Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Electronic Supplementary Information

Polystyrene micro- and nanoplastics affect locomotion and daily activity of *Drosophila melanogaster*

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Fluorescence imaging of plastic spheres *in situ*. Particle suspensions and spiked food media were prepared as described in the method section. Slides were prepared by spreading 2 µL aliquots of suspension or food media between a clean glass slide and coverslip. Slides were mounted on the stage of a fluorescence microscope (Olympus IX71) and monitored using a 40x objective (LUCPlanFI, numerical aperture 0.6, working distance 4 mm). Exposure time was 30 ms and 200 ms for red and green fluorescent images, respectively.

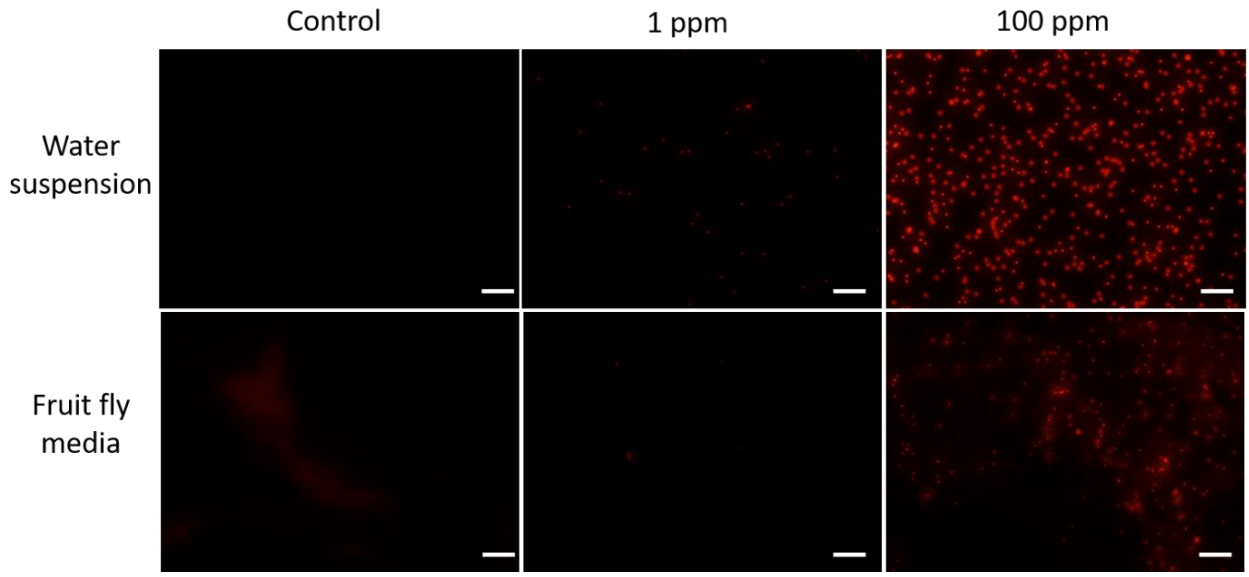

Figure S1. Fluorescence imaging of 1 µm red fluorescent carboxylated polystyrene spheres (ThermoFisher) in water suspension and mixed into Ward's Instant Drosophila Medium. The white scale bar represents 20 µm. Particles show strong fluorescence compared to fluorescence from food (seen in control) and are clearly visible. There is no significant change in particle shape or size when incorporated into food.

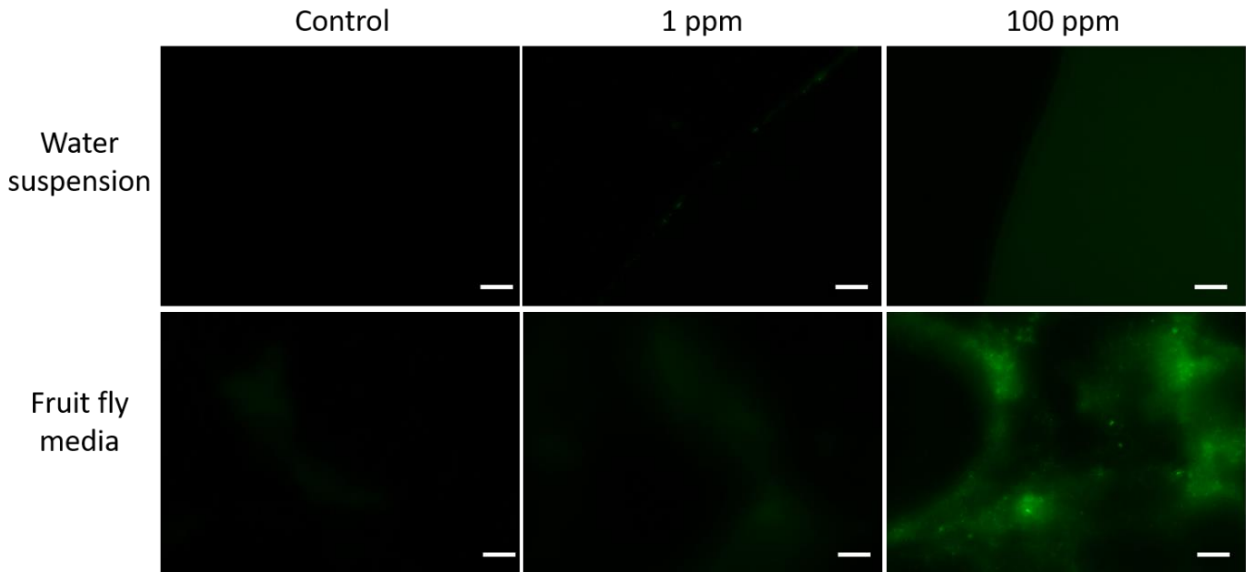

Figure S2. Fluorescence imaging of 20 nm green-yellow fluorescent carboxylated polystyrene spheres (ThermoFisher) in water suspension and mixed into Ward's Instant Drosophila Medium. The white scale bar represents 20 µm. Some aggregates of nanoplastics were observed at high concentrations when mixed into food media, with the largest aggregates being approximately 1 µm.

