ELECTRONIC SUPPLEMENTARY INFORMATION

SPECIES-SPECIFIC SENSITIVITY OF *EISENIA* EARTHWORMS TOWARDS NOBLE METAL NANOPARTICLES: A MULTIPARAMETRIC *IN VITRO* STUDY

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Materials and methods

UV-visible light spectrophotometry

Aggregation state and particle morphology of 10 nm AgNPs and AuNPs (40 μg/mL) were studied by quantifying the light absorbance profile characteristic to the localized surface plasmon resonance within the wavelength regime of 300-800 nm scanned on a UV-visible Spectrophotometer (Jasco V-660 UV/VIS Spectrophotometer). Following 24 h incubation (at RT, in the dark) in different solutions (H_2O, PBS, RPMI-1640, RPMI-1640 with 1% and 10% heat-inactivated FBS) each sample was diluted in 1:200 in the appropriate solution prior to the measurement.

Transmission electron microscopy (TEM)

The AgNPs and AuNPs (with a nominal primary size of 10 nm) were imaged under a JEOL-1400 transmission electron microscope. For the characterization of NPs-protein complexes, free (unbound) proteins were removed through 3-times washing in ddH_2O by centrifugation (30 min, 16 kRCF, RT). After re-suspending the NPs in approximately 20 μL ddH_2O, they were further diluted twice and dropped directly onto the middle of 300 mesh grids coated with colloid ion film (Hatfield, PA, USA). Grids were dried at the ambient atmosphere for at least one day. Several grids were prepared for all treatments, and more than 15 images were captured for estimation of the size distributions.

Quantification of dissolved ion content

AgNPs and AuNPs were incubated at the highest test concentration (40 μg/mL) under the exposure conditions and after ultra-centrifugation (1 h, 164-192 kRCF, 4 ^{o}C). For quantitative

metal analysis a quadrupole Agilent 7700X inductively coupled plasma mass spectrometer (ICP-MS) was used. The sample introduction system consisted of an Agilent I-AS autosampler and a Micro Mist pneumatic nebulizer equipped with a Peltier-cooled Scott-type spray chamber. The sample uptake rate was 400 μL/min. Instrumental parameters were set up as follows: 1550 W RF power, 15.0 L/min plasma gas flow rate, 1.05 L/min carrier gas flow rate, 10.0 mm sampling depth. Measurements were carried out by monitoring the signal of ^{197}Au and ^{107}Ag isotopes. In all ICP-MS experiments trace-quality de-ionized labwater (MilliPoreElix 5 with Synergy, Merck) was used for preparing solutions and dilution. Ultra trace quality HCl and HNO_3 acids (VWR Chemicals, USA) were used for sample preparation. To determine the total metal concentrations in the NP suspensions from which the supernatants were collected by ultracentrifugation, hot HNO_3 acid (for Ag) or hot aqua regia (for Au) were used to completely dissolve the NPs for 1 h at 180 °C. ICP-MS tuning was performed prior to the analytical measurements using tuning solutions supplied by Agilent (No. G1820-60410). Calibration was performed using the Agilent Multi-Element Calibration Standard-3 (for gold) and Inorganic Ventures IV-ICPMS-71A (for silver). The 99.996% purity argon gas was purchased from Messer Hungarogáz (Hungary). ICP-MS data processing was performed within the Agilent Mass Hunter software.

Extrusion of coelomocytes and in vitro exposure conditions

Coelomocytes were harvested from *Eisenia andrei* and *E. fetida* as we described earlier[1] and washed twice (5 min, 100 RCF) with *Lumbricus* Balanced Salt Solution (LBSS: 71.5 mM NaCl, 4.8 mM KCl, 1.1 mM $MgSO_4 \times 7H_2O$, 0.4 mM KH_2PO_4, 0.3 mM NaH_2PO_4, 4.2 mM $NaHCO_3$, pH: 7.3). Coelomocyte numbers were enumerated by a dead-cell exclusion method using 0.14 % trypan-blue dye.

