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1 **Species-specific Cd-detoxification mechanisms in lumbricid earthworms *Eisenia andrei*,**
2 ***E. fetida* and their hybrids**

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23 **Abstract:**

24 Hermaphroditic lumbricid *Eisenia* sp. earthworms are ubiquitous and highly resistant to a
25 variety of environmental stressors, including heavy metals. Among the progeny of laboratory
26 mated inter-specific pairs of *Eisenia fetida* (*Ea*) and *Eisenia andrei* (*Ef*) there are fertile *Ha*
27 hybrids derived from *Ea* ova fertilized by *Ef* spermatozoa and very rare sterile *Hf* hybrids
28 from *Ef* ova fertilized by *Ea* spermatozoa. The aim of the first part of the experiment was to
29 compare the life traits and whole body accumulation of cadmium in adult earthworms from
30 genetically defined *Ea*, *Ef* and their hybrids (*Ha*) exposed for four weeks to commercial soil
31 either unpolluted (control) or cadmium-spiked leading to moderate (M) or high (H) soil
32 pollution (M=425 and H=835 mg kg⁻¹ dry soil weight). Such exposure impaired cocoon
33 production but not affected earthworm viability despite the massive Cd bioaccumulation in
34 the whole earthworm bodies reaching at M and H groups 316-454, 203-338, 114-253, and
35 377-309 mg kg⁻¹ dry body weights of *Ea*, *Ef1*, *Ef2*, and *Ha*, respectively, surprisingly reaching
36 maximum accumulation quantities in hybrids. The second part of the experiment aimed to
37 investigate cadmium-related defense mechanisms at transcriptomic level in coelomocytes
38 non-invasively extruded from coelomic cavities of the new sets of *Ea*, *Ef*, *Ha*, and *Hf*
39 earthworms exposed to Cd in microcosms for 0 days (control), 2 days, and 7 days (M=425 mg
40 kg⁻¹). Expression level of stress-induced *Cd-metallothionein* (*mt*) and *superoxide dismutase*
41 (*sod*) were gradually up-regulated, while the immune-connected lysenin (*lys*) was rapidly
42 down-regulated; the expression of glutathione S-transferase (*gst*) and phytochelatin synthase
43 (*pcs*) remained unaffected. *Mt* and *sod* gene up-regulation and *lys* gene down-regulation were
44 especially pronounced in *Ea*-derived hybrids. In sum, capacity of cadmium bioaccumulation
45 and detoxification mechanisms is more efficient in interspecific hybrids than in the pure *Ea*
46 and *Ef* species.

47 **Keywords:** *Eisenia* sp.; hybrids; cadmium; Gene expression; detoxification; Bioaccumulation

48 **1. Introduction**

49 Cadmium (Cd) contamination of soils is a ubiquitous environmental problem that has resulted
50 from uncontrolled industrialization, unsustainable urbanization, and intensive agricultural
51 practices (Khan et al., 2017). The dispersion of cadmium in the environment had been
52 increasing in the past few decades all over the world. As an example, currently, Cd
53 contamination can be found as a direct result of most industrial activities such as, mining,
54 smelting, transport, burning of fossil fuels, wastewater irrigation systems, and as a by-product
55 of synthetic phosphate fertilizers (Grobela et al., 2019). Being a toxic element, Cd is a high
56 threat to soil quality, food safety, and human health (Shahid et al., 2016). To date, numerous
57 studies have investigated the toxic impact of Cd in plants and animals, including humans.
58 Research focused on human exposure to cadmium shows that Cd induces several types of
59 cancers, neurotoxic effects as well as kidney damage, bone demineralization, and the
60 disruptions of the immune system (Buha et al., 2019). Overall, industrial activities involved in
61 a large scale dispersal of heavy metals to the environment are associated with genotoxic and
62 carcinogenic hazards, which creates a crucial challenge for regulatory authorities (Eom et al.,
63 2017). Improvements in the technologies of contaminant detection and quantification in
64 environmental samples are essential (Mahajan and Kaushal, 2018). Nevertheless, novel
65 approaches that could take into account not only chemical hazards but its biological and
66 ecological effects are currently needed for accurate safety evaluation (Khan et al., 2017).

67 Earthworms, living in close contact with soil, are constantly confronted with different
68 pollutants, among the others high cadmium concentrations (Wang et al., 2019). Moreover,
69 they are a crucial part of soil fauna, which helps to maintain soil structure and organic matter
70 cycling via soil ingestion and excretion, making them key regulators of soil processes. Hence,
71 earthworms are widely used as model organisms for the evaluation of the hazardous effects of
72 different terrestrial toxicants (Yu et al., 2019).

73 Compared to other lumbricid species, composting earthworms from *Eisenia* genus are very
74 resistant to most pollutants, including heavy metal contamination. Nevertheless, most of the
75 early studies are focused on the *Eisenia* sp., not taking into account the distinction between *E.*
76 *fetida* and *E. andrei* (Rombke et al., 2016, Suleiman et al., 2017). Proper distinction of the red
77 earthworm *E. andrei* and striped *E. fetida* on basis of morphological criteria is sometimes
78 unequivocal, thus shall be connected with species delimitation by the species-specific DNA
79 sequences of the mitochondrial and/or nuclear genes. Such molecular markers revealed a
80 clear distinction between *Ea* and *Ef* species, and at least two distinct mitochondrial lineages of
81 the latter, described as *Ef1* and *Ef2* (e.g. Pérez-Losada et al. 2005; Otomo et al. 2008; Rombke
82 et al. 2016). Combined analysis of sequences of the haploid mitochondrial genes (of the
83 maternal origin) and diploid nuclear genes of maternal-paternal origin made possible to find
84 out hybrids between laboratory-mated (*Ea+Ef1*) or (*Ea+Ef1*) pairs (Plytycz et al. 2018a,b;
85 2020; Podolak et al. 2020).

86 Among the progeny of hermaphroditic *Ea+Ef* pairs appeared both specimens of the pure *Ea*
87 or *Ef* species (derived by self-fertilization) and hybrids derived from *Ea* ova fertilized by *Ef*
88 spermatozoa (called here *Ha*), but non *Hf* hybrids from *Ef* ova fertilized by *Ea* spermatozoa.
89 These later appeared only among progeny of *Ha* hybrids mated with *Ef* pure species but were
90 very rare and sterile. The *Ha* hybrids were relatively common and fertile, giving offspring
91 with *Ea* species consisting off second (and then third) generations of *Ha* hybrids and pure *Ea*
92 specimens (Plytycz et al. 2018a,b; Podolak et al. 2020), but fertility of hybrids was gradually
93 diminished in the subsequent generations (Plytycz et al. 2020). The question arose whether
94 hybrids are more sensitive to stressing factors than specimens of the pure parental species?

95 Our previous laboratory studies on effects of cadmium were performed on *E. fetida* (Brulle et
96 al. 2008, Bernard et al. 2010) and more recently on *E. andrei* (Homa et al. 2015; Takacs et al.
97 2016, Rorat et al. 2017). We have documented that exposure of *E. andrei* earthworms to soil

98 spiked with high concentration of cadmium chloride (500 mg kg⁻¹ air-dried soil), caused
99 massive cadmium bioaccumulation in earthworm bodies without lethal effects; it caused
100 inhibition of maturation of juveniles, inhibition of cocoon production by adults without
101 adverse effects on regenerative processes (Takacs et al. 2016). Cadmium exposure even
102 enhanced regeneration of amputated posterior segments that corresponded with induction of
103 expression of metallothionein gene of this species (Rorat et al. 2017).

104 In our previous studies, the mechanisms of detoxification of Cd were investigated on
105 transcriptomic level and some genes were identified as involved or potentially involved in
106 those processes, namely, metallothionein (*mt*), glutathione S-transferase (*gst*), lysenin (*lys*),
107 superoxide dismutase (*sod*), phytochelatin synthase (*pcs*) (Brulle et al. 2008, Bernard et al.
108 2010, Homa et al. 2015). Among the others, it has been shown that the protective mechanisms
109 were mainly based on a strong induction of a gene coding the cadmium metallothionein (*mt2*)
110 in earthworms (Homa et al. 2015, Engelmann et al., 2016). The implication of the other genes
111 mentioned before is not fully elucidated. Even if the two *Eisenia* species are very resistant to
112 stressors and show similar responses overall, we noted specificities, in particular at the
113 molecular level, which we decided to investigate in this work.

114 Therefore, it was reasonable to compare effects of cadmium exposure on *E. andrei*, two
115 distinct lineages of *E. fetida* (*Ef1* and *Ef2*) and on hybrids appearing among progeny of *Ea+Ef*
116 pairs mated in the laboratory settings (Plytycz et al. 2018a,b, 2020; Podolak et al. 2020). For
117 this, the accumulation of Cd and the level of expression of genes directly or indirectly
118 involved in the response to Cd were measured in pure species and their hybrids investigated at
119 the same laboratory conditions.