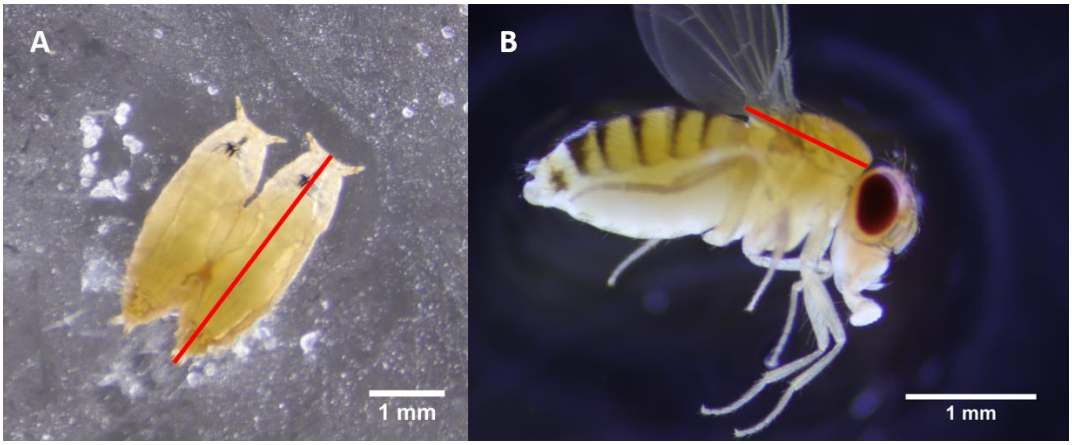

Figure S3. Body measurements were taken to compare the growth and development of treatment groups. **A)** The length of the pupal case was measured on day 6 of exposure, and **B)** the thorax length of adults on the final day.

Quantitative Real-Time PCR. Stress-response genes (*HSP70, CAT, SOD2*) and genotoxic stress response gene (*P53*) were selected, and primers were designed with Benchling (https://benchling.com) (**Table S1**) and targets were verified by NCBI Primer-Blast. Fifteen males and 15 females from each condition were stored in groups of 5 in RNA*later* (Thermo Fisher) at -4 °C, giving a total of 6 biological replicates per condition. Total RNA was isolated from flies using Purelink RNA Mini Kit (Thermo Fisher); the amount of RNA in each sample was determined using a spectrophotometer ($OD_{260/280}$ 1.8; concentration > 5 ng/uL), and the RNA quality was analyzed using agarose gel electrophoresis (1%) in TAE buffer prepared in-house. First-strand cDNA was prepared from 300-500 ng of total RNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) in 20 µL reactions and then diluted to a concentration of 2 ng/µL with nuclease-free, RNAse free water. Real-time quantitative PCR (RT-PCR) was performed in triplicate for each sample with a 7900HT Fast Real-Time PCR

System (Applied Biosystems) following manufacturer's instructions and using SYBR Green-based detection of PCR products. Melting curves were examined after amplification to exclude the presence of unspecific products. For each gene, 10 ng of cDNA was mixed with 10 µL of Power SYBR Green PCR Master Mix (Thermo Fisher), 1 µL of 1 mM of gene-specific primers, and 3 µL of water in a 394 well-plate. The RT-PCR was performed with the following protocol: 1 cycle of denaturation at 95 °C for 10 min; 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. Following the amplification reaction, a melting curve program (60-95 °C) was carried out and fluorescence data were collected at 0.5 °C intervals. Relative expression was calculated through the $2^{-\Delta\Delta Ct}$ method and normalized to the transcript levels for alpha-tubulin.

Table S1. Primers for quantitative real-time PCR.

Gene	**GeneBank Acc. No**	**F**	**R**	**Size**	**Average gene efficiency**
HSP70	NM_169441.2	AGGGTCAGATCCACGACATC	CGTCTGGGTTGATGGATAGG	117	97
CAT	NM_080483.3	GATGCGGCTTCCAATCAGTTG	GCAGCAGGATAGGTCCTCG	139	78
P53	NM_206544.2	TGCGGACACAAATCGCAACTGCT	ACGACGCGGACTTGTGAAGACTC	79	105
SOD2	NM_057577.3	CTCCTGCCCTGCGTTTCA	GTCAGCGTGGTCAGCTCCTT	160	98
TUB	NM_057424	TGTCGCGTGTGAAACACTTC	AGCAGGCGTTTCCAATCTG	96	86

Measuring daily activity and circadian rhythm. The daily activity and circadian rhythm of adult flies were assessed using a DAM2 Drosophila Activity Monitor (TriKinetics) following a protocol previously described by Chiu et al.[38] with some modifications. Food with 0, 10, 50, and 100 ppm microplastics or nanoplastics were prepared as previously described and approximately 0.6 mL was injected into glass tubes (5 mm × 65 mm, TriKinetics) with a small syringe before being sealed with parafilm. Two-day old adult male fruit flies were then anesthetized with carbon dioxide, placed in tubes individually, and capped with 100% cotton string to allow air circulation in the tube. Two monitors were loaded with 32 tubes (16 flies per treatment, 64 in total) and placed in an incubator at 25 ˚C, 60% humidity for 8 days. For the first 4 days, flies were subjected to 12 h/12 h light/dark cycle (LD) and then a 24 h dark cycle (DD) for the remaining 4 days. The dark conditions were created within the incubator by using a cardboard box with small ventilation holes. The light intensity in the incubator and in the dark box was 30 and 0 µmol/m^2s, respectively, when measured using a UV detector (Apogee, MU-200). An activity event was defined as a break in the infrared beam that crosses the center of the activity tube and the number of events was recorded over 1-min intervals. Data were analyzed using Shiny-R software[39] to extract daily activity accounts, sleep events, bout lengths, and circadian rhythms. The software used the standard definition of sleep as a continuous period of inactivity lasting at least 5 min and dead flies were defined as those with < 50 counts per day. The first day of both cycles was considered to be a period of acclimatization and excluded from the final statistical analysis along with data from dead flies.

Fluorescence imaging of live larvae. Fluorescent images were taken using an Olympus SZX16 stereomicrope at 2X magnification and GFP (5-SX810) or FRFP2 (5-SX822) filter. Images were captured with an EOS Rebel SL2 camera with ISO 800 and exposure time of 5 and 4 seconds for GFP and FRFP2 filters, respectively.

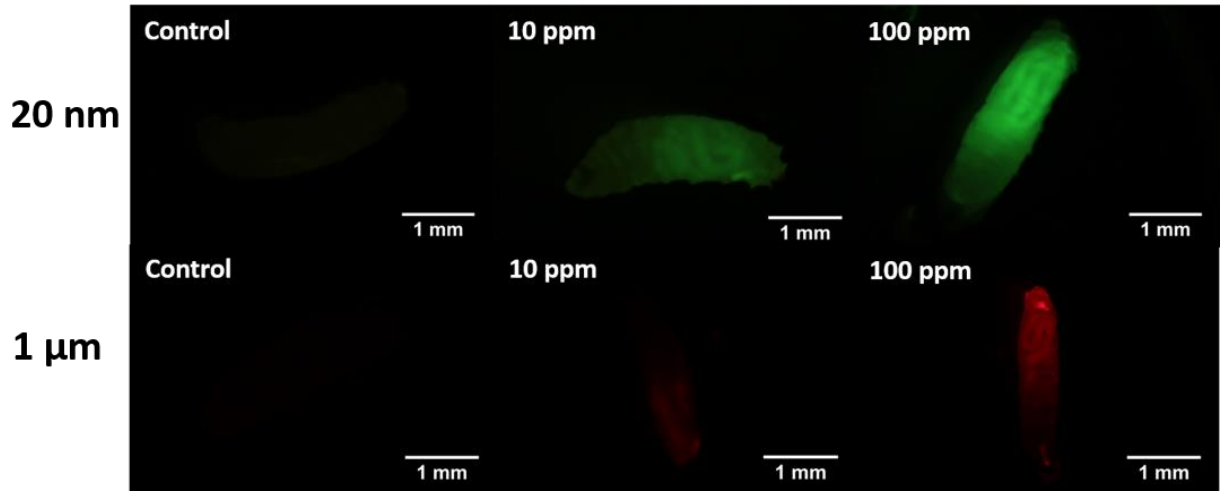

Figure S4. Fluorescence microscopy images of fruit fly larvae exposed to various concentrations of 20 nm yellow-green or 1 µm red fluorescent polystyrene spheres for 3 days.