Evaluation of concentration response curve by flow cytometry

Coelomocytes were exposed to a concentration series of NPs (1.25-40 µg/mL) or $AgNO_3$ (0.05-1.35 µg/mL) for 24 h at RT (as mentioned in the main article). Following treatments, coelomocytes were washed twice in LBSS (5 min, 100 RCF). 7-aminoactinomycin D (7-AAD, 1 µg/ml, Biotium) was added to each sample just prior the flow cytometry measurements. Amoebocytes (a subpopulation of coelomocytes) were gated and the fluorescent signal of 7-AAD was measured in FL3 (670 LP filter). Concentration-response results were fitted to a 4-parameter logistic non-linear regression curve fit to derive LC_x values using Prism v5.0 (GraphPad Software, La Jolla, CA USA).

Evaluation of oxidative and mitochondrial stress by flow cytometry

Intracellular ROS profile was investigated for early time points (1, 2 and 4 h) using 2́,7́-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Sigma-Aldrich) for staining and hydrogen peroxide (100 µM, H_2O_2) as a positive ROS-inducer control in phenol red-free cell culture media. After exposure, coelomocytes were collected, spun down and loaded with 10 µM H_2DCF-DA for 25 min in the dark at RT. Thereafter the cells were washed twice with LBSS (5 min, 100 RCF) prior to flow cytometry. Fluorescence signals were detected in FL1 (530/30 filter) gated on the population with intact cell membranes, as determined by 7-AAD in FL3 (670 LP filter).

For nitric oxide (NO) measurements, coelomocytes were collected at 4 and 24 h time points, washed with LBSS (5 min, 100 RCF), and stained with 4-Amino-5-methylamino-2′,7′-difluorescein (10 µM DAF-FM-DA, **S**igma-Aldrich) for 20 minutes in the dark at RT. After incubation, coelomocytes were washed with LBSS (5 min, 100 RCF) and subsequently monitored by flow cytometry in FL1 (530/30 filter) along with of 7-AAD in FL3 (670 LP filter). Caspase-3 activation and mitochondrial damages were studied likewise using a dual NucView488

(detected in FL1, 530/30 filter) and MitoView633 (detected in FL4, 661/16 filter) Apoptosis Assay Kit (Biotium).

RNA isolation, cDNA synthesis, qPCR

Total RNA was extracted from coelomocytes applying NucleoSpin® RNA isolation kit (Macherey-Nagel GmBH, Düren, Germany) according to the manufacturer's protocol (including DNase I digestion). The quantities of total RNA were measured at 260 nm by NanoDrop 1000 spectrometer (Thermo Scientific) and the qualities inspected by 1% agarose gel electrophoresis. Total RNA samples were stored at -80 ^{o}C.

The cDNA was reverse-transcribed following DNase I digestion (Sigma-Aldrich) using High-Capacity cDNA reverse transcription kit (Thermo Scientific) in 20 µl total volume and stored at -20 ^{o}C. Maxima SYBR Green/Low Rox MasterMix reagents (Thermo Scientific) were used on an ABI Prism 7500 instrument (Applied Biosystems, Warrington, UK). The amplification profile started with pre-denaturation at 95 ^{o}C for 10 min followed by 40 cycles of denaturation at 95 ^{o}C for 35 s, annealing at 58 ^{o}C for 35 s and elongation at 72 ^{o}C for 1 min.

In-gel digestion

The excised gel bands of interest were subjected to in-gel trypsin digestion[2]. Briefly, after destaining, reduction was performed using 20 mM dithiothreitol (Bio-Rad, Hercules, CA, USA) for 1 h at 56 °C followed by alkylation with 55 mM iodoacetamide (Bio-Rad) for 45 min at RT in the dark. The trypsin digestion was carried out using stabilized MS grade TPCK-treated bovine trypsin (Sciex, Framingham, MA, USA) at 37 °C overnight. The digested peptides were extracted, lyophilized and re-dissolved in 10 µL 1% formicacid (VWR Ltd., Radnor, PA, USA).

LC-MS/MS analysis

Peptides were separated with a flow rate of 300 nL/min on a 180 min water/acetonitrile gradient using an EASY-nLC 1200 nano UHPLC (Thermo Scientific, Waltham, MA, USA). The peptide mixture was desalted on an Acclaim PepMap 100 C18 trap column (20 × 75 μm, 3 μm particle size, 100 Å poresize, Thermo Scientific, Waltham, MA, USA), followed by separation on Acclaim PepMap RSLC C18 analytical column (150 mm × 50 μm 2 μm particle size, 100 Å poresize, Thermo Scientific, Waltham, MA, USA).