120 The main goal of the first part of our experiments was to compare the life traits and the
121 capacity of cadmium accumulation in whole bodies of adult earthworms from genetically
122 defined *Ea*, *Ef1*, *Ef2* and their hybrids (*Ha*). Earthworms were exposed for four weeks to

123 commercial soil either unpolluted (control) or moderately or highly spiked with cadmium
124 chloride at the same concentrations as that used during our previous studies (Takacs et al.
125 2016; Rorat et al. 2017). The second part aimed for gaining insight into cadmium-related
126 defense mechanisms at transcriptomic level in coelomocytes extruded from a new set of *Ea*,
127 *Ef1*, *Ef2*, and *Ha/Hf* earthworms exposed for 0 days (control), 2 days or 7 days to moderately
128 Cd-polluted soil in mesocosms. Overall, the upregulation of *mt* and *sod* genes and down-
129 regulation of *lys* genes were more pronounced in hybrids than in parental species.

130 **2. Materials and methods**

131 **2.1. Earthworms**

132 Adult composting *E. andrei* (*Ea*) and *E. fetida* (*Ef*) earthworms deriving from laboratory
133 stocks at the University in Lille (France) were cultured for a decade in the laboratory of the
134 Institute of Zoology and Biomedical Research of the Jagiellonian University (Krakow,
135 Poland), and in parallel for last four years in the laboratories of Rzeszow University (Poland).
136 Earthworms were cultured in boxes with commercial soil at room temperature and fed *ad*
137 *libitum* on boiled/dried tea, nettle and dandelion leaves. For several years some of them were
138 used for studies on progeny of laboratory-paired genotyped virgin specimens giving in
139 offspring specimens of *E. andrei* (*Ea*), two mitochondrial lineages of *E. fetida* (*Ef1* and *Ef2*)
140 species, and interspecific hybrids (*H*) derived either from *Ea* ova (*Ha*) or *E1/Ef2* ova (*Hf*),
141 each of them individually delimited by species-specific sequences of the mitochondrial COI
142 gene and diploid nuclear 28S rRNA gene (Plytycz et al. 2018a; 2020; Podolak et al. 2020).

143 Some of these genetically delimited adult (clitellate) specimens were used for experiments
144 performed in the same laboratory conditions (Plytycz et al. 2020; Podolak et al. 2020). One
145 group of earthworms (54 individuals) was used for the measurement of cadmium

146 accumulation in the whole earthworm bodies, the second group (50 individuals) served as a
147 source of coelomocytes for the measurement of the expression of stress-related genes.

148 **2.2. Soil preparation**

149 The air-dried commercial soil (PPUH BIOVITA, Tenczynek, Poland organic soil, 51.7%
150 organic matter, pH=6.11) was spiked either with water (control) or with cadmium chloride
151 (CHEMPUR, Piekary Slaskie, Polska), 500 mg kg⁻¹, as described previously (Takacs et al.
152 2016; Rorat et al. 2017). Control and Cd-polluted soils were kept separately or mixed 1:1,
153 giving samples of unpolluted control soil, and those with high (425 ± 45.66 mg kg⁻¹) or very
154 high (835 ± 35.712 mg kg⁻¹) cadmium concentration.

155 **2.3. Experimental design**

156 *Experiment 1 Cd accumulation in earthworms:* In total 54 earthworms were exposed either to
157 unpolluted control soil or to soil with high cadmium concentration during 4 weeks in
158 mesocosms. Briefly, four individuals from each group of species: *Ea*, *Ef1*, *Ef2* and *Ha* were
159 placed in the plastic boxes (of 9 cm diameter) with perforated lids. At the end of the exposure
160 period, the earthworms were depurated overnight in boxes with wet lignin substrate (Paso-
161 Trading, Polska), and then sacrificed by freezing and prepared for the metal accumulation
162 analysis.

163 *Experiment 2: Gene expression in Cd-exposed earthworms:* In total 50 individuals were used
164 as a source of coelomocytes, i.e. the immune cells present in earthworm coelomic cavity, non-
165 invasively retrieved from coelomic cavity by a mild electric shock as described in 2.4.3.1. *E.*
166 *andrei*, *E. fetida*, and their *Ea*-derived (*Ha*) and *Ef*-derived hybrids (*Hf*) were exposed for 2
167 and 7 days to highly polluted soil in mesocosms (boxes with with perforated lids, 9 cm
168 diameter). The level of gene expression of glutathione S-transferase (*gst*), lysenin (*lys*),

169 metallothionein (*mt*), superoxide dismutase (*sod*) and phytochelatin synthase (*pcs*) was
170 assessed in extruded coelomocytes, and related to the expression of two stable house-keeping
171 genes – actin (*act*) and ribo13 (*r13*).

172 **2.4. Analyses**

173 **2.4.1 Earthworm genotyping**

174 Supravivally amputated tail tips of numerically coded adult earthworms were used for DNA
175 extraction and genetic analysis using oligonucleotide primers amplifying species-specific
176 variants of mitochondrial COI genes and nuclear 28S rRNA genes, as described earlier
177 (Plytycz et al. 2018a,b; 2020; Podolak et al. 2020).

178 As previously established, mitochondrial species-specific sequences of the COI gene were
179 called either ‘a’ or ‘f’/‘f2’ for *Ea* and *Ef1/Ef2*, respectively, while the diploid species-specific
180 nuclear markers of the 28S rRNA gene were called either ‘A’ or ‘F’ for *Ea* and *Ef*,
181 respectively. In a results, the investigated specimens were genotyped either as pure species,
182 *Ea* (*aAA*), *Ef1* (*fFF*), *Ef2* (*f2FF*), or inter-specific hybrids, either *Ea*-derived *aAF*, *Ef*-derived
183 *fFA* with the two first letters (*aA*-, *fF*-, *f2F*) representing ova-derived genes, i.e. maternity,
184 and the last letter (*A* or *F*) representing spermatozoa-derived genes, i.e. paternity.

185 **2.4.2. Cadmium content**

186 Cadmium content in soil and in whole earthworm bodies was measured by slightly modified
187 methods used in our previous studies (Takacs et al. 2016; Rorat et al. 2017). Soil samples
188 were dried at 105°C for 24 h and then divided into subsamples about 0.5 g of dry soil mass of
189 each. Cadmium concentration in soil was determined after half open wet digestion in a quartz
190 glass with 10 mL of mixture of concentrated HNO₃ (69.0-70.0% to trace metal analysis,
191 Baker Instra-Analyzed) and HClO₄ (70% a.r, Chem-Lab) (7:1 v/v), using hot plates.

192 Cadmium concentrations were measured by atomic absorption spectrometry (AAS) with a
193 flame and atomic absorption spectrometry with a graphite furnace nebulizer (Perkin-Elmer) to
194 achieve the desired method sensitivity. Accuracy was verified by analyzing four blanks and
195 three replicates of standard certified material (Loamy Sand 10 CRM033-50G) with the
196 samples.

197 Prior to the analysis of cadmium concentration in whole earthworm bodies, earthworms were
198 kept for 2 days on wet filter papers to remove their gut content, frozen, and then dried in
199 vacuum drier in 70°C for 24 h to achieve constant dry mass. For determination of cadmium
200 concentration in earthworm bodies, the wet digestion method was applied but to reach the
201 required ratio 1: 20 only 4 mL of concentrated HNO₃ (69.0-70.0% to trace metal analysis,
202 Baker Instra-Analyzed) were used. Cadmium concentrations in earthworm bodies were
203 measured by atomic absorption spectrometry methods with four blanks and three replicates of
204 standard certified material (Bovine Liver SRM 1577c) with the sample in digestion.

205 The cadmium concentrations were calculated on a dry mass of each sample of soil or
206 earthworms and are presented in mg kg⁻¹ (µg g⁻¹).

207

208 **2.4.3. Gene expression analysis**

209 2.4.3.1. Extrusion of coelomocytes extrusion via electrostimulation

210 After 24 h depuration on wet lignin (Paso-Trading, Poland), earthworms were placed
211 individually on petri dishes and stimulated for 30 s with a 4.5 V electric current to expel
212 coelomic fluid with suspended coelomocytes through the dorsal pores (Homa et al. 2015;
213 Rorat et al. 2017). Pellets of coelomocyte samples were resuspended in 2 mL of TriReagent
214 (Molecular Research Center Inc., USA) for RNA extraction.

215 2.4.3.2. Extraction of RNA and reverse transcription

216 Expression of five target and two house-keeping genes (HKG) was performed on the total
217 RNA extracted from coelomocytes by TRI Reagent® (Molecular Research Center, USA),
218 according to manufacturer's instructions. Overall, coelomocytes' pellets from each sample
219 were suspended in 2 mL of Tri Reagent and 1 mL was later used for each extraction. Samples
220 were then treated with DNase I, RNase-free (1 U/μL) (Thermo Fisher Scientific, USA)
221 according to manufacturer's instructions. The quantity and quality of isolated RNA were
222 analyzed spectrophotometrically using spectrometer with a LVis nano plate (SPECTROstar
223 Nano, BMG LABTECH, USA, LVis Plate, BMG LABTECH, USA). RNA integrity was
224 confirmed on 2% agarose gel stained with ethidium bromide (Ethidium Bromide Solution (10
225 mg mL⁻¹), Thermo Scientific, USA). The amount of isolated RNA allowed us to perform the
226 reverse transcription of 1.5μg cDNA using QIAGEN OMNISCRIPT RT KIT (QIAGEN,
227 Germany), according to manufacturer instructions. cDNA samples were then stored in -20°C
228 until further analyses.