Table S2. Mortality and development of *D. melanogaster*. The development and mortality of fruit flies exposed to nano- and microplastics were tracked by marking the timing and success of life-stage transitions, body size, and mortality. The average and standard deviations of the endpoints are shown in this table. No statistically significant difference was found between control and experimental groups in all conditions (ANOVA with post-hoc Tuckey test, significance p-value < 0.05).

	20 nm polystyrene						**1 µm polystyrene**					
Concentration (ppm)	**Control**	**0.01**	**1**	**10**	**50**	**100**	**Control**	**0.01**	**1**	**10**	**50**	**100**
Day of first pupae	4	4	4	5	4	4	4	5	5	4	4	4
Day of first adult emergence	9	9	9	9	8	8	7	8	9	8	9	9
Pupae length (mm) n=30	3.12 ±0.13	3.03 ±0.19	3.19 ±0.22	3.15 ±0.24	3.00 ±0.21	3.24 ±0.13	3.06 ±0.022	2.92 ±0.21	2.97 ±0.23	3.05 ±0.22	2.88 ±0.15	3.09 ±0.17
Female thorax length (mm) n=30	1.03 ±0.04	1.04 ±0.05	1.04 ±0.05	1.04 ±0.05	1.01 ±0.05	1.10 ±0.16	1.03 ±0.07	1.03 ±0.06	0.99 ±0.06	1.06 ±0.06	1.01 ±0.05	1.05 ±0.06
Male thorax length (mm) n=30	0.91 ±0.04	0.90 ±0.05	0.92 ±0.03	0.92 ±0.04	0.91 ±0.04	0.92 ±0.04	0.92 ±0.04	0.90 ±0.08	0.89 ±0.05	0.901 ±0.05	0.91 ±0.05	0.91 ±0.04
Eclosion rate (%) n=6	89 ± 7	93± 3	93 ± 5	96 ±2	97 ±2	97 ±2	97 ±4	97 ±5	93 ±3	97 ±1	98 ±3	98 ±2
Mortality (%) n=6	4 ± 3	4 ±3	5 ± 6	2 ±2	4 ± 4	4 ± 2	6 ±3	6 ±5	4 ±1	3 ±2	6 ±4	5 ±4

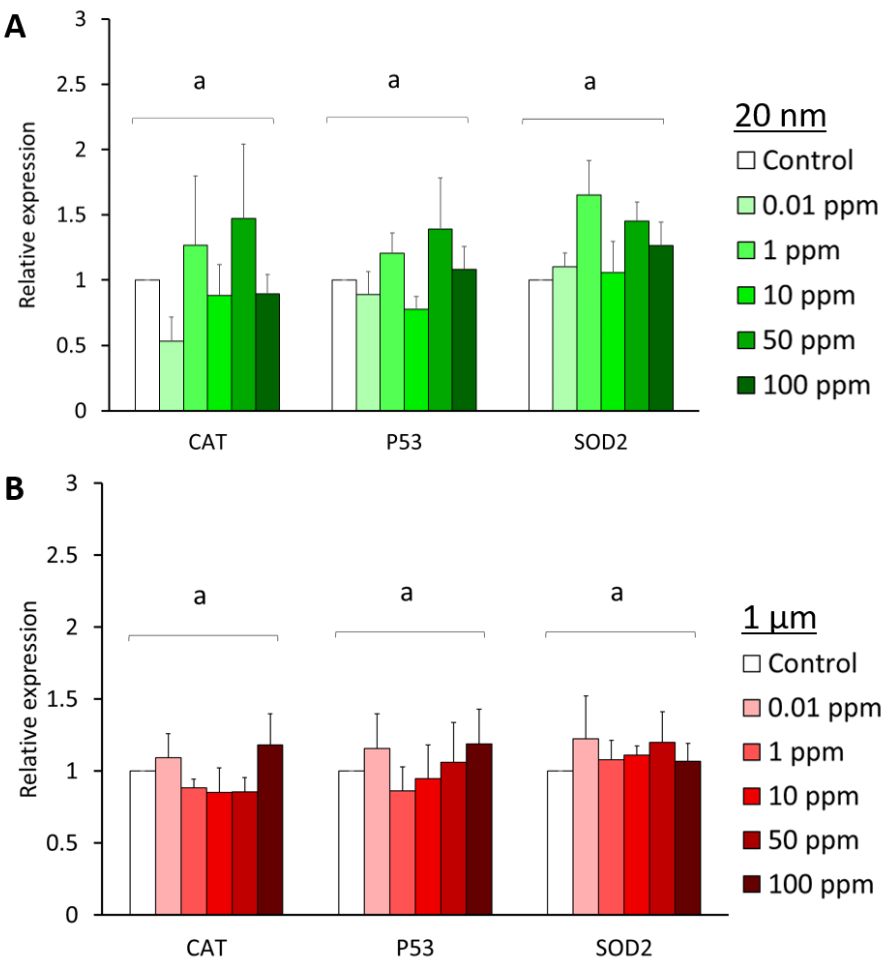

Figure S5. Gene expression in fruit flies. The relative abundance of stress-response genes (*CAT*, *SOD2*) and genotoxicity (*P53*) was determined from total RNA extracts of groups of 5 individuals per replicate (n = 6). Tubulin served as the reference gene. There were no significant differences in one-way ANOVA ($P > 0.05$).