Data-dependent analyses were carried out on an Orbitrap Fusion mass spectrometer (Thermo Scientific, Waltham, MA, USA). The 14 most abundant multiply-charged ions were selected from each survey MS scan using a scan range of 350-1600 m/z for MS/MS analyses (Orbitrap analyse resolution: 60000, AGC target: 4.0e5, acquired in profile mode). CID fragmentation was performed in the linear ion trap with 35% normalized collision energy (AGC target: 2.0e3, acquired in centroid mode).

The acquired LC-MS/MS spectra were used for protein identification with the help of MaxQuant 1.6.2.10 software[3] searching against the *Eisenia* proteins downloaded from the UniProt knowledge base (33430 sequence entries). Cys carbamidomethylation was set as fixed modification; Met oxidation and N-terminal acetylation were set as variable modifications. Maximum 2 missed cleavage sites were allowed. Proteins were accepted with at least 3 identified peptides using 1% FDR criteria.

Western blot

The *ex-situ* protein corona or protein secretion samples were transferred onto nitrocellulose membranes following SDS-PAGE. Nitrocellulose membranes were incubated in

blocking buffer (1×PBS, 0.1 % Tween-20, 1 % BSA) for 1 h at RT, and then with polyclonal anti-lysenin antibody (rabbit, Pepta Nova GmbH, Sandhausen, Germany) diluted (1:1500) in blocking buffer for overnight at 4 ^{o}C. Next, blots were washed in PBS-T for 30 min and incubated with HRP-conjugated anti-rabbit IgG secondary antibody diluted (1:1000) in PBS-T for 1 h at RT. Finally, ECL detection reagent (Super Signal West Pico Plus, Thermo Scientific) was used for visualization.

References

1 P. Engelmann, L. Pálinkás, E. L. Cooper, and P. Németh, Monoclonal antibodies identify four distinct annelid leukocyte markers, *Dev. Comp. Immunol.,* 2005, **29**, 599–614.

2 G. Kalló, M. Emri, Z. Varga, B. Ujhelyi, J. Tőzsér, A. Csutak, and É. Csősz, Changes in the chemical barrier composition of tears in Alzheimer's disease reveal potential tear diagnostic biomarkers, *PloS One*, 2016, **11**, e0158000.

3 J. Cox, and M. Mann, MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification, *Nat. Biotechnol.,* 2008, **26**, 1367-1372.

Tables

Table S1. Lists of primers and GenBank Accession numbers used for qPCR analysis

Target Gene	Gene Bank Accession #	Sequence (5'-3')[a]	Amplicon size (bp)
RPL 17	BB998250	ATT GTG TCA AAC GCC TTC GC GTCGGCGATCTCTTCCAACA	159
TLR	JX898685	ATT GTG TCA AAC GCC TTC GC GTCGGCGATCTCTTCCAACA	123
Lysenin	D85846	CTT GTG AGC GAT GTC GGC TA TGA TCC ACA CTG GTG CTT CC	117
Lumbricin	KX816866	ACT CGG AAC GCA AGA ACC AA GGT TCT GCG TGA CCT CCT TC	139
LuRP	KX816867	GGT CGA GAG AAT CAA CCC AAC TA TGC GAG TAC AGG CTC GTT AAC	133
MT	AJ236886	GCT GTG GAA AAT CTA CCT GCG CAC ATT TGC CCT TCT CAG CG	129
Cu/Zn SOD	KR106132	TGC CAA GTT TGA AGT GAC GG TTT GCC AAG ATC GTC CAC CA	103

[a]Upper and lower primer sequences indicate forward and reverse primers.

Table S2. Estimated LC_x values

Species	Treatment	LC_{20} (µg/mL)	LC_{50} (µg/mL)	Hill-Slope
E. andrei	AgNP	2.76±0.64	6.72±1.71	1.37
E. fetida	AgNP	2.04±0.53	5.24±1.22	1.7
E. andrei	$AgNO_3$	0.206±0.04	0.48±0.10	1.49
E. fetida	$AgNO_3$	0.207±0.01	0.41±0.01	3.13

LC_x values of 24 h exposures represent the mean ± SEM (n=3).