229 2.4.3.3. Real-time qPCR amplification.

230 The mRNA levels of chosen genes were determined by real-time quantitative PCR after
231 reverse transcription. For qPCR, Agilent Mx3000P QPCR System (Agilent Technologies,
232 USA) with 2X Takyon for SYBR Assay - No ROXqPCR Kits for SYBR Assay (Eurogentec,
233 Belgium) were used, and reactions were performed on 96-well qPCR plates (Eppendorf
234 twin.tec® PCR Plates, Eppendorf AG, Germany). All qPCR reactions were performed as
235 follows: 95°C 5 min and 40 cycles at 95°C 30 s, 55°C 30 s, and 72°C 60 s. After 40 cycles,
236 the last step of the program consisted of 72°C for 5 min. The *Eisenia* sp. specific primes were
237 used for actin (*act*), ribosomal protein S13 (*r13*), metallothionein (*mt*), glutathione S-
238 transferase (*gst*), phytochelatin synthase (*pcs*), lysenin (*lys*) and superoxide dismutase (*sod*)
239 (Brulle et al., 2008, Homa et al. 2015) after the validation of their compatibility with hybrids.
240 The selected primers are presented in Table 1. Expression levels were determined as the

241 number of cycles needed for the amplification to reach a threshold fixed in the exponential
242 phase of PCR reaction (Ct). Levels of expression and amplification effectiveness of target
243 genes were compared to the expression of two constitutively expressed and validated house-
244 keeping genes. qPCR efficiency of each target gene was calculated according to the method
245 previously described (Chaâbene et al., 2017). Overall, the relative expression level was
246 calculated according to the following formula:

247
$$R = 2^{\Delta Ct(\text{target gene}) - \Delta Ct(\text{reference gene})}$$

248

249 Table 1. qPCR primer sequences

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Amplicon size (bp)	Accession number
<i>r13</i>	5'CGCACGGTTTTAGTTTCT3'	5'CCATGCGAGTCTCGAAG3'	148	MG076964
<i>act</i>	5'CCATCCATCGTCCACAG 3'	5'GCATGTGTGAGTCCTAATT3'	147	EY893028.1
<i>mt</i>	5'AAAGTGAGTGCTTGCC3'	5'ACTGATGACAGAGTTCCG3'	120	KP770991.1
<i>gst</i>	5'TGGAAGTGGAGGTTTCCTTG3'	5'TGAGGTGGCCAAGTCTTTTC3'	161	EY892760.1
<i>pcs</i>	5'TCATGGTCCTGAACACG3'	5' CACAAGTTGCCGAAACTC 3'	154	KP770990.1
<i>lys</i>	5'CGGCAACAAACGTCTAC3'	5'GTGAAATACAGGCAGAAGC 3'	163	MG076962.1
<i>sod</i>	5'GGTGCTCACTTCAACCCATT3'	5'AAGATCGTCCACCAGCTCAT3'	167	DQ286712.1

250

251 2.5. Data analysis

252 HKG stability was quantified using two software's: BestKeeper and NormFinder. All results
 253 are expressed as means \pm standard deviation, n=3-6. Descriptive statistics and statistical
 254 analyses were produced using the OriginPro 2015 software and Statistica. Different letters
 255 "a", "b" "c", "d" on top of bars indicate statistically significant differences according to the 1
 256 one-way analysis of variance.

257 3. Results

258 3.1. Genetic species delimitation

259 Cytoplasm-rich ova with plenty mitochondria are fertilized by spermatozoa with very scarce
 260 cytoplasm and a few mitochondria. DNA barcoding based on specific sequences of
 261 mitochondrial haploid COI gene shows three distinct branches; 'a' for *Ea* or 'f'/'f2' for two

262 mitochondrial lineages of *Ef*, namely *Ef1* and *Ef2* (Plytycz et al. 2020). Species-specific
263 maternal-paternal diploid nuclear sequences of 28S rRNA gene, are either 'AA' (for *Ea*) or
264 'FF' for *Ef* or 'AF' for interspecific hybrids. Fertilization of 'aA' ovum by 'A' spermatozoon
265 gives aAA zygote of the pure *Ea* species, while 'aA' ovum fertilized by 'F' spermatozoon
266 gives *Ea*-derived aAF hybrid. The 'fF' or 'f2F' ova fertilized by 'A' spermatozoa give fFA or
267 f2FA hybrids, respectively (Figure 1). All sequences are deposited in GenBank under the
268 following accession numbers: MT646836-MT646899 and MT649303-MT649399. All the
269 earthworms survived the experiment but cadmium exposure inhibited cocoon production
270 almost completely (data not shown).

271 **3.2. Cadmium content in soil and in whole earthworm bodies**

272 The cadmium concentration in the commercial unpolluted soil was very low (L; 0.11 ± 0.107
273 mg kg^{-1}); in soil spiked with cadmium chloride was very high (H; $835 \pm 35.712 \text{ mg kg}^{-1}$ dry
274 weight) and intermediate in 1:1 mixture of these soil samples (M; $425 \pm 45.66 \text{ mg kg}^{-1}$ dry
275 weight) (Table 2). Cadmium concentrations in earthworms living in unpolluted soil were low
276 ($0.69 - 1.12 \text{ mg kg}^{-1}$) and did not differ significantly between *Ea*, *Ef1*, *Ef2*, and *Ha* groups of
277 earthworms.

278 Cadmium concentrations in whole bodies of earthworms kept in soil with M or H cadmium
279 pollution were overlapping ($113.79 - 376.73$ and $252.68 - 454.18 \text{ mg kg}^{-1}$, respectively), with
280 lowest accumulation capacity in *Ef2* earthworms (113.79 and $252.68 \text{ mg kg}^{-1}$ in M and H soil,
281 respectively), but these values were not significantly different from those in the second
282 lineage of the *Ef* species, i.e. *Ef1* (203.43 and 337.66 respectively). The whole body Cd
283 bioaccumulation in *Ea* reached $316.43 \text{ mg kg}^{-1}$ in the M soil and was significantly higher in
284 the *Ea* from the H soil ($454.19 \text{ mg kg}^{-1}$). Cadmium bioaccumulation was most efficient in the
285 *Ha* hybrids reaching plateau already in the M soil, as the values at M and H soil were similar
286 (376.73 and $309.246 \text{ mg kg}^{-1}$, respectively).

287 Table 2. Concentration of Cd in soil and earthworms' bodies after 4-week exposure in
 288 unpolluted (L), intermediate (M) or very high (H) soil. Results shown as means \pm SD, n=3 - 6.
 289 Mean concentrations in earthworm bodies not sharing the same small letters (in columns) or
 290 capital letters (in rows) are statistically significantly different according to ANOVA with post
 291 hoc Tukey's test.
 292

Groups	Cd concentration in soil and earthworm bodies [mg kg ⁻¹]			
	L	M	H	
Soil	0.11 \pm 0.107	425 \pm 45.66	835 \pm 35.712	
<i>Earthworms</i>	<i>Ea</i>	1.09 \pm 0.38 aA	316.43 \pm 33.09 bB	454.19 \pm 37.58 bC
	<i>Ef1</i>	0.76 \pm 0.26 aA	203.43 \pm 82.7 abB	337.66 \pm 115.12 abB
	<i>Ef2</i>	0.69 \pm 0.15 aA	113.79 \pm 28.16 aB	252.68 \pm 62.59 aC
	<i>Ha</i>	1.12 \pm 0.10 aA	376.73 \pm 136.99 bB	309.23 \pm 59.50 abB

293 3.3. Gene expression in earthworms

294 3.3.1. Reference genes

295 For determination of the stability of chosen housekeeping genes, the NormFinder and
 296 BestKeeper software were used. Overall, the expression of both chosen reference genes was
 297 stable across all experimental conditions and species (Appendix 1).
 298

299 3.3.2. Expression of *gst* and *pcs* genes

300 The expression level of *pcs* and *gst* was undetectable in coelomocytes of *E. andrei* in both
 301 clean and Cd-contaminated soil. However, a negligible expression of those genes was
 302 observed for *Ef1*, *Ef3* and *Ha* exposed to soil contaminated by cadmium (Figures 2A and 2B).

303 3.3.3 Expression of *mt*, *sod*, and *lys* genes

304 Only two rare *Ef*-drived hybrids (*Hf**) were used for analysis of *mt*, *sod*, and *lys* expression. In
305 unpolluted soil (i.e. on day 0 of cadmium exposure), the expression of *mt* and *sod* was very
306 low while it was relatively in a case of *lys*. In each instance, it was similar to the expression of
307 these genes in other specimens from unpolluted soil, i.e. on day 0 of cadmium exposure (Fig.
308 3, right columns of rows A, B, and C).

309 In all tested organisms, a significant induction of the expression of *mt* and *sod* was observed
310 after exposure to cadmium (Figure 4, rows A and B), while expression of *lys* was drastically
311 diminished (Fig. 3, row C).

312 3.3.3.1 Expression of *mt*

313 The expression rate of metallothionein was negligible in earthworms exposed to clean,
314 uncontaminated soil. In coelomocytes of *Ef1*, *Ef2* and *Ha*, the expression of *mt* was
315 significantly ($p < 0.05$) induced after already two days of exposure, and in *Ef1* and *Ha*
316 continued to increase further until the 7-day timepoint. However, in coelomocytes of *E.*
317 *andrei*, the induction of *mt* expression occurred later than for other earthworms, and was
318 significant only after 7 days post exposure (Figure 3, row A, left column). At each time point
319 the interspecies differences were statistically insignificant (Fig. 3, row A, right column).