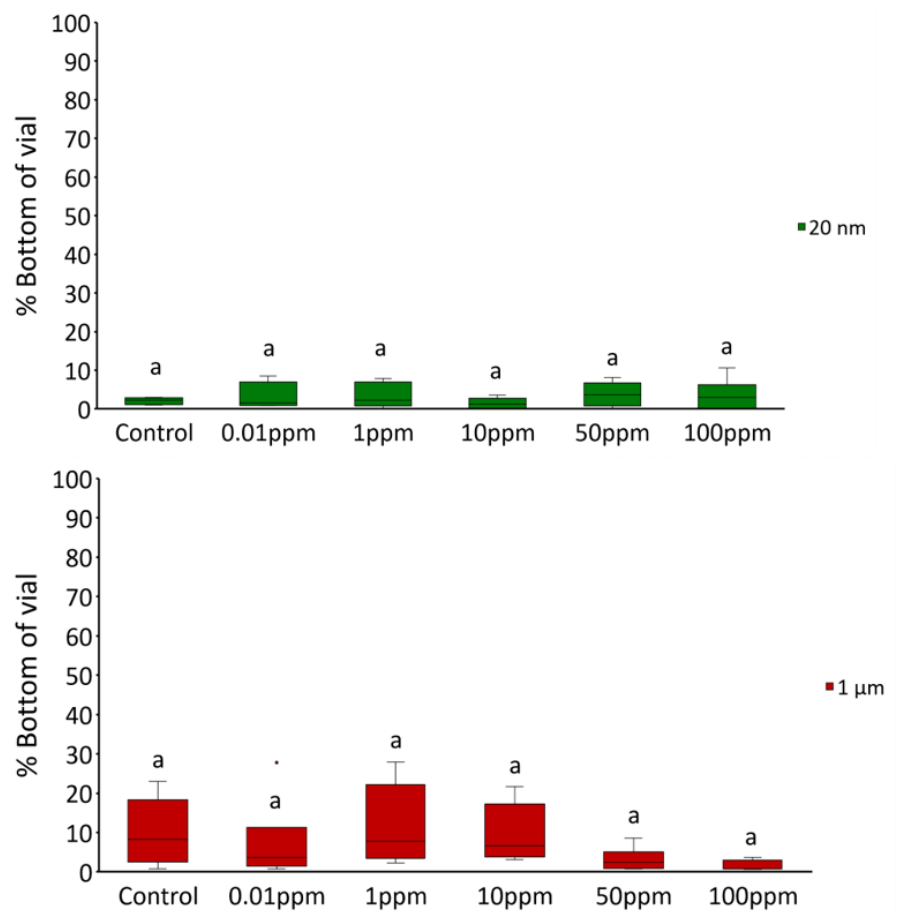

Figure S6. The locomotion of adult *D. melanogaster* was assessed via climbing assays after exposure to 20 nm green or 1 µm red fluorescent polystyrene spheres for 13 days (larvae to adult). There were no significant differences in one-way ANOVA ($P > 0.05$).

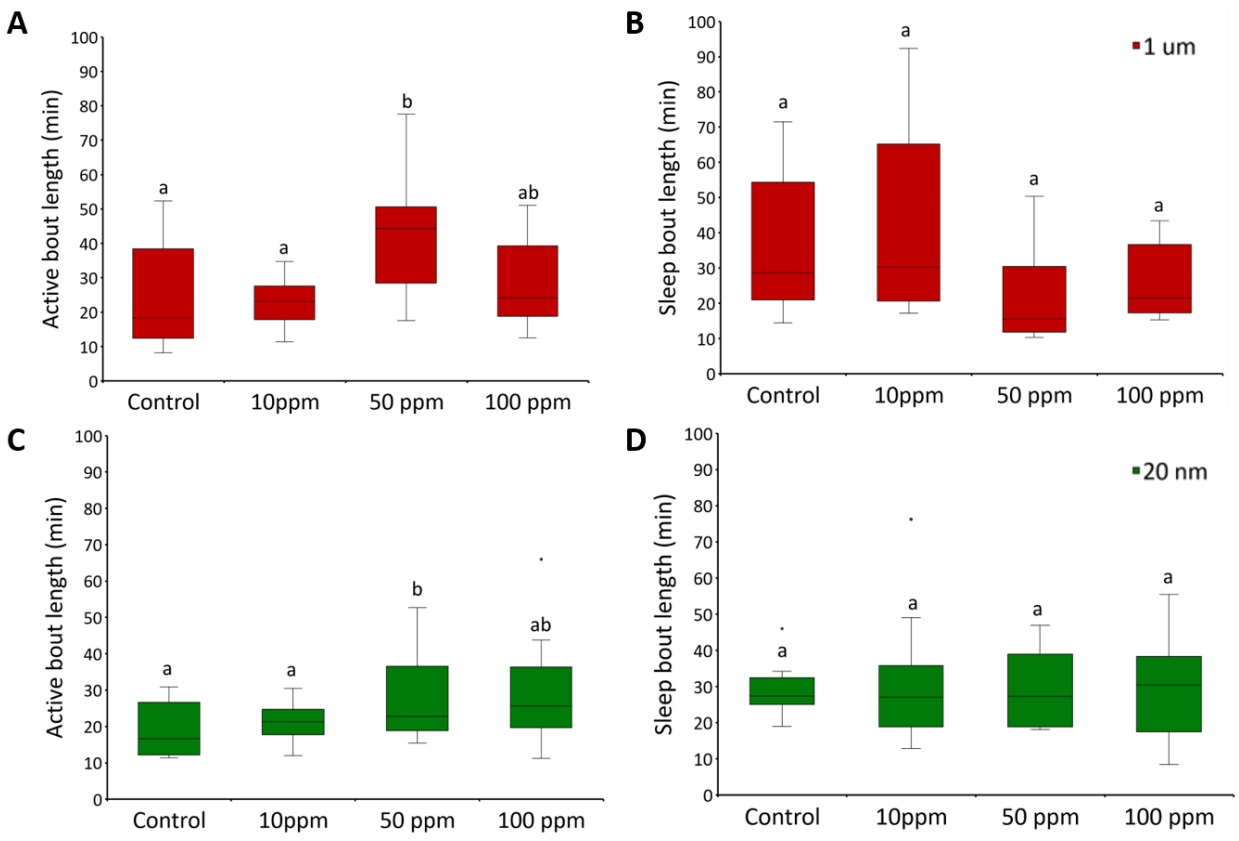

Figure S7. Activity and sleep bout lengths. The daily activity of two-day-old adult male fruit flies was recorded by a Drosophila Activity Monitor 2 with activity counts being recorded in 1 min time intervals. The raw data were processed with Shiny-R software[39] to extract individual (**A and C**) daily active bout lengths and (**B and D**) sleep bout lengths (≥ 5 min of inactivity). Letters correspond to significant differences according to one-way ANOVA with concentration as factors, n=16 for each concentration. There is a range of sample size as flies that died during the experiment were excluded from the analysis. Mortality was ≤ 12% in all controls.

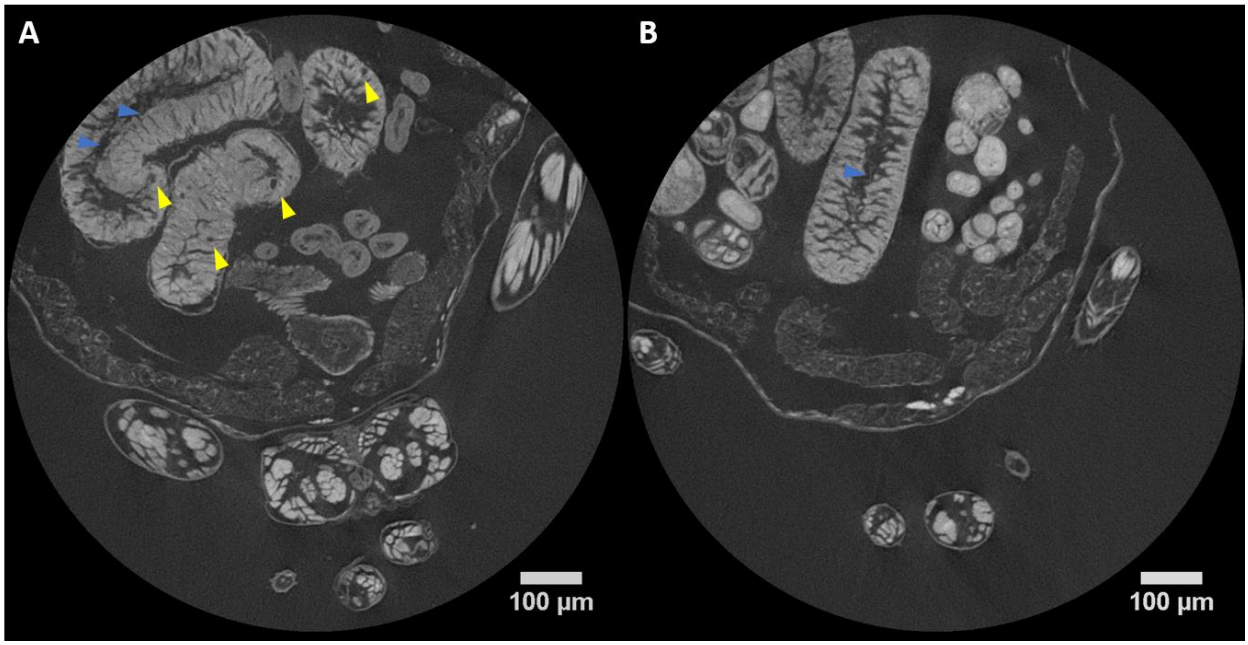

Figure S8. Computed tomography (CT) scanned images of **A)** midgut and **B**) hindgut adult female fruit flies from the control of developmental assay, which consisted of a 13-day exposure to 20 nm dialyzed yellow-green fluorescent polystyrene particles from larval to adult stage. Yellow arrows indicate secretory enteroendocrine cells and blue, the peritrophic matrix. Scan was acquired at 1 µm resolution with 4x objective lens with 2x2 camera binning, LE1 filter with 2.7 sec exposure. 2401 projections were taken over a 360 degree-rotation scan, at 60 kVp and 82 µA.

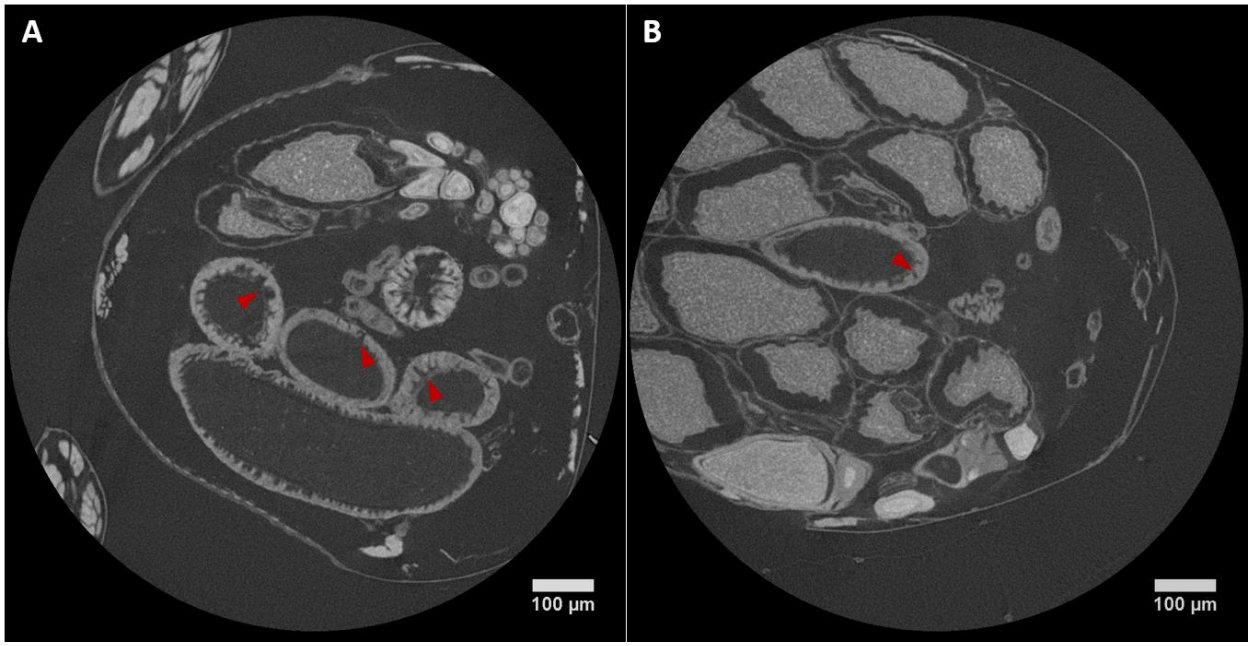

Figure S9. Computed tomography (CT) scanned images of **A)** midgut and **B**) hindgut adult female fruit flies from 100 ppm treatment of developmental assay, which consisted of a 13-day exposure to 20 nm dialyzed yellow-green fluorescent polystyrene particles from larval to adult stage. There are signs of intestinal damage such as a thinner epithelial layer, a lack of secretory enteroendocrine cells and peritrophic matrix, and deep crypts which are indicated by red arrows. Scan was acquired at 1 µm resolution with 4x objective lens with 2x2 camera binning, LE2 filter with 3.5 sec exposure. 2401 projections were taken over a 360 degree-rotation scan, at 60 kVp and 82 µA.