Table S3. Basal gene expression levels of *E. fetida* / *E. andrei* (where the value of >1 indicates a higher expression level in *E. fetida*).

	TLR	*Lumbricin*	*LuRP*	*Lysenin*	*SOD*	*MT*
2 h	0.465	0.248	0.184	0.469	0.419	0.413
12 h	1.426	0.158	2.096	0.179	0.828	12.170
24 h	2.496	1.167	6.583	0.332	0.420	25.917
All	**1.183**	0.358	**1.364**	0.303	0.526	**5.068**

Values were calculated by taking a geometric mean of the control R_0 values at each time point or all time points.

Table S4. Tandem mass spectrometry-based identification of *E. andrei* and *E. fetida* coelomic proteins enriched by AgNPs after 24 h incubation.

SDS-PAGE band range (kDa)	Unique peptides #	Unique sequence coverage (%)	Protein name (accession #)	Species	Mw[a] (kDa)	pI[a]	Functions[b]
35-55	10	37.4	**Lysenin** (O18423)	*Eisenia fetida*	33.44	5.86	Hemolysis Cytolysis Ion transport Antimicrobial
35-55	11	38.3	**Lysenin-related protein 2** (O18425)	*Eisenia fetida*	34.142	5.64	Hemolysis Cytolysis Ion transport Antimicrobial
35-55	34	73.1	**Actin** (A0A2I7YV73)	*Eisenia fetida*	41.919	5.30	Cytoskeleton Transport mechanisms

All samples listed here contained BSA as a non-specific background protein source.
[a]Before post-transitional modification according to the Protein knowledgebase (UniProtKB) from the whole amino acid sequences and pI were calculated by Compute pI/MW (ExPASy).
[b]Functions are derived from Protein knowledgebase (UniProtKB)

Figures

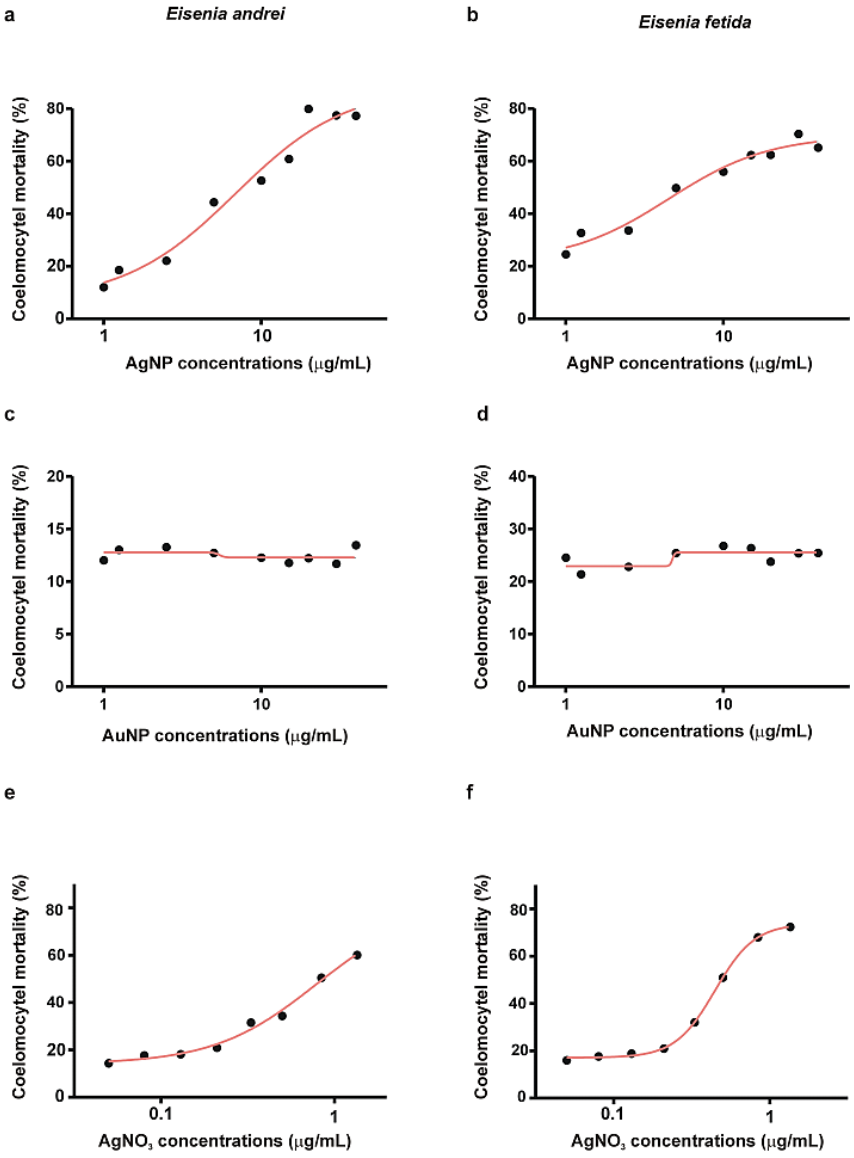

Figure S1. Concentration-dependent coelomocyte mortality curves for the concentration ranges of 1.25-40 µg/mL AgNP (a, b), AuNP (c, d) and 0.05-1.35 µg/mL $AgNO_3$ (e, f). Cytotoxicity was determined by the 7-AAD live/dead cell assay on flow cytometry following 24 h exposure. Dots represent the mean of three independent measurements.

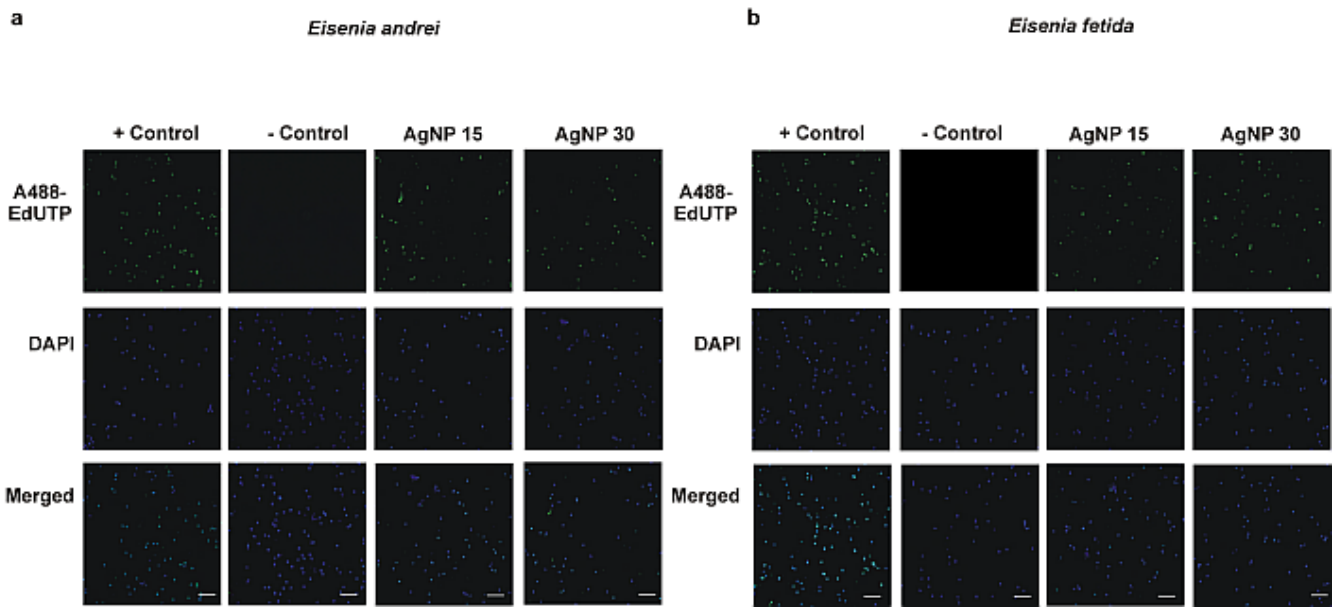

Figure S2. Representative images of one TUNEL-assay in coelomocytes of *E. andrei* (a) and *E. fetida* (b) earthworms after 24 h exposure to 10 nm AgNP (15 µg/mL, 30 µg/mL). Unexposed coelomocytes were applied for TUNEL-controls. The positive controls were treated with DNase-I for 30 min, negative controls did not receive TdT-enzyme (following the manufacturer's instructions). Each assay was repeated three independent times. Scale bars: 50 µm.

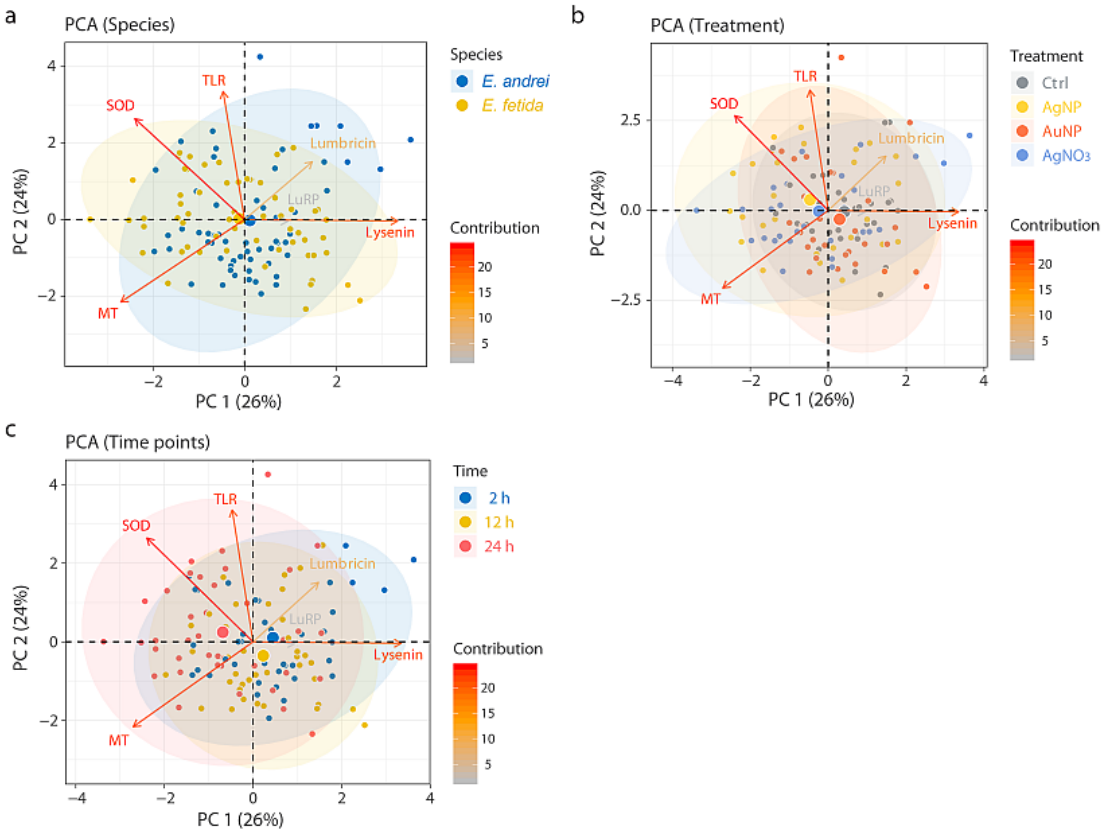

Figure S3. Principal component analysis (PCA) on species-pooled gene expression datasets. The same PCA biplot is shown with color codes differentiating (a) species, (b) treatments and (c) time points. Note that the same PCA biplot is also shown in the main article, Fig. 5, with a color scheme for treatments × time points. Individual samples (small dots) and mean points (large dots) are plotted according to the coordinates in the first two principal components (PCs), overlaid with variable coefficients (arrows). Percentage contributions of the 6 variables (genes) to the two PCs are color-scaled from grey to red. Concentration ellipses are drawn for each sample feature as a guide to identify overall trends along with the mean points (the distance from the center representing the degree of bias).

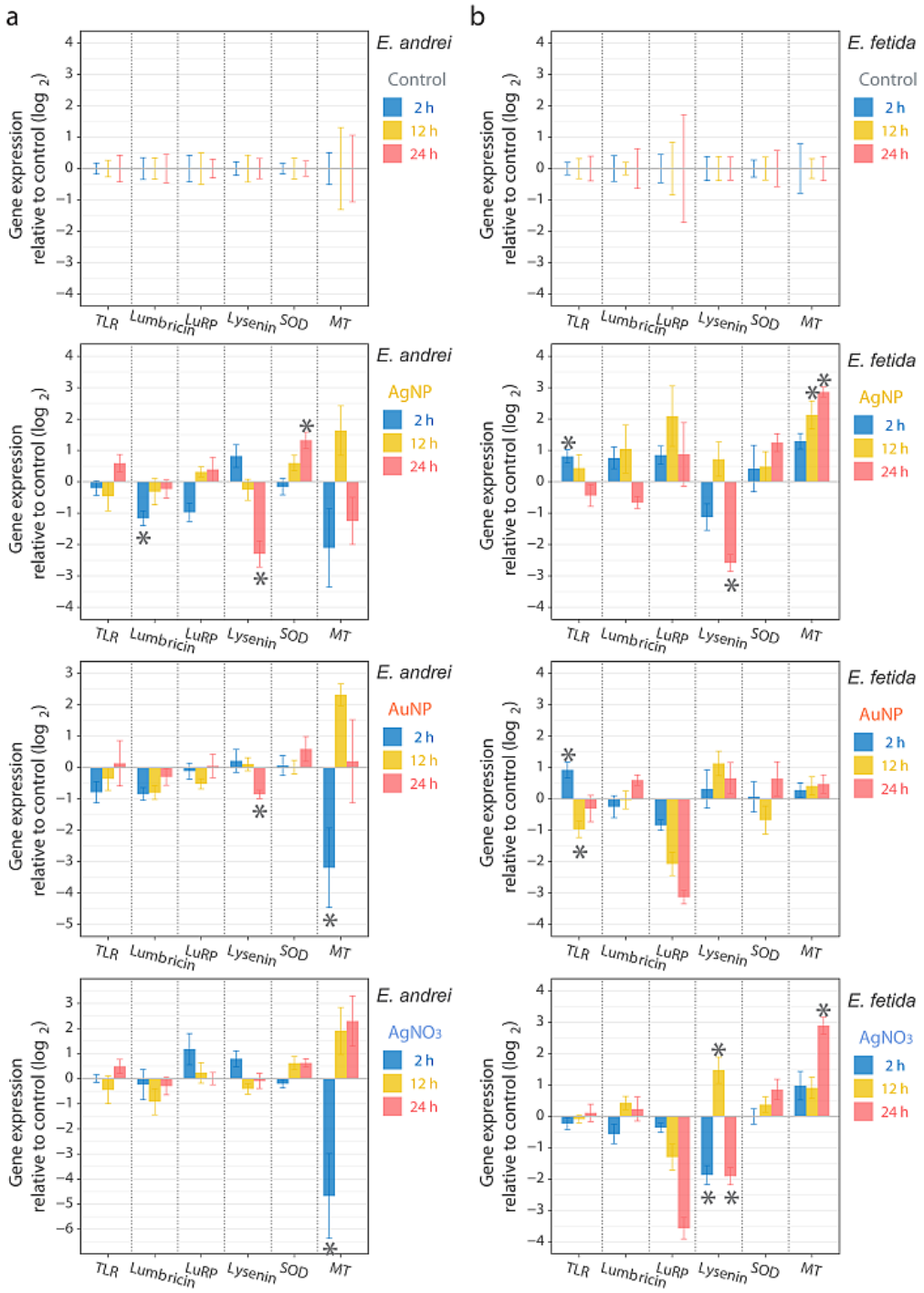

Figure S4. Univariate statistics. The $\log_2$-transformed relative expression values are plotted as mean ± SEM (n = 5). Asterisks (*) denote a significant difference between the control and the treatment at the specified time point (Student's t-test, $p < 0.05$).