320 3.3.3.2 Expression of *sod*

321 Similar results were observed for the expression rate of *sod* (Figure 3, row B). Once again, the
322 expression rate for all organisms living in clean soil was almost undetectable while the
323 exposure to cadmium caused a significant increase in *sod* expression. However, two days
324 post-exposure, the expression rate of *sod* in *E. andrei* was still almost no detectable, whereas
325 for all other organisms was already significantly ($p < 0.05$) induced. At day 7 day after
326 cadmium exposure, expression of *sod* significantly increased versus day 2 in *Ea*, *Ef2*, and *Ha*

327 (Fig. 3, row B, left column). The interspecies differences were insignificant on day 0 and day
328 2, while on day 7 was significantly higher in *Ha* than in *Ea*, *Ef1*, and *Ef2* (Fig. 3, row B, right
329 column).

330 3.3.3.3 Expression of *lys*

331 The expression of lysenin gene was highly influenced by exposure to different soil conditions
332 across all tested organisms. In all cases, its expression was at its highest in coelomocytes of
333 earthworms exposed to clean soil and the Cd contamination caused a severe and significant
334 ($p < 0.05$) downregulation of that expression (Fig. 3, row C). Moreover, for *Ef2* and *Ha*, the
335 downregulation was significant for both, 2 and 7 day post exposure, whereas for *Ea* and *Ef1* it
336 was downregulated by 2 days post-exposure, and then the expression rate stabilized and
337 stayed at the same level without further downregulation (Figure 3, row C, left column).
338 Interspecies comparisons revealed the lack of statistically significant differences in day zero
339 and day 2 after cadmium exposure while on day 7 *lys* expression differed significantly
340 between species, being the highest in *Ef1*, and then in *Ea*, *Ef2* and *Ha* (Fig. 3, row C, right
341 part).

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350 4. Discussion

351 Adult specimens of hermaphroditic lumbricid earthworms *E. andrei* and *E. fetida*, the latter
352 with two distinct lineages *Ef1* and *Ef2*, and interspecific Ha hybrids developed from *Ea* ova
353 fertilized by *Ef* spermatozoa were fully viable during four week exposure to cadmium-
354 polluted soil ($425 \pm 45.66 \text{ mg kg}^{-1}$ or $835 \pm 35.712 \text{ mg kg}^{-1}$) despite a high cadmium
355 accumulation in the whole earthworm bodies. Bioaccumulation was dose-dependent, similar
356 in *Ef1/Ef2* lineages of *Ef*, and lower than that in *Ea*. In contrast, *Ea*-derived hybrids reached
357 plateau of Cd-accumulation capacity already at lower cadmium concentration in soil.
358 Hypothetically, all investigated specimens from genus *Eisenia* are able to survive in Cd-
359 contaminated environments due to adapting the same or similar detoxification mechanisms to
360 those described previously at transcriptomic level in representatives of *E. andrei* and *E. fetida*
361 (Homa et al. 2015, Rorat et al. 2017), while data concerning hybrids were vastly unexplored.
362 Therefore comparative studies on the expression of genes involved in Cd detoxification were
363 imperative. Second part of previous investigations revealed that the expression of stress-
364 related genes upon exposure to Cd-polluted soil at $425 \pm 45.66 \text{ mg kg}^{-1}$ was either up-
365 regulated (*mt* and *sod*), down-regulated (*lys*) or unchanged (*gst* and *psc*).

366

367 *Expression of genes for metallothionein and phytochelatin synthase*

368 In a study by Brulle et al. (2008) performed on *E. fetida*, the induction pattern of genes
369 encoding phytochelatin synthase was significantly increased in earthworms exposed to a low
370 concentration of cadmium (8 mg kg^{-1}). However, in higher concentrations, no difference was
371 noticed between Cd-exposed earthworms and earthworms not exposed to any contamination,
372 which suggests that *pcs* expression is only induced during low exposure to cadmium.
373 Similarly to our study, expression of metallothionein was highly up-regulated after Cd
374 exposure, and in the mentioned Brulle et al. (2008) study, it was also shown to be dose-

375 dependent in high Cd concentrations. Such relation between different responses of the
376 expression of *pcs* and *mt* genes is of interest when considering their role in metal
377 detoxification in animals. In low concentrations of Cd, the expression of *pcs* was shown to be
378 significant and responsible for metal chelation in worm cells (probably via the synthesis of
379 Phytochelatins (PCs) although the presence of PCs has not been demonstrated), whereas such
380 induction was only noticed at a small exposure level. This is on the contrary to the effects of
381 high concentrations of Cd, where similarly to our study, *pcs* expression patterns did not show
382 any change of their level over control animals, whereas the expression rate of *mt* was
383 substantially upregulated. In another study, focused on *Caenorhabditis elegans*, the
384 toxicogenomic analysis after exposure to cadmium also showed no effect on the expression of
385 *pcs-1* gene (Cui et al., 2007). Moreover, a recent transcriptomic study on the same species: *C.*
386 *elegans*, found similar results – after exposure of nematods to cadmium (300µM) and zinc
387 (500µM), the expression change of *pcs-1* was determined via qPCR followed by
388 normalization by the invariant ribosomal *rla-1*. The results showed, that the *pcs* response to
389 Zn and Cd contamination was at best negligible, and the change in comparison to control
390 animals was not significant ($p < 0.05$) (Essig et al., 2016). By combining these studies, we can
391 see that phytochelatins and metallothioneins possibly act together to protect the cells against
392 Cd. Phytochelatins have more of an immediate role at lower concentrations and
393 metallothioneins coming later with higher induction. In addition, a comparison between the
394 chosen gene expression profiles in earthworms exposed or unexposed to Cd showed up-
395 regulation of two genes for all tested earthworms after Cd exposure – metallothionein and
396 superoxide dismutase as well as down-regulation of lysenin. Such alterations between the
397 expressions of those genes suggest that earthworms can oppose and reduce the toxic effects of
398 Cd through several different pathways. Moreover, in the presented study, we noticed that the
399 *mt* overexpression due to Cd exposure occurs not only in *E. fetida* but also in *E. andrei* and

400 hybrids between those species, which suggest, that this biomarker is suitable for both species.
401 However, results suggest that *E. andrei* (*aAA*) activate its detoxification mechanisms slower
402 than *E. fetida* (*fFF*) and their hybrids (*aAF*). Moreover, fast up regulation of expression of *mt*
403 genes in hybrids suggests, that they obtained the nuclear allele responsible for a fast reaction
404 from *E. fetida*, which showed similar tendencies, and not from *E. andrei*. In addition, such
405 results could indicate that perhaps, *Ef* earthworms possess another detoxification mechanism
406 or a specific regulation mechanism that is responsible for faster *mt* expression in comparison
407 to *Ea*. Thus, by cloning of the genes coding *mt* in both species and studying of the regulation
408 of the gene is needed to broaden our understanding of such response to metal exposure.
409 Similar to our study, the induction of overexpression of the *mt* gene was recently shown in *E.*
410 *andrei* earthworms after exposure to copper, which is a widely used fungicide, especially in
411 organic farming (Mincarelli et al., 2019). Moreover, an induction of *mt* expression was also
412 noticed in other earthworms including *Dendrobaena octaedra* and *Lumbricus rubellus*
413 exposed to Cu (Mustonen et al., 2014, Höckner et al., 2015). The population of *Dendrobaena*
414 *octaedra* exposed to Cu-Ni contaminated soil was shown to have higher *mt* expression levels
415 in comparison to earthworms not exposed to metals. What is even more interesting, during
416 artificial exposure experiment, exposure affected the *mt* expression, but only in the
417 earthworms collected from the clean, unpolluted soil, showing that there is a delay in the *mt*
418 response of earthworms which were not exposed to metals in the past. On the contrary,
419 earthworms collected from the Cu-Ni smelting site showed not only higher but also constant
420 levels of *mt* expression (Mustonen et al., 2014). Such results reflect on the role of the *mt*
421 expression and the homeostasis of several metals and protection against oxidant damage.
422
423 *Superoxide dismutase and Glutathione S-transferase*

424 Glutathione S-transferase (GST) is an enzyme known to take part in the metabolism of
425 lipophilic organic contaminants and in cellular protection against oxidative stress. A study by
426 Mkhinini et al. (2019) showed an induction of *gst* expression after the exposure of *E. andrei*
427 to treated wastewater (TWW) containing several trace metals. However, such effects were
428 observed in earthworms exposed to TWW for more than 20 days. Our study showed no effect
429 of cadmium exposure to the expression of this gene in a 7-day period, which could suggest
430 that the *gst* mechanism is induced after prolonged, chronic exposure to heavy metals, but it is
431 not an immediate response to the presence of contamination. In another study, the antioxidant
432 gene expression of *E. fetida* was explored after worm exposure to various concentrations of
433 hexabromocyclododecane. The results showed a significant up-regulation of the *sod* gene but
434 no significant differences in the expression rate of *gst*, which further suggests its involvement
435 in protection against the oxidative damage occurs during a prolonged period of time (Shi et
436 al., 2018). Moreover, a recent study of Maity et al. (2018) focused on the oxidative stress
437 response of *E. fetida* and *Eityphoeus waltoni* exposed to Cd, the overexpression of the *gst*
438 gene increased significantly post-exposure and was dose-dependent. However, in *E. fetida*,
439 after 2 days post-exposure, the expression of *gst* remained the same as in control animals,
440 which also suggest its late-onset. In a transcriptomic study by Chai et al. (2019), focused on
441 cadmium toxicity in *E. fetida*, a significant up-regulation of *sod* genes was also observed in
442 earthworms exposed to 10, 30, 60 mg/kg of Cd²⁺ in dry soil, respectively.

443

444 *Lysenin*

445 Lysenin is a protein involved in immunity processes produced by coelomocytes and
446 chloragocytes (Munguira et al., 2017). The presence of lysenin and lysenin-related proteins
447 was documented in *E. andrei* and *E. fetida* and their putative hybrids (Swiderska et al. 2016).
448 In the present study, the expression rate of *lys* gene was significantly down-regulated post Cd

449 exposure in all tested animals (*Ea*, *Ef*, *Ha*). A similar decrease in *lys* expression was noticed
450 by Rorat et al. (2017) in *E. andrei* and Brulle et al. (2011) in *E. fetida*. However, the
451 mechanisms behind such response are still unknown. In a study by Wang et al. (2010) the
452 expression of *lys* was also shown to diminish after earthworms were exposed to *E. coli*.
453 Besides, Wu et al. (2013), discovered that lysenin-related protein was significantly up-
454 regulated post-exposure to PAH phenanthrene. Hence, lysenin expression in earthworms can
455 have a complex response to exposure to different contaminants. Overall, the results imply the
456 existence of a complex regulatory network that needs to be further explored in future studies.

457

458 *Hybrids response to environmental stressors*

459 We have shown previously that *Eisenia andrei* (*Ea*) and *E. fetida* (*Ef*) of French provenance
460 are capable of hybridization, as viable offspring appeared among progeny of laboratory-mated
461 inter-specific *Ea+Ef* pairs. Recently we have shown that hybridization is not unique for
462 French *Ea/Ef* earthworms, but is shared by laboratory-mated earthworms from French,
463 Hungarian, and Polish laboratory cultures (Podolak et al. 2020; Plytycz et al. 2020), but
464 fertility of hybrids is gradually diminished in the subsequent generations (Plytycz et al. 2020).
465 Inter-specific hybrids were detected in natural populations of *Ea* and *Ef* from Scandinavia
466 (Martinsson and Erseus, 2018) thus further data on hybridization in natural environment, even
467 such highly polluted, including compost heaps, are expected.

468 Both *E. andrei* and *E. fetida* earthworms as well as their hybrids are perfect cadmium
469 bioaccumulators and can survive in heavily contaminated soil due to efficient protective
470 mechanisms. *Mt* and *sod* upregulation and *lys* down-regulation are especially pronounced in
471 hybrids. Therefore we shall exclude such a possibility that the improper functioning of
472 investigated stress-related genes is the main factor responsible for gradual impairment of
473 hybrid fertility in the subsequent generations (Plytycz et al. 2020).

474 Interestingly, similar to our results, recent research on several species of plants indicated that
475 hybrids can outperform their parental species after exposure to different stressors (both biotic
476 and abiotic). As an example, a couple of types of poplar hybrids had been shown to better
477 adapt to suboptimal temperatures and demonstrate superior growth, particularly under the less
478 favorable, suboptimal temperature conditions (Guet et al., 2015). Overall, the interspecific
479 hybridization has recently become of-interest to forest geneticists for tree improvement by
480 assessing the possible feasibility of transferring desirable traits such as higher resistance to
481 contamination, droughts, or diseases, among species (Juranović-Cindrić et al., 2018). Apart
482 from trees, another study showed a variation between Cd uptake in maize hybrids, which can
483 be used for phytoremediation of Cd-contaminated soil as well as for the selection and
484 breeding of new, Cd-tolerant genotypes of maize (Akhtar et al., 2016, Wang et al., 2015).
485 Similar tendencies had been observed for the accumulation of Cr in sunflower hybrids, which
486 is currently used in the selection and breeding of Cr-tolerant genotypes of sunflowers (Farid et
487 al., 2017). However, the molecular basis of such differential accumulation and the differences
488 in the genetic makeup of both hybrids and parent species are scarcely researched and need to
489 be further investigated in both, plants and earthworms exposed to contamination (Wang et al.,
490 2015, Naeem et al., 2016, Juranović-Cindrić et al., 2018).

491

492 **5. Conclusions**

493 The insights gained in the presented study have improved our understanding of metal
494 resistance of *E. fetida*, *E. andrei* and their hybrids. The results indicate that all of them are
495 very efficient cadmium bio-accumulators with similar mechanisms of Cd detoxication.
496 Consistently, upregulation of *mt* and *sod* expression was fastest in hybrids, slower in *Ef* and
497 slowest in *Ea*. Hypothetically *Ef* and *Ea* differ in their mechanisms controlling expression of

498 stress-related genes and immunity-connected genes thus further studies of these genes and
499 their interactions in hybrids are necessary.

500

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506 References

- 507 Akhtar, T., Zia-ur-Rehman, M., Naeem, A., Nawaz, R., Ali, S., Murtaza, G., ... Rizwan,
508 M. (2016). Photosynthesis and growth response of maize (*Zea mays* L.) hybrids exposed to
509 cadmium stress. *Environmental Science and Pollution Research*, 24(6), 5521–5529.
510 doi:10.1007/s11356-016-8246-0
- 511 Brulle, F., Cocquerelle, C., Wamalah, A. N., Morgan, A. J., Kille, P., Leprêtre, A., &
512 Vandebulcke, F. (2008). cDNA cloning and expression analysis of *Eisenia fetida*
513 (*Annelida: Oligochaeta*) phytochelatin synthase under cadmium exposure. *Ecotoxicology*
514 and *Environmental Safety*, 71(1), 47–55. doi:10.1016/j.ecoenv.2007.10.032
- 515 Buha, A., Jugdaohsingh, R., Matovic, V., Bulat, Z., Antonijevic, B., Kerns, J. G., ...
516 Powell, J. (2019). Bone mineral health relates to environmental cadmium exposure-
517 experimental and human data. *Environmental Research*, 108539.
518 doi:10.1016/j.envres.2019.108539
- 519 Chaâbene, Z., Rorat, A., Rekik Hakim, I., Bernard, F., Douglas, G. C., Elleuch, A., ...
520 Mejdoub, H. (2018). Insight into the expression variation of metal-responsive genes in the
521 seedling of date palm (*Phoenix dactylifera*). *Chemosphere*, 197, 123–134.
522 doi:10.1016/j.chemosphere.2017.12.146
- 523 Chai, L., Yang, Y., Yang, H., Zhao, Y., & Wang, H. (2019). Transcriptome analysis of
524 genes expressed in the earthworm *Eisenia fetida* in response to cadmium exposure.
525 *Chemosphere*, 124902. doi:10.1016/j.chemosphere.2019.124902
- 526 Cui, Y., McBride, S. J., Boyd, W. A., Alper, S., Freedman, J. H. (2007). Toxicogenomic
527 analysis of *Caenorhabditis elegans* reveals novel genes and pathways involved in the
528 resistance to cadmium toxicity. *Genome Biology*, 8(6). doi:10.1186/gb-2007-8-6-r122
- 529 Engelmann, P., Hayashi, Y., Bodó, K., Ernszt, D., Somogyi, I., Steib, A., Molnár, L.
530 (2016). Phenotypic and functional characterization of earthworm coelomocyte subsets:

531 Linking light scatter-based cell typing and imaging of the sorted populations.
532 *Developmental and Comparative Immunology*, 65, 41-52. doi:10.1016/j.dci.2016.06.017
533 Eom, S.-Y., Seo, M.-N., Lee, Y.-S., Park, K.-S., Hong, Y.-S., Sohn, S.-J., ... Park, J.-D.
534 (2017). Low-Level Environmental Cadmium Exposure Induces Kidney Tubule Damage in
535 the General Population of Korean Adults. *Archives of Environmental Contamination and*
536 *Toxicology*, 73(3), 401–409. doi:10.1007/s00244-017-0443-4
537 Essig, Y., Webb, S., & Stürzenbaum, S. (2016). Deletion of Phytochelatin Synthase
538 Modulates the Metal Accumulation Pattern of Cadmium Exposed *C. elegans*. *International*
539 *Journal of Molecular Sciences*, 17(2), 257. doi:10.3390/ijms17020257
540 Farid, M., Ali, S., Akram, N. A., Rizwan, M., Abbas, F., Bukhari, S. A. H., & Saeed, R.
541 (2017). Phyto-management of Cr-contaminated soils by sunflower hybrids: physiological
542 and biochemical response and metal extractability under Cr stress. *Environmental Science*
543 *and Pollution Research*, 24(20), 16845–16859. doi:10.1007/s11356-017-9247-3
544 Grobelak, A., Świątek, J., Murtaś, A., Jaskulak, M. (2019). Cadmium-induced oxidative
545 stress in plants, cadmium toxicity, and tolerance in plants: from physiology to remediation.
546 *Cadmium toxicity and tolerance in plants*, 213-231
547 Guet, J., Fabbrini, F., Fichot, R., Sabatti, M., Bastien, C., Brignolas, F. (2015). Genetic
548 variation for leaf morphology, leaf structure and leaf carbon isotope discrimination in
549 European populations of black poplar (*Populus nigra* L.). *Tree Physiology*, 35(8), 850-863.
550 doi:10.1093/treephys/tpv056
551 Höckner, M., Dallinger, R., & Stürzenbaum, S. R. (2015). Metallothionein gene
552 activation in the earthworm (*Lumbricus rubellus*). *Biochemical and Biophysical Research*
553 *Communications*, 460(3), 537–542. doi:10.1016/j.bbrc.2015.03.065
554 Homa, J., Rorat, A., Kruk, J., Cocquerelle, C., Plytycz, B., & Vandebulcke, F.
555 (2015). Dermal exposure of *Eisenia andrei* earthworms: Effects of heavy metals on

556 metallothionein and phytochelatin synthase gene expressions in coelomocytes.
557 Environmental Toxicology and Chemistry, 34(6), 1397-1404. doi:10.1002/etc.2944
558 Juranović-Cindrić, I., Zeiner, M., Starčević, A., Liber, Z., Rusak, G., Idžojtić, M., &
559 Stinger, G. (2018). Influence of F 1 hybridization on the metal uptake behaviour of pine
560 trees (*Pinus nigra x Pinus thunbergiana* ; *Pinus thunbergiana x Pinus nigra*). Journal of
561 Trace Elements in Medicine and Biology, 48, 190–195. doi:10.1016/j.jtemb.2018.04.009
562 Khan, M. A., Khan, S., Khan, A., & Alam, M. (2017). Soil contamination with cadmium,
563 consequences and remediation using organic amendments. Science of The Total
564 Environment, 601-602, 1591–1605. doi:10.1016/j.scitotenv.2017.06.030
565 Mahajan, P., & Kaushal, J. (2018). Role of Phytoremediation in Reducing Cadmium
566 Toxicity in Soil and Water. Journal of Toxicology, 2018, 1–16. doi:10.1155/2018/4864365
567 Maity, S., Banerjee, R., Goswami, P., Chakrabarti, M., & Mukherjee, A. (2018).
568 Oxidative stress responses of two different ecophysiological species of earthworms
569 (*Eutyphoeus waltoni* and *Eisenia fetida*) exposed to Cd-contaminated soil. Chemosphere,
570 203, 307–317. doi:10.1016/j.chemosphere.2018.03.189
571 Martinsson, S., & Erséus, C. (2018). Hybridisation and species delimitation of
572 Scandinavian Eisenia spp. (Clitellata: Lumbricidae). European Journal of Soil Biology, 88,
573 41-47. doi:10.1016/j.ejsobi.2018.06.003
574 Mincarelli, L., Tiano, L., Craft, J., Marcheggiani, F., & Vischetti, C. (2019). Evaluation
575 of gene expression of different molecular biomarkers of stress response as an effect of
576 copper exposure on the earthworm *Eisenia Andrei*. Ecotoxicology. doi:10.1007/s10646-019-
577 02093-3
578 Mkhinini, M., Boughattas, I., Alphonse, V., Livet, A., Bousserhine, N., & Banni, M.
579 (2019). Effect of treated wastewater irrigation in East Central region of Tunisia (Monastir

580 governorate) on the biochemical and transcriptomic response of earthworms *Eisenia andrei*.
581 Science of The Total Environment, 647, 1245–1255. doi:10.1016/j.scitotenv.2018.07.449
582 Munguira, I. L. B., Takahashi, H., Casuso, I., & Scheuring, S. (2017). Lysenin Toxin
583 Membrane Insertion Is pH-Dependent but Independent of Neighboring Lysenins.
584 Biophysical Journal, 113(9), 2029–2036. doi:10.1016/j.bpj.2017.08.056
585 Mustonen, M., Haimi, J., Väisänen, A., & Knott, K. E. (2014). Metallothionein gene
586 expression differs in earthworm populations with different exposure history. Ecotoxicology,
587 23(9), 1732–1743. doi:10.1007/s10646-014-1338-z
588 Naeem, A., Saifullah, Rehman, M. Z., Akhtar, T., Ok, Y. S., & Rengel, Z. (2016).
589 Genetic Variation in Cadmium Accumulation and Tolerance among Wheat Cultivars at the
590 Seedling Stage. Communications in Soil Science and Plant Analysis, 47(5), 554-562.
591 doi:10.1080/00103624.2016.1141918
592 Otomo, V. P., van Vuuren, B. J., & Reinecke, S. A. (2008). Usefulness of DNA
593 Barcoding in Ecotoxicological Investigations: Resolving Taxonomic Uncertainties Using
594 *Eisenia Malm 1877* as an Example. Bulletin of Environmental Contamination and
595 Toxicology, 82(3), 261–264. doi:10.1007/s00128-008-9585-4
596 Pérez-Losada, M., Eiroa, J., Mato, S., & Domínguez, J. (2005). Phylogenetic species
597 delimitation of the earthworms *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei* Bouché,
598 1972 (Oligochaeta, Lumbricidae) based on mitochondrial and nuclear DNA sequences.
599 Pedobiologia, 49(4), 317–324. doi:10.1016/j.pedobi.2005.02.004
600 Plytycz, B., Bigaj, J., Osikowski, A., Hofman, S., Falniowski, A., Panz, T.,
601 Vandembulcke, F. (2018a). The existence of fertile hybrids of closely related model
602 earthworm species, *Eisenia andrei* and *E. fetida*. Plos One, 13(1).
603 doi:10.1371/journal.pone.0191711

604 Plytycz, B., Bigaj, J., Panz, T., & Grzmil, P. (2018b). Asymmetrical hybridization
605 and gene flow between *Eisenia andrei* and *E. fetida* lumbricid earthworms. Plos One, 13(9).
606 doi:10.1371/journal.pone.0204469

607 Plytycz, B., Bigaj, B., Rysiewska, A., Osikowski, A., Hofman, S., Podolak, A., Grzmil,
608 P. (2020). Impairment of reproductive capabilities in three subsequent generations of
609 asymmetric hybrids between *Eisenia andrei* and *E. fetida* from French, Hungarian and
610 Polish laboratory colonies. Plos One, in press.

611 Podolak, A., Kostecka, J., Hofman, S., Osikowski, A., Bigaj, J., & Plytycz, B.
612 (2020). Annual Reproductive Performance of *Eisenia andrei* and *E. fetida* 2 in Intra- and
613 Inter-Specific Pairs and Lack of Reproduction of Isolated Virgin Earthworms. Folia
614 Biologica, 68(1), 1-6. doi:10.3409/fb_68-1.01

615 Rorat, A., Vandenbulcke, F., Gałuszka, A., Klimek, B., Plytycz, B. (2017). Protective
616 role of metallothionein during regeneration in *Eisenia andrei* exposed to cadmium.
617 Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 203,
618 39-50. doi:10.1016/j.cbpc.2017.10.002

619 Shahid, M., Dumat, C., Khalid, S., Niazi, N. K., & Antunes, P. M. C. (2016). Cadmium
620 Bioavailability, Uptake, Toxicity and Detoxification in Soil-Plant System. Reviews of
621 Environmental Contamination and Toxicology Volume 241, 73–137.
622 doi:10.1007/398_2016_8

623 Shi, Y., Xu, X., Chen, J., Liang, R., Zheng, X., Shi, Y., & Wang, Y. (2018). Antioxidant
624 gene expression and metabolic responses of earthworms (*Eisenia fetida*) after exposure to
625 various concentrations of hexabromocyclododecane. Environmental Pollution, 232, 245–
626 251. doi:10.1016/j.envpol.2017.09.039

627 Suleiman, H., Rorat, A., Grobelak, A., Grosser, A., Milczarek, M., Plytycz, B., Kacprzak,
628 M., Vandenbulcke, F. (2017). Determination of the performance of vermicomposting

629 process applied to sewage sludge by monitoring of the compost quality and immune
630 responses in three earthworm species: *Eisenia fetida*, *Eisenia andrei* and *Dendrobaena*
631 *veneta*. *Bioresource Technology*. 241: 103-112. doi: 10.1016/j.biortech.2017.05.104

632 Swiderska, B., Kedracka-Krok, S., & Plytycz, B. (2016). Data on proteins of *lysenin*
633 family in coelomocytes of *Eisenia andrei* and *E. fetida* obtained by tandem mass
634 spectrometry coupled with liquid chromatography. *Data in Brief*, 9, 629-634.
635 doi:10.1016/j.dib.2016.09.035

636 Rombke, J., Aira, M., Backeljau, T., Breugelmans, K., Dumonguez, J., Funke, E. (2016).
637 DNA barcoding of earthworms (*Eisenia fetida/andrei* complex) from 28 ecotoxicological
638 test laboratories. *Appl Soil Ecol*. 104: 3–11. <https://doi.org/10.1016/j.apsoil.2015.02.010>

639 Takacs, V., Molnar, L., Klimek, B., Gałuszka, A., Morgan, A. J., & Plytycz, B.
640 (2016). Exposure of *Eisenia andrei* (*Oligochaeta; Lumbricidae*) to Cadmium Polluted Soil
641 Inhibits Earthworm Maturation and Reproduction but not Restoration of Experimentally
642 Depleted Coelomocytes or Regeneration of Amputated Segments. *Folia Biologica*, 64(4),
643 275-284. doi:10.3409/fb64_4.275

644 Wang, X., Chang, L., Sun, Z., Zhang, Y., & Yao, L. (2010). Analysis of earthworm
645 *Eisenia fetida* proteomes during cadmium exposure: An ecotoxicoproteomics approach.
646 *PROTEOMICS*, 10(24), 4476–4490. doi:10.1002/pmic.201000209

647 Wang, A., Wang, M., Liao, Q., & He, X. (2015). Characterization of Cd
648 translocation and accumulation in 19 maize cultivars grown on Cd-contaminated soil:
649 Implication of maize cultivar selection for minimal risk to human health and for
650 phytoremediation. *Environmental Science and Pollution Research*, 23(6), 5410-5419.
651 doi:10.1007/s11356-015-5781-z

652 Wang, G., Wang, L., Ma, F., You, Y., Wang, Y., & Yang, D. (2019). Integration of
653 earthworms and arbuscular mycorrhizal fungi into phytoremediation of cadmium-

654 contaminated soil by *Solanum nigrum* L. Journal of Hazardous Materials, 121873.
655 doi:10.1016/j.jhazmat.2019.121873

656 Wu, S., Xu, X., Zhao, S., Shen, F., & Chen, J. (2013). Evaluation of phenanthrene
657 toxicity on earthworm (*Eisenia fetida*): An ecotoxicoproteomics approach. Chemosphere,
658 93(6), 963-971. doi:10.1016/j.chemosphere.2013.05.062

659 Yu, Y., Li, X., Yang, G., Wang, Y., Wang, X., Cai, L., & Liu, X. (2019). Joint toxic
660 effects of cadmium and four pesticides on the earthworm (*Eisenia fetida*). Chemosphere.
661 doi:10.1016/j.chemosphere.2019.04.064

662

663 **Figure Captions**

664 Figure 1. Maximum-likelihood phylogram for the species-specific sequences ‘a’ or ‘f’/‘f2’ of
665 the mitochondrial COI gene, with additional information on the ‘A’ or ‘F’ nuclear sequences
666 of 28S rRNA nuclear gene of the same individuals with the same code, showing the pure aAA
667 (*Ea*) and fFF/f2FF (*Ef1/Ef2*) species and their aAF or f2FA hybrids.

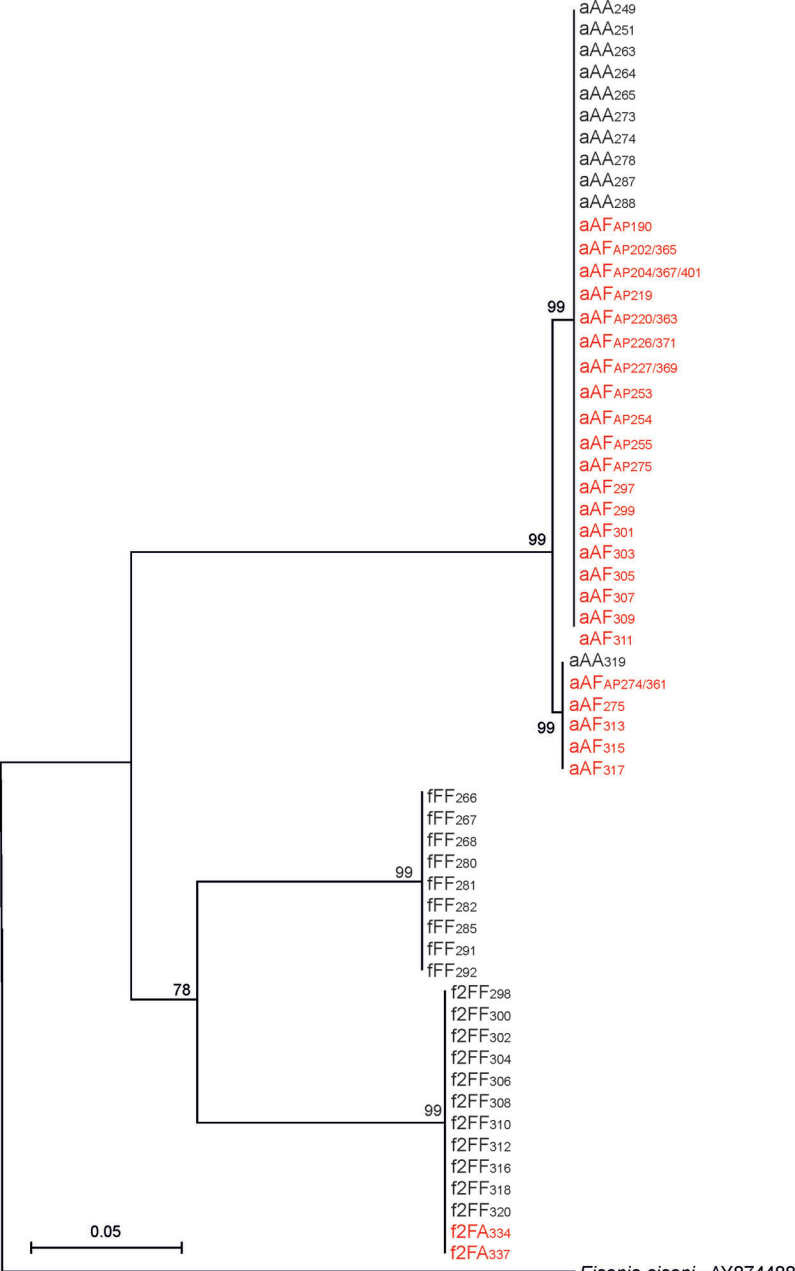
668 Figure 2. Relative expression level of *gst* (A) and *pcs* (B) in coelomocytes of groups of
669 earthworms exposed to cadmium polluted soil concentration for 0 days (control), 2 days, or 7
670 days: Effects of time of exposure. Different letters “a”, “b” “c”, “d” on top of bars indicate
671 statistically significant differences according to the Kruskal–Wallis one-way analysis of
672 variance ($p < 0.05$).

673 Figure 3. Relative expression level of *mt* (row A), *sod* (row B) and *lys* (row C) in
674 coelomocytes of groups of earthworms exposed to cadmium polluted soil concentration for 0
675 days (control), 2 days, or 7 days; Left column - effects of time of exposure; right column
676 interspecies comparisons between *Ea*, *Ef1*, *Ef2*, and *Ha* earthworms at particular time points.
677 Results shown as means \pm SD, $n=6$; *Hf** - mean from two specimens only. Different letters
678 “a”, “b” “c”, “d” on top of bars indicate statistically significant differences according to the
679 Kruskal–Wallis one-way analysis of variance ($p < 0.05$).

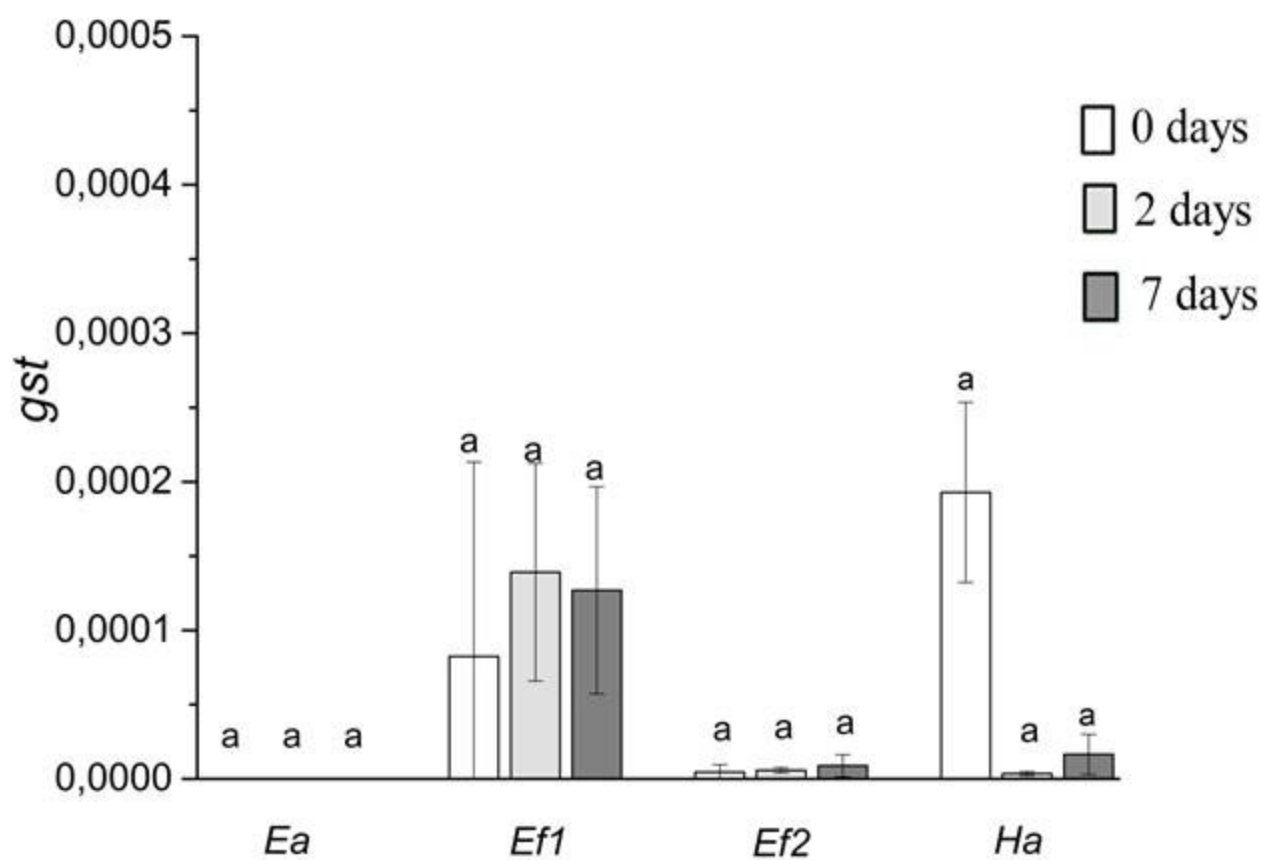
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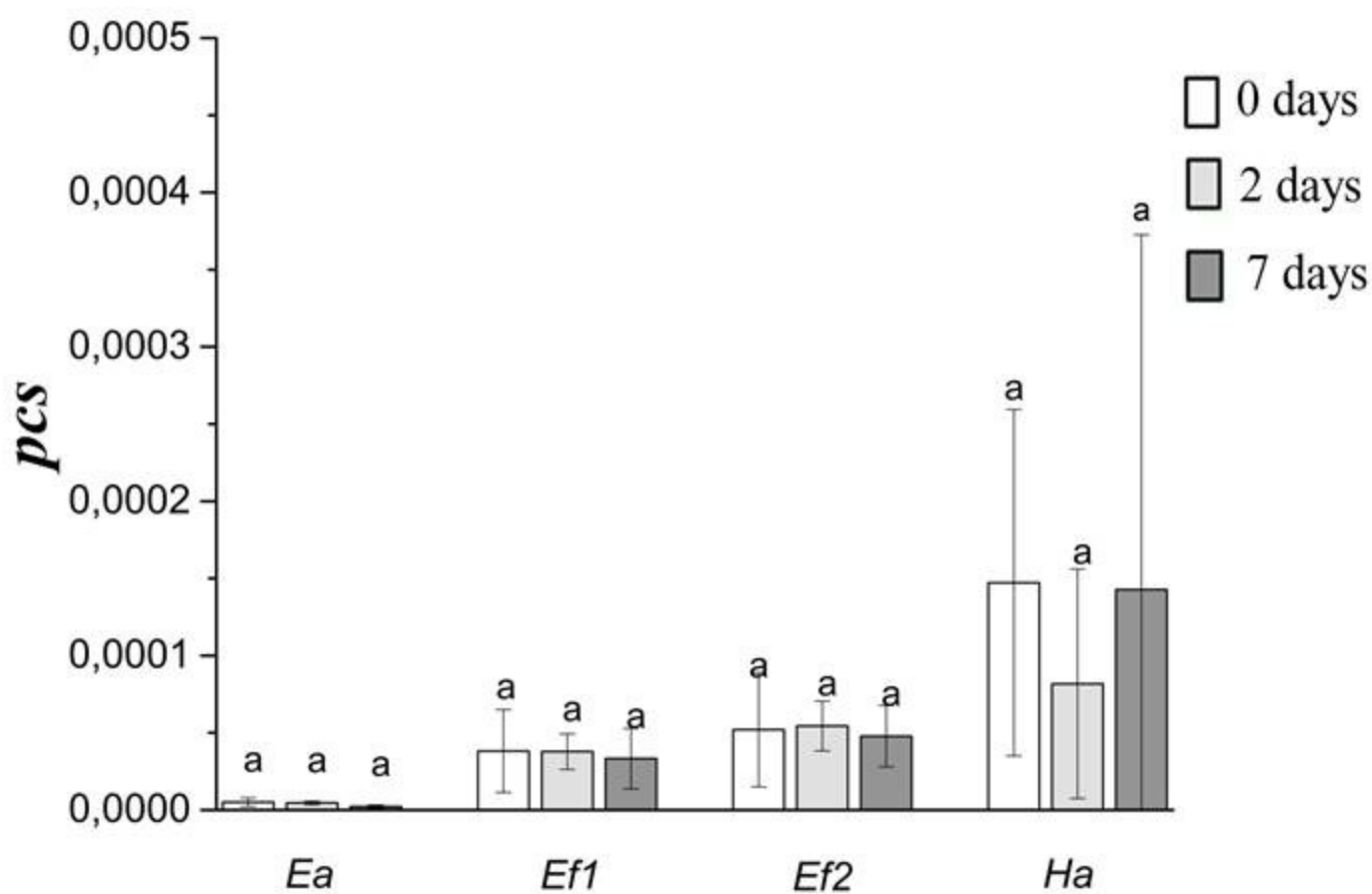
682



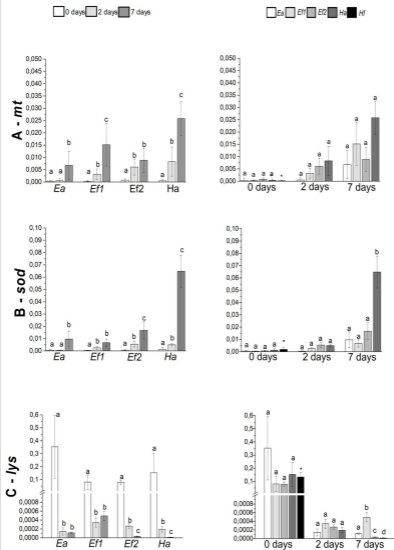
A)



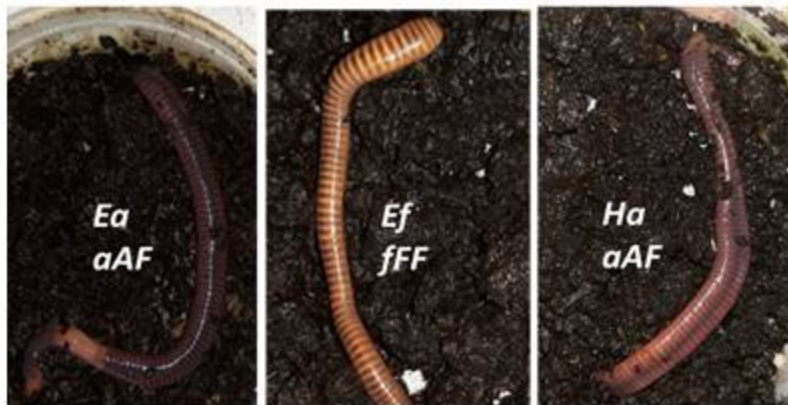
B)



Relative expression level



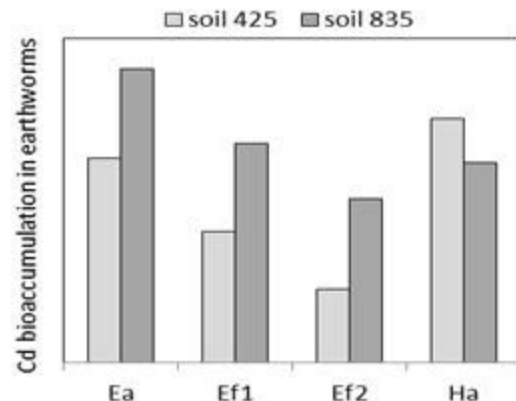
Exposure on Cd
(425 or 835
mg/kg)



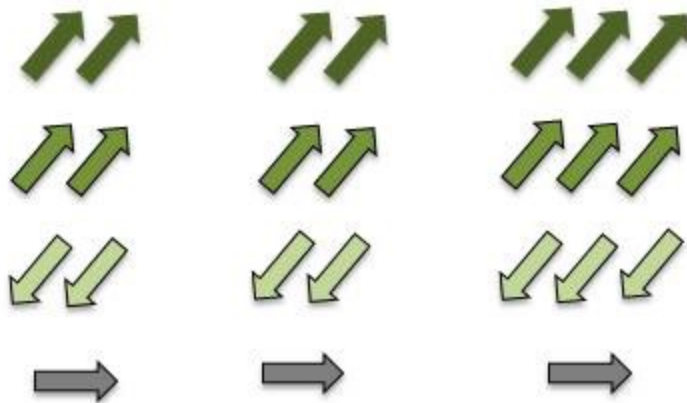
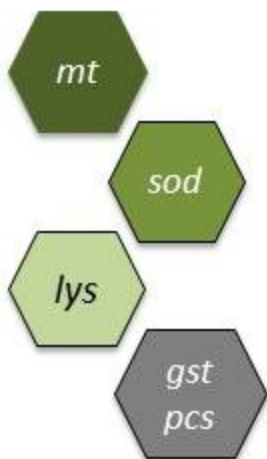
Cd accumulation



4 weeks Cd bioaccumulation



Gene expression



Cd detoxication