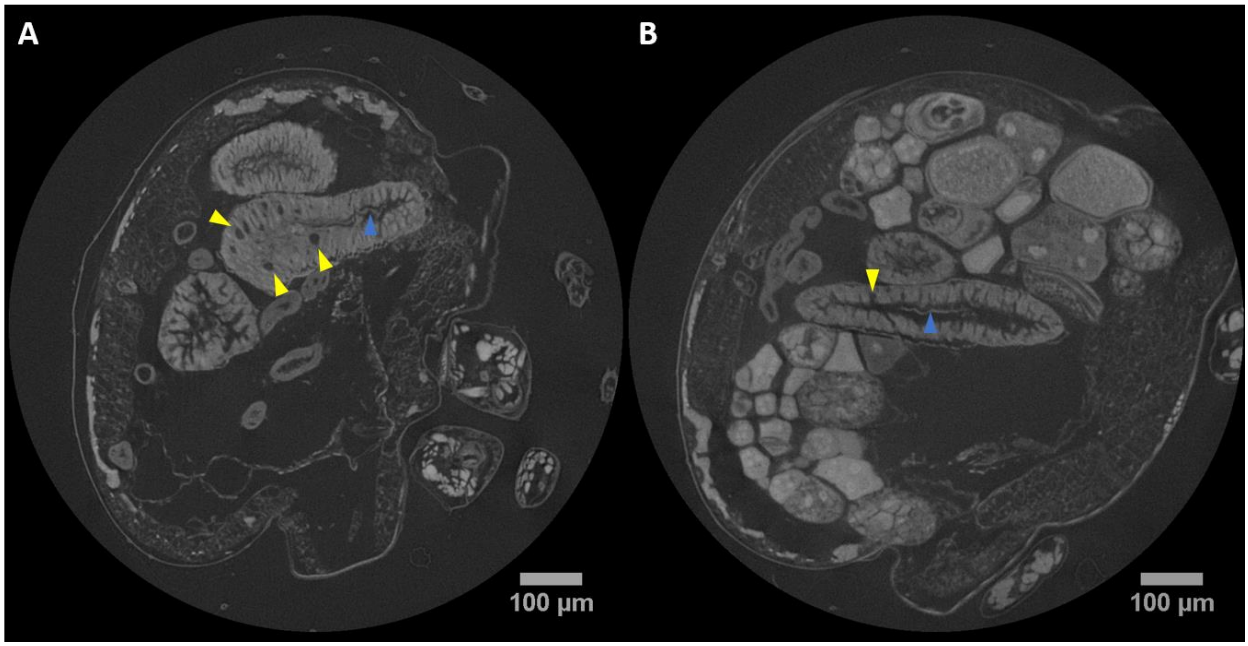

Figure S10. Computed tomography (CT) scanned images of **A)** midgut and **B**) hindgut adult female fruit flies from the control of developmental assay, which consisted of a 13-day exposure to 1 µm dialyzed red fluorescent polystyrene particles from larval to adult stage. Yellow arrows indicate secretory enteroendocrine cells and blue, the peritrophic matrix. Scan was acquired at 1 µm resolution with 4x objective lens with 2x2 camera binning, LE1 filter with 2.7 sec exposure. 2401 projections were taken over a 360 degree-rotation scan, at 60 kVp and 82 µA.

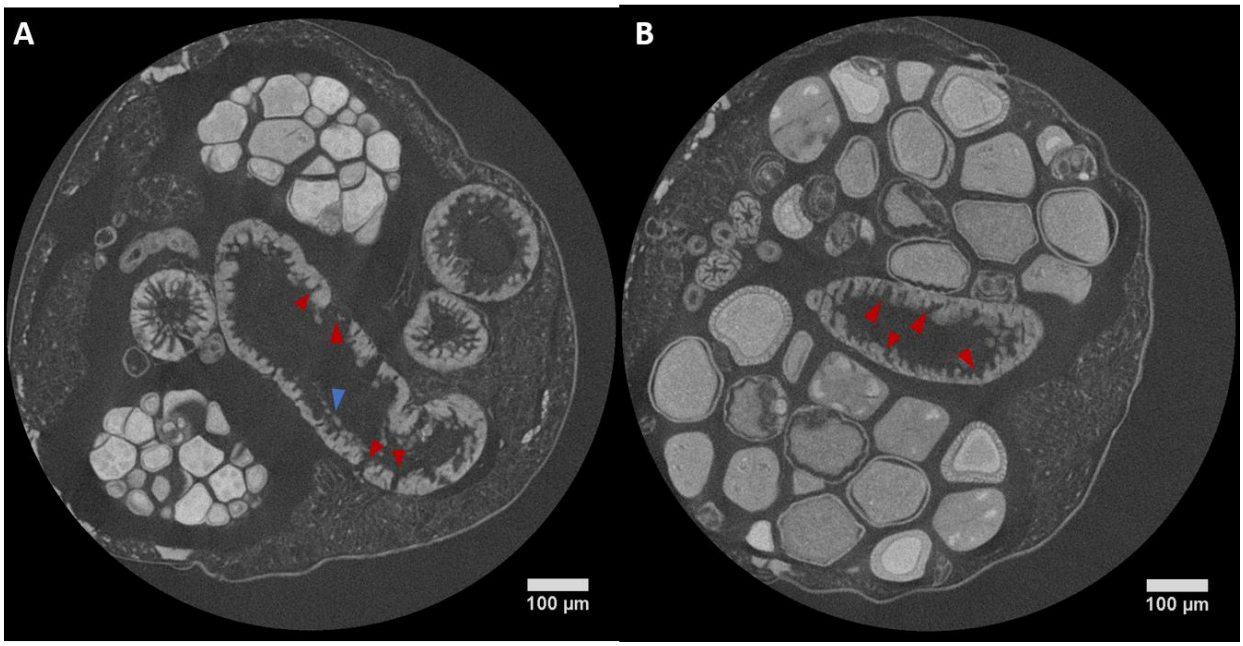

Figure S11. Computed tomography (CT) scanned images of **A)** midgut and **B**) hindgut adult female fruit flies from 100 ppm of developmental assay, which consisted of a 13-day exposure to 1 µm dialyzed red fluorescent polystyrene particles from larval to adult stage. Blue arrows indicated the peritrophic matrix. There are signs of intestinal damage such as a thinner or missing epithelial layer, shrunken enteroendocrine cells and peritrophic matrix, and deep crypts which are indicated by red arrows. Scan was acquired at 1 µm resolution with 4x objective lens with 2 x 2 camera binning, LE1 filter with 2.7 sec exposure. 2401 projections were taken over a 360 degree-rotation scan, at 60 kVp and 82 µA.

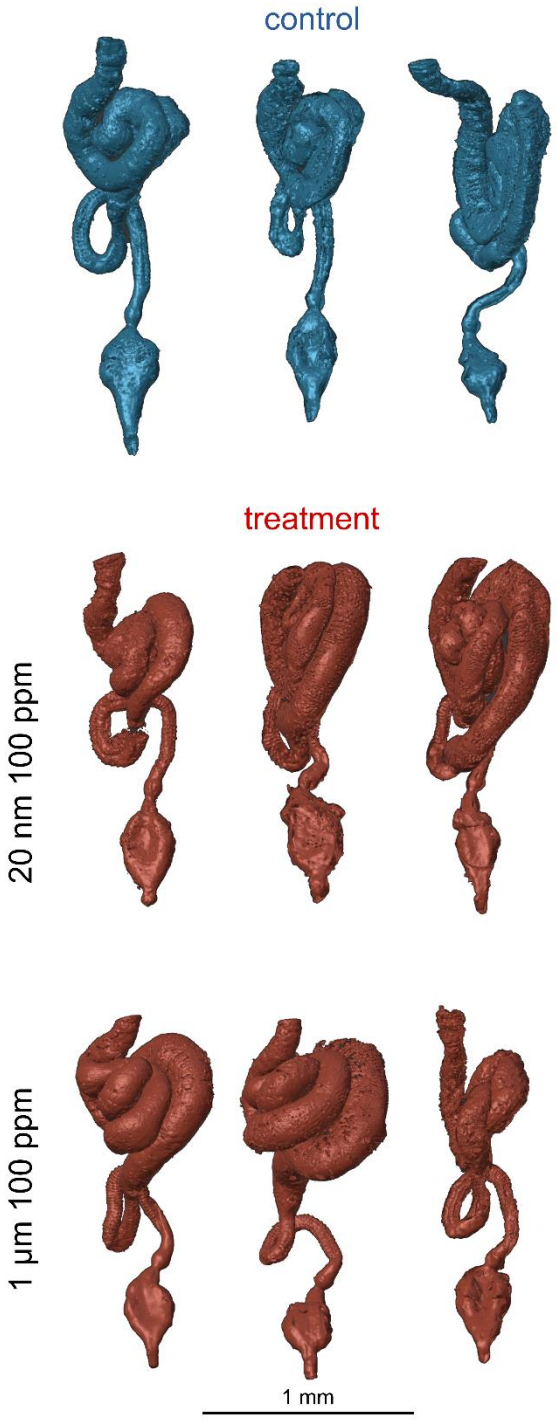

Figure S12. Reconstructed adult gastrointestinal tracts of *D. melanogaster* exposed to 100 ppm concentrations of 20 nm or 1 µm polystyrene particles. GITs are represented to the same scale from the anterior midgut to the ampulla and shown in ventral view with anterior toward the top.

Table S3. Summary table of the P-values and significance levels (*,**, ***) for factorial ANOVAs testing the effects of the variables Treatment (*i.e*. particle concentrations, 1-way) and Time (*i.e.* duration of exposure, 2-ways) for two particle sizes, 20 nm and 1 µm. *F* = Fischer calculated values, followed by degree of freedom and residual degree of freedom. Significance was set at $P < 0.05$.

Endpoint	**Factor**	**20 nm**	**1 µm**
Fertility - Offspring	Treatment Time	$F_{5,201} = 9.48$, $P = 0.136$ $F_{4, 201} = 0.842$, $P = 0.660$	$F_{5, 219} = 4.754$, $P = 0.000382$ *** $F_{4, 219} = 41.122$, $P <$ 2e-16 ***
Ind.Larv.Crawl	Treatment	$F_{5, 160} = 1.777$, $P = 0.12$	$F_{5, 165} = 11.12$, $P =$ 3.01e-09 ***
Group.Larv.Crawl	Treatment	$F_{5, 160} = 14.89$, $P =$ 5.47e-12 ***	$F_{5, 167} = 15.6$, $P =$ 1.38e-12 ***
Climbing - Pass	Treatment	$F_{5, 25} = 4.458$, $P = 0.00485$ **	$F_{5, 28} = 2.268$, $P = 0.075$
Climbing - Fail	Treatment	$F_{5, 25} = 0.53$, $P = 0.752$	$F_{5, 28} = 2.868$, $P = 0.0326$ *
Daily activity count	Treatment	$F_{3, 43} = 4.128$, $P = 0.0117$ *	$F_{3, 55} = 5.108$, $P = 0.00344$ **
Total sleep average	Treatment	$F_{3, 55} = 2.506$, $P = 0.0685$	$F_{3, 41} = 9.845$, $P =$ 5.13e-05 ***
Sleep bout length	Treatment	$F_{3, 43} = 1.835$, $P = 0.155$	$F_{3, 55} = 0.363$, $P = 0.78$
Activity bout length	Treatment	$F_{3, 55} = 3.23$, $P = 0.0292$ *	$F_{3, 43} = 5.71$, $P = 0.0221$ **
HSP70	Treatment	$F_{5, 22} = 0.383$, $P = 0.855$	$F_{5, 26} = 2.883$, $P = 0.0335$ *
CAT	Treatment	$F_{5, 24} = 2.131$, $P = 0.0962$	$F_{5, 21} = 0.701$, $P = 0.629$
P53	Treatment	$F_{5, 23} = 0.220$, $P = 1.353$	$F_{5. 21} = 0.363$, $P = 0.868$
SOD	Treatment	$F_{5, 23} = 1.926$, $P = 0.129$	$F_{5, 21} = 0.09$, $P = 0.993$

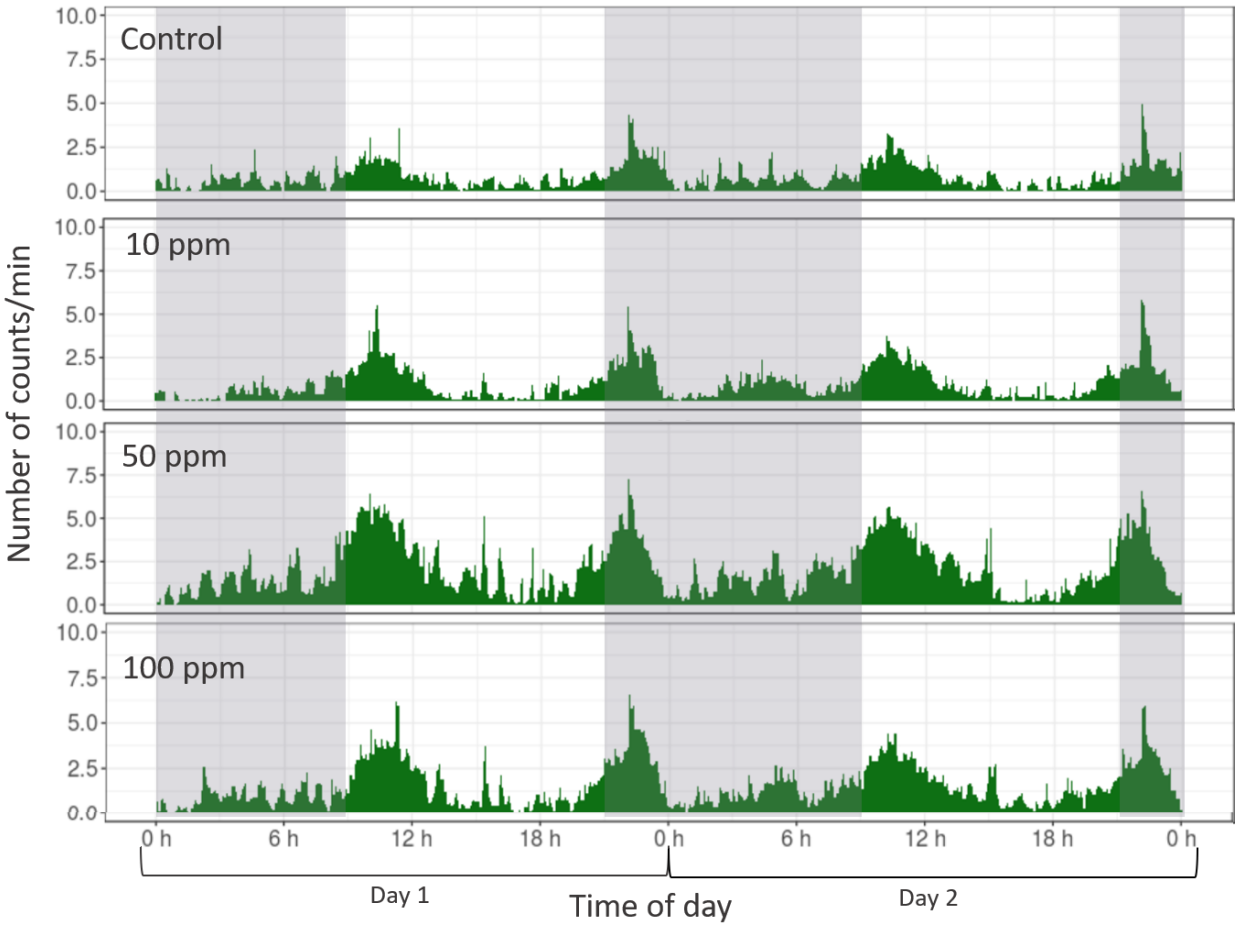

Figure S13. Actograms of two-day-old adult male fruit flies exposed to various concentration of 20 nm yellow-green fluorescent polystyrene spheres. The activity was recorded by a Drosophila Activity Monitor 2 in 1 min time intervals. Light periods are indicated by a white background and dark periods by grey. There were 16 flies in each treatment but there is a range of sample size as flies that died during the experiment were excluded from the analysis.

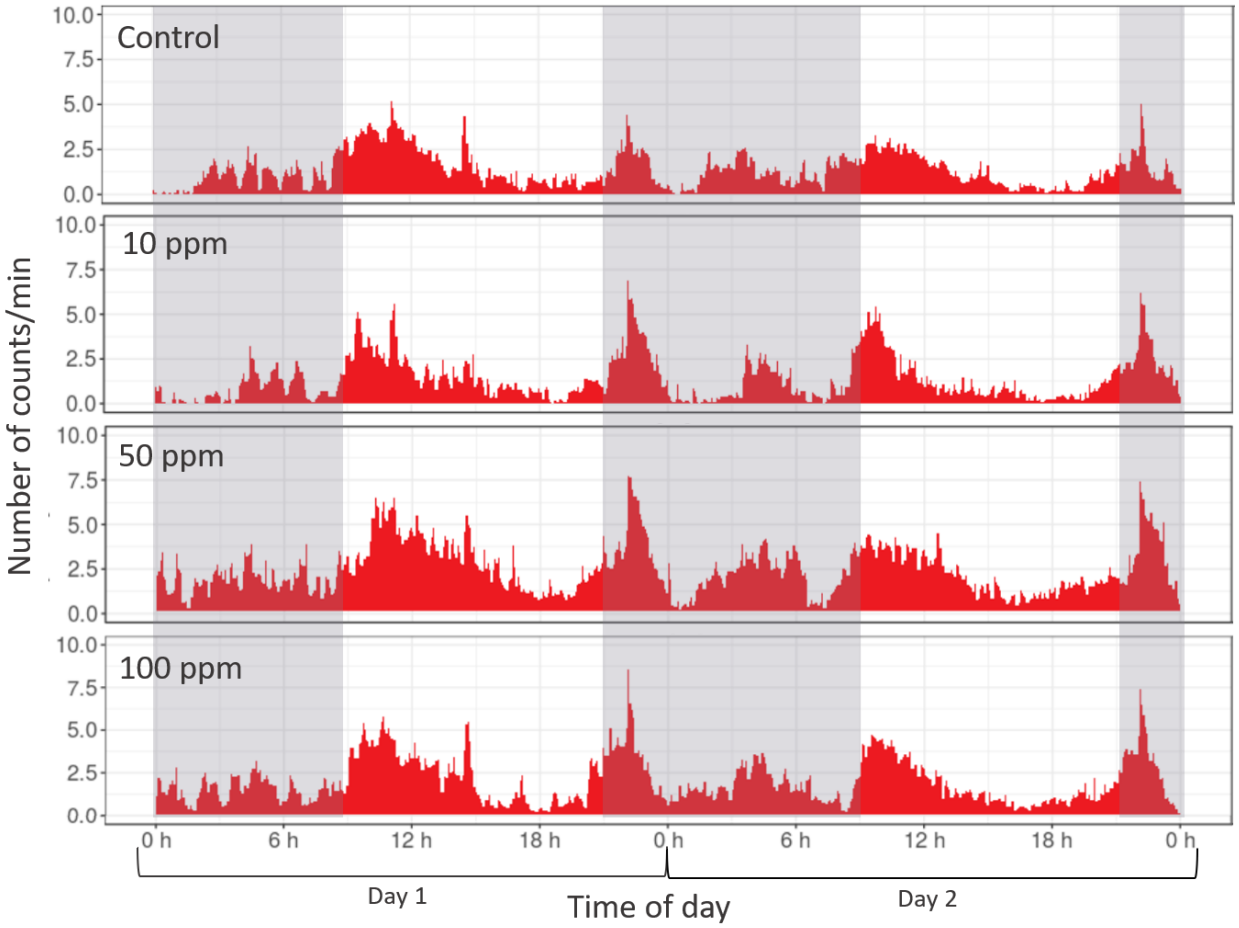

Figure S14. Actograms of two-day-old adult male fruit flies exposed to various concentration of 1 µm red fluorescent polystyrene spheres. The activity was recorded by a Drosophila Activity Monitor 2 in 1 min time intervals. Light periods are indicated by a white background and dark periods by grey. There were 16 flies in each treatment but there is a range of sample size as flies that died during the experiment were excluded from the analysis.