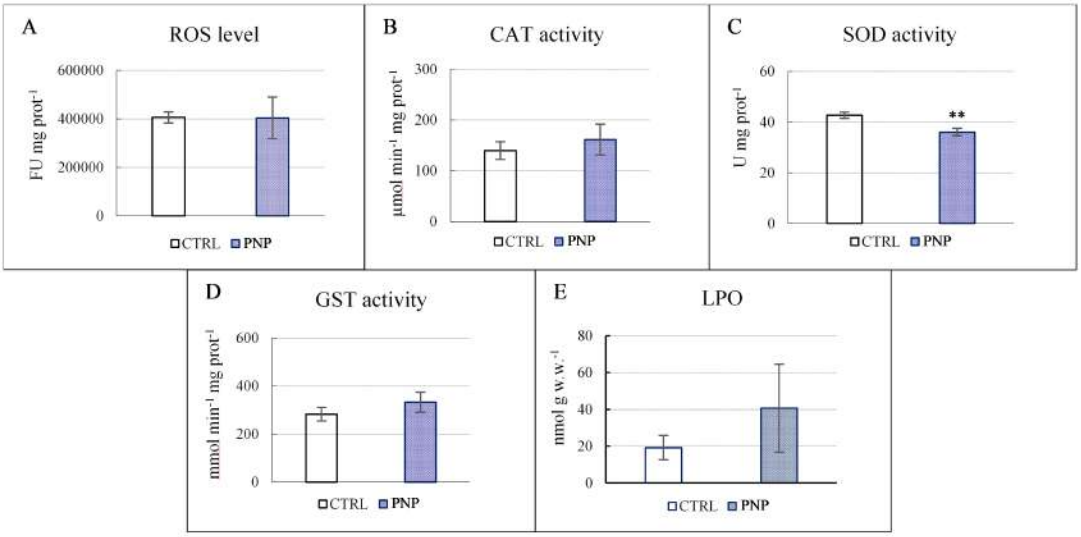

- First study on nanoplastic uptake and effects in the silkworm *Bombyx mori*
- Presence of nanoplastics in tissues and haemolymph
- Nanoplastic ingestion alters larval locomotor behaviour

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: