Ecotoxicology and Environmental Safety 208 (2021) 111425

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

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

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ARTICLE INFO

Edited by: Dr Yong Liang

Keywords:
Eisenia Sp.
Hybrids
Cadmium
Gene expression
Detoxification
Bioaccumulation

ABSTRACT

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

1. Introduction

Cadmium (Cd) contamination of soils is a ubiquitous environmental problem that has resulted from uncontrolled industrialization, unsustainable urbanization, and intensive agricultural practices (Khan et al., 2017). The dispersion of cadmium in the environment had been increasing in the past few decades all over the world. As an example, currently, Cd contamination can be found as a direct result of most industrial activities such as, mining, smelting, transport, burning of fossil fuels, wastewater irrigation systems, and as a by-product of synthetic phosphate fertilizers (Grobelak et al., 2019). Being a toxic element, Cd is a high threat to soil quality, food safety, and human health (Shahid et al., 2016). To date, numerous studies have investigated the toxic impact of Cd in plants and animals, including humans. Research focused on human exposure to cadmium shows that Cd induces several types of cancers, neurotoxic effects as well as kidney damage, bone demineralization, and the disruptions of the immune system (Buha et al., 2019). Overall, industrial activities involved in a large scale dispersal of heavy metals to the environment are associated with genotoxic and carcinogenic hazards, which creates a crucial challenge for regulatory authorities (Eom

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https://doi.org/10.1016/j.ecoenv.2020.111425
Received 9 July 2020; Received in revised form 8 September 2020; Accepted 27 September 2020
Available online 15 October 2020

et al., 2017). Improvements in the technologies of contaminant detection and quantification in environmental samples are essential (Mahajan and Kaushal, 2018). Nevertheless, novel approaches that could take into account not only chemical hazards but its biological and ecological effects are currently needed for accurate safety evaluation (Khan et al., 2017).

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

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

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

Our previous laboratory studies on effects of cadmium were performed on *E. fetida* (Brulle et al., 2008, Bernard et al., 2010) and more recently on *E. andrei* (Homa et al., 2015; Takacs et al., 2016; Rorat et al., 2017). We have documented that exposure of *E. andrei* earthworms to soil spiked with high concentration of cadmium chloride (500 mg kg^{-1} air-dried soil), caused massive cadmium bioaccumulation in earthworm bodies without lethal effects; it caused inhibition of maturation of juveniles, inhibition of cocoon production by adults without adverse effects on regenerative processes (Takacs et al., 2016). Cadmium exposure even enhanced regeneration of amputated posterior segments that corresponded with induction of expression of metallothionein gene of this species (Rorat et al., 2017).

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

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

The main goal of the first part of our experiments was to compare the life traits and the capacity of cadmium accumulation in whole bodies of adult earthworms from genetically defined *Ea, Ef1, Ef2* and their hybrids (*Ha*). Earthworms were exposed for four weeks to commercial soil either unpolluted (control) or moderately or highly spiked with cadmium chloride at the same concentrations as that used during our previous studies (Takacs et al., 2016; Rorat et al., 2017). The second part aimed for gaining insight into cadmium-related defense mechanisms at transcriptomic level in coelomocytes extruded from a new set of *Ea*, *Ef1*, *Ef2*, and *Ha/Hf* earthworms exposed for 0 days (control), 2 days or 7 days to moderately Cd-polluted soil in mesocosms. Overall, the upregulation of *mt* and *sod* genes and down-regulation of *lys* genes were more pronounced in hybrids than in parental species.

2. Materials and methods

2.1. Earthworms

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

Some of these genetically delimited adult (clitellate) specimens were used for experiments performed in the same laboratory conditions (Plytycz et al., 2020; Podolak et al., 2020). One group of earthworms (54 individuals) was used for the measurement of cadmium accumulation in the whole earthworm bodies, the second group (50 individuals) served as a source of coelomocytes for the measurement of the expression of stress-related genes.

2.2. Soil preparation

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

2.3. Experimental design

2.3.1. Experiment 1 Cd accumulation in earthworms

In total 54 earthworms were exposed either to unpolluted control soil or to soil with high cadmium concentration during 4 weeks in

Table 1
qPCR primer sequences.

Gene	Forward primer 5′– 3′	Reverse primer 5′– 3′	Amplicon size (bp)	Accession number
r13	5′CGCACGGTTTTAGTTTCT3’	5′CCATGCGAGTCTCGAAG3’	148	MG076964
act	5′CCATCCATCGTCCACAG 3′	5’GCATGTGTGAGTCCTAATTT3’	147	EY893028.1
mt	5’AAAGTGAGTGCTTGCC3’	5’ACTGATGACAGAGTTCCG3’	120	KP770991.1
gst	5′TGGAAGTGGAGGTTTCCTTG3’	5′TGAGGTGGCCAAGTCTTTTC3’	161	EY892760.1
pcs	5′TCATGGTCCTGAACACG3’	5′ CACAAGTTGCCGAAACTC 3′	154	KP770990.1
lys	5′CGGCAACAAACGTCTAC3’	5′GTGAAATACAGGCAGAAGC 3′	163	MG076962.1
sod	5′GGTGCTCACTTCAACCCATT3’	5′AAGATCGTCCACCAGCTCAT3’	167	DQ286712.1

mesocosms. Briefly, four individuals from each group of species: *Ea*, *Ef1*, *Ef2* and *Ha* were placed in the plastic boxes (of 9 cm diameter) with perforated lids. At the end of the exposure period, the earthworms were depurated overnight in boxes with wet lignin substrate (Paso-Trading, Polska), and then sacrificed by freezing and prepared for the metal accumulation analysis.

2.3.2. Experiment 2: gene expression in Cd-exposed earthworms

In total 50 individuals were used as a source of coelomocytes, i.e. the immune cells present in earthworm coelomic cavity, non-invasively retrieved from coelomic cavity by a mild electric shock as described in 2.4.3.1. *E. andrei, E. fetida*, and their *Ea*-derived (*Ha*) and *Ef*-derived hybrids (*Hf*) were exposed for 2 and 7 days to highly polluted soil in mesocosms (boxes with perforated lids, 9 cm diameter). The level of gene expression of glutathione S-transferase (*gst*), lysenin (*lys*), metallothionein (*mt*), superoxide dismutase (*sod*) and phytochelatin synthase (*pcs*) was assessed in extruded coelomocytes, and related to the expression of two stable house-keeping genes – actin (*act*) and ribo13 (*r13*).

2.4. Analyses

2.4.1. Earthworm genotyping

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

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

2.4.2. Cadmium content

Cadmium content in soil and in whole earthworm bodies was measured by slightly modified methods used in our previous studies (Takacs et al., 2016; Rorat et al., 2017). Soil samples were dried at 105 °C for 24 h and then divided into subsamples about 0.5 g of dry soil mass of each. Cadmium concentration in soil was determined after half open wet digestion in a quartz glass with 10 mL of mixture of concentrated HNO_3 (69.0–70.0% to trace metal analysis, Baker Instra-Analyzed) and $HClO_4$ (70% a.r, Chem-Lab) (7:1 v/v), using hot plates. Cadmium concentrations were measured by atomic absorption spectrometry (AAS) with a flame and atomic absorption spectrometry with a graphite furnace nebulizer (Perkin-Elmer) to achieve the desired method sensitivity. Accuracy was verified by analyzing four blanks and three replicates of standard certified material (Loamy Sand 10 CRM033–50G) with the samples.

Prior to the analysis of cadmium concentration in whole earthworm bodies, earthworms were kept for 2 days on wet filter papers to remove their gut content, frozen, and then dried in vacuum drier in 70 °C for 24 h to achieve constant dry mass. For determination of cadmium concentration in earthworm bodies, the wet digestion method was applied but to reach the required ratio 1: 20 only 4 mL of concentrated HNO_3 (69.0–70.0% to trace metal analysis, Baker Instra-Analyzed) were used. Cadmium concentrations in earthworm bodies were measured by atomic absorption spectrometry methods with four blanks and three replicates of standard certified material (Bovine Liver SRM 1577c) with the sample in digestion.

The cadmium concentrations were calculated on a dry mass of each sample of soil or earthworms and are presented in mg kg^{-1} (μg g^{-1}).

2.4.3. Gene expression analysis

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

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

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

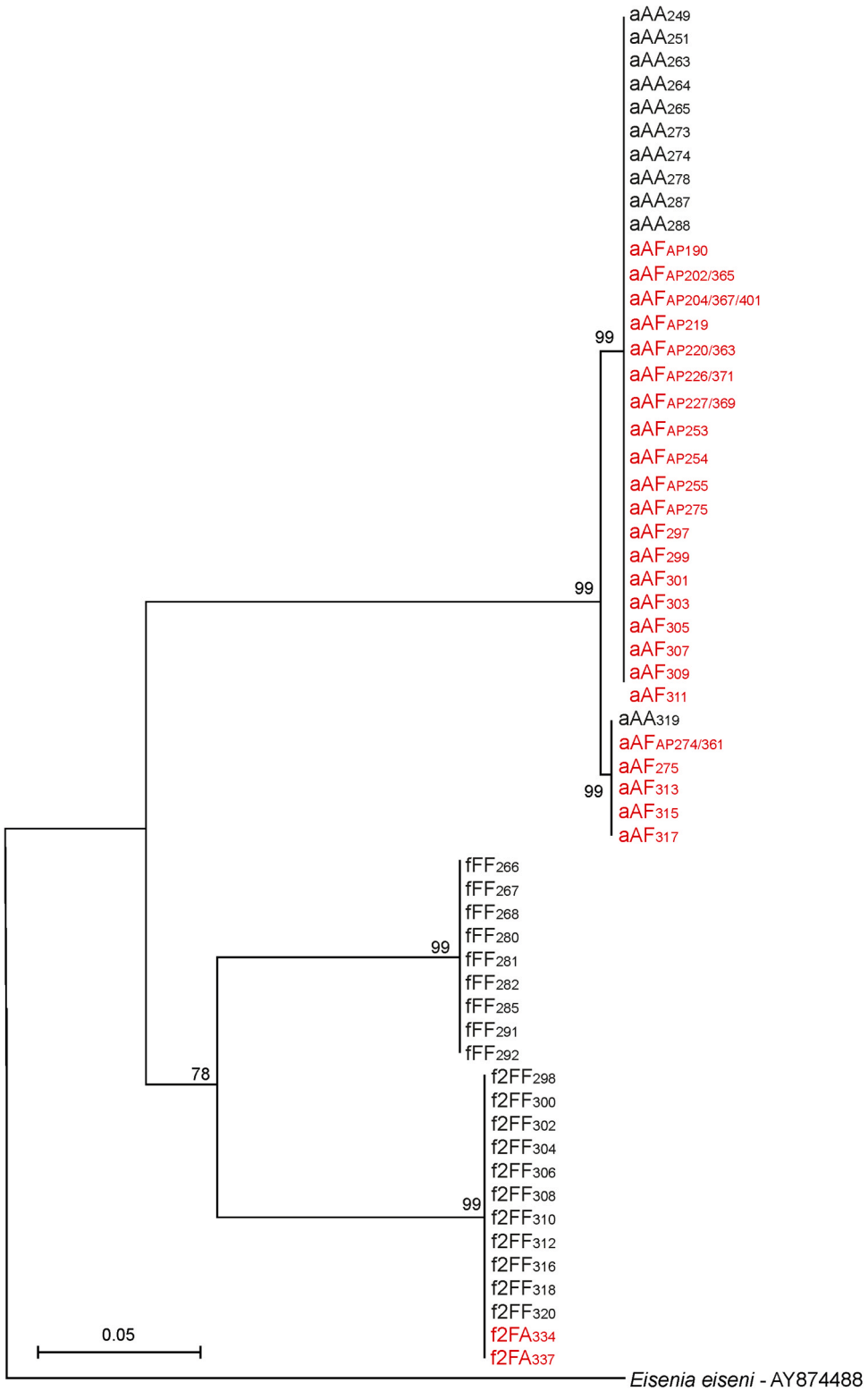


Fig. 1. Maximum-likelihood phylogram for the species-specific sequences 'a' or 'f'/'f2' of the mitochondrial COI gene, with additional information on the 'A' or 'F' nuclear sequences of 28S rRNA nuclear gene of the same individuals with the same code, showing the pure aAA (*Ea*) and fFF/f2FF (*Ef1/Ef2*)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

were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of PCR reaction (Ct). Levels of expression and amplification effectiveness of target genes were compared to the expression of two constitutively expressed and validated house-keeping genes. qPCR efficiency of each target gene was calculated according to the method previously described (Chaâbene et al., 2018). Overall, the relative expression level was calculated according to the following formula:

$$R = 2^{\Delta Ct(targetgene) - \Delta Ct(referencegene)}$$

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

Groups		Cd concentration in soil and earthworm bodies [mg kg^{-1}]		
		L	M	H
Soil		0.11 ± 0.107	425 ± 45.66	835 ± 35.712
Earthworms	*Ea*	1.09 ± 0.38 aA	316.43 ± 33.09 bB	454.19 ± 37.58 BCE
	Ef1	0.76 ± 0.26 aA	203.43 ± 82.7 abB	337.66 ± 115.12 abB
	Ef2	0.69 ± 0.15 aA	113.79 ± 28.16 aB	252.68 ± 62.59 aC
	Ha	1.12 ± 0.10 aA	376.73 ± 136.99 bB	309.23 ± 59.50 abB

2.5. Data analysis

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

3. Results

3.1. Genetic species delimitation

Cytoplasme-rich ova with plenty mitochondria are fertilized by spermatozoa with very scarce cytoplasm and a few mitochondria. DNA barcoding based on specific sequences of mitochondrial haploid COI gene shows three distinct branches; 'a′ for *Ea* or 'f′/'f2′for two mitochondrial lineages of *Ef*, namely *Ef1* and *Ef2* (Plytycz et al., 2020). Species-specific maternal-paternal diploid nuclear sequences of 28S rRNA gene, are either '*AA*' (for *Ea*) or '*FF*' for *Ef* or '*AF*' for interspecific hybrids. Fertilization of '*aA*' ovum by 'A′ spermatozoon gives *aAA* zygote of the pure *Ea* species, while '*aA*' ovum fertilized by 'F′ spermatozoon gives *Ea*-derived *aAF* hybrid. The '*fF*' or '*f2F*' ova fertilized by 'A′ spermatozoa give *fFA* or *f2FA* hybrids, respectively (Fig. 1). All sequences are deposited in GenBank under the following accession numbers: MT646836-MT646899 and MT649303-MT649399. All the earthworms survived the experiment but cadmium exposure inhibited cocoon production almost completely (data not shown).

3.2. Cadmium content in soil and in whole earthworm bodies

The cadmium concentration in the commercial unpolluted soil was very low (L; 0.11 ± 0.,107 mg kg^{-1}); in soil spiked with cadmium chloride was very high (H; 835 ± 35.712 mg kg^{-1} dry weight) and intermediate in 1:1 mixture of these soil samples (M; 425 ± 45.66 mg kg^{-1}dry weight) (Table 2). Cadmium concentrations in earthworms living in unpolluted soil were low (0.69 −1.12 mg kg^{-1}) and did not differ significantly between *Ea*, *Ef1*, *Ef2*, and *Ha* groups of earthworms.

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

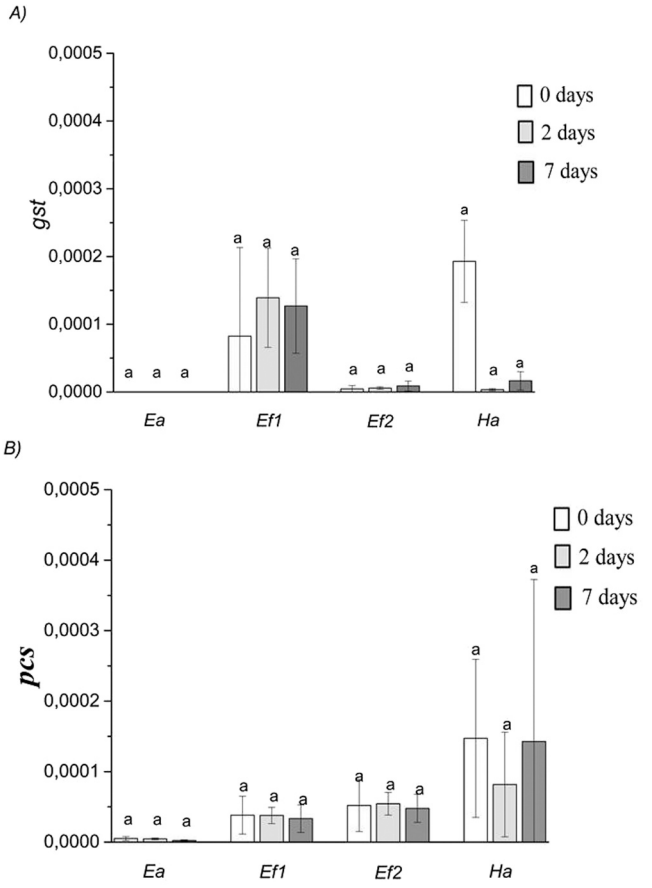


Fig. 2. Relative expression level of *gst* (A) and *pcs* (B) in coelomocytes of groups of earthworms exposed to cadmium polluted soil concentration for 0 days (control), 2 days, or 7 days: Effects of time of exposure. Different letters "a", "b" "c", "d" on top of bars indicate statistically significant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

3.3. Gene expression in earthworms

3.3.1. Reference genes

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

3.3.2. Expression of gst and pcs genes

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

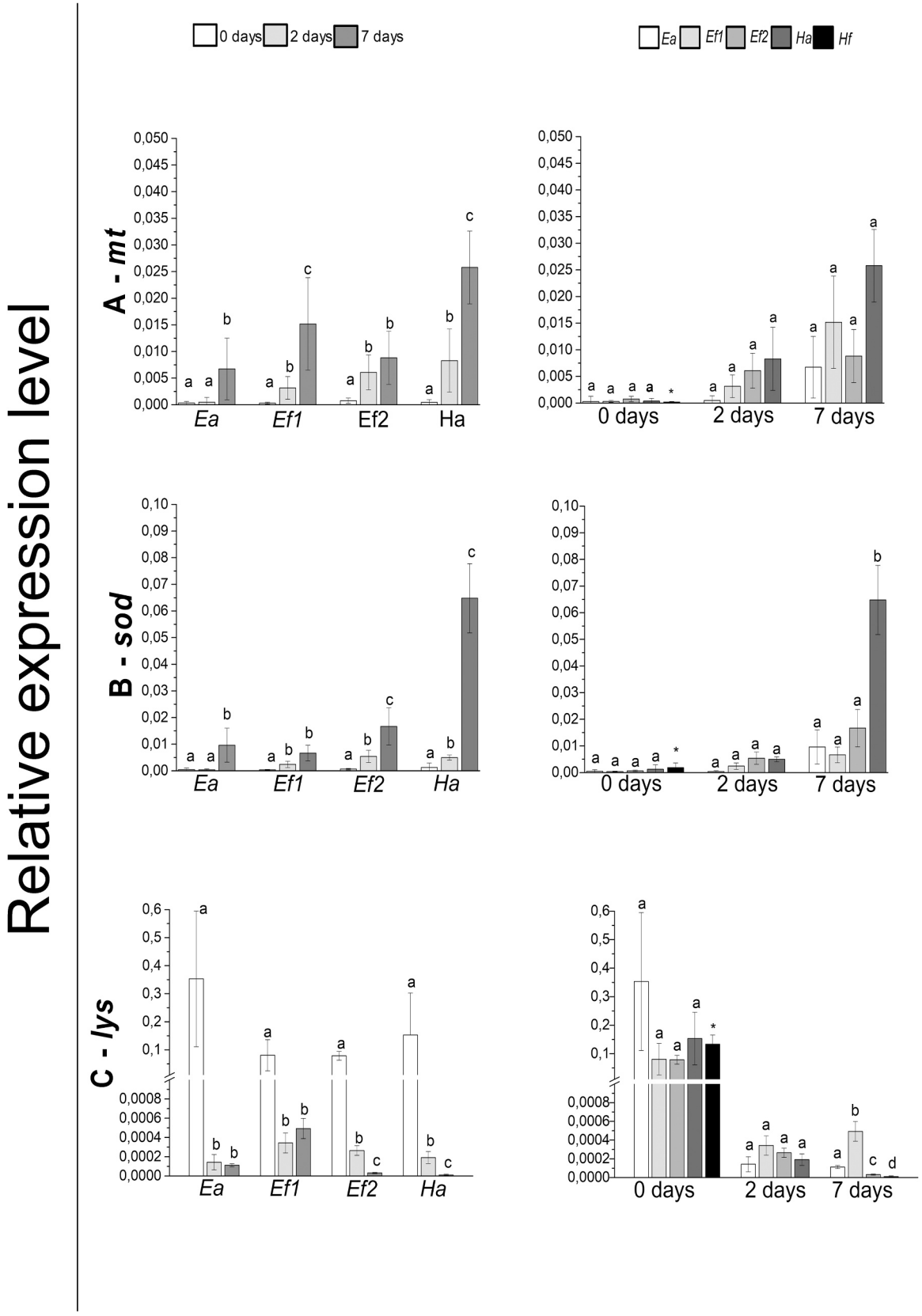


Fig. 3. Relative expression level of *mt* (row A), *sod* (row B) and *lys* (row C) in coelomocytes of groups of earthworms exposed to cadmium polluted soil concentration for 0 days (control), 2 days, or 7 days; Left column - effects of time of exposure; right column interspecies comparisons between *Ea,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

3.3.3. Expression of mt, sod, and lys genes

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

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

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

3.3.3.2. Expression of sod. Similar results were observed for the expression rate of *sod* (Fig. 3, row B). Once again, the expression rate for all organisms living in clean soil was almost undetectable while the exposure to cadmium caused a significant increase in *sod* expression. However, two days post-exposure, the expression rate of *sod* in *E. andrei* was still almost no detectable, whereas for all other organisms was already significantly ($p < 0.05$) induced. At day 7 day after cadmium exposure, expression of *sod* significantly increased versus day 2 in *Ea*, *Ef2*, and *Ha* (Fig. 3, row B, left column). The interspecies differences were insignificant on day 0 and day 2, while on day 7 was significantly higher in *Ha* than in *Ea*, *Ef1*, and *Ef2* (Fig. 3, row B, right column).

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

4. Discussion

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

4.1. Expression of genes for metallothionein and phytochelatin synthase

In a study by Brulle et al. (2008) performed on *E. fetida*, the induction pattern of genes encoding phytochelatin synthase was significantly increased in earthworms exposed to a low concentration of cadmium (8 mg kg^{-1}). However, in higher concentrations, no difference was noticed between Cd-exposed earthworms and earthworms not exposed to any contamination, which suggests that *pcs* expression is only induced during low exposure to cadmium. Similarly to our study, expression of metallothionein was highly up-regulated after Cd exposure, and in the mentioned Brulle et al. (2008) study, it was also shown to be dose-dependent in high Cd concentrations. Such relation between different responses of the expression of *pcs* and *mt* genes is of interest when considering their role in metal detoxification in animals. In low concentrations of Cd, the expression of *pcs* was shown to be significant and responsible for metal chelation in worm cells (probably via the synthesis of Phytochelatins (PCs) although the presence of PCs has not been demonstrated), whereas such induction was only noticed at a small exposure level. This is on the contrary to the effects of high concentrations of Cd, where similarly to our study, *pcs* expression patterns did not show any change of their level over control animals, whereas the expression rate of *mt* was substantially upregulated. In another study, focused on *Caenorhabditis elegans*, the toxicogenomic analysis after exposure to cadmium also showed no effect on the expression of *pcs-1* gene (Cui et al., 2007). Moreover, a recent transcriptomic study on the same species: *C. elegans*, found similar results – after exposure of nematods to cadmium (300 μM) and zinc (500 μM), the expression change of *pcs-1* was determined via qPCR followed by normalization by the invariant ribosomal *rla-1*. The results showed, that the *pcs* response to Zn and Cd contamination was at best negligible, and the change in comparison to control animals was not significant ($p < 0.05$) (Essig et al., 2016). By combining these studies, we can see that phytochelatins and metallothioneins possibly act together to protect the cells against Cd. Phytochelatins have more of an immediate role at lower concentrations and metallothioneins coming later with higher induction. In addition, a comparison between the chosen gene expression profiles in earthworms exposed or unexposed to Cd showed up-regulation of two genes for all tested earthworms after Cd exposure – metallothionein and superoxide dismutase as well as down-regulation of lysenin. Such alterations between the expressions of those genes suggest that earthworms can oppose and reduce the toxic effects of Cd through several different pathways. Moreover, in the presented study, we noticed that the *mt* overexpression due to Cd exposure occurs not only in *E. fetida* but also in *E. andrei* and hybrids between those species, which suggest, that this biomarker is suitable for both species. However, results suggest that *E. andrei* (*aAA*) activate its detoxification mechanisms slower than *E. fetida* (*fFF*) and their hybrids (*aAF*). Moreover, fast up regulation of expression of *mt* genes in hybrids suggests, that they obtained the nuclear allele responsible for a fast reaction from *E. fetida,* which showed similar tendencies, and not from *E. andrei*. In addition, such results could indicate that perhaps, *Ef* earthworms possess another detoxification mechanism or a specific regulation mechanism that is responsible for faster *mt* expression in comparison to *Ea*. Thus, by cloning of the genes coding *mt* in both species and studying of the regulation of the gene is needed to broaden our understanding of such response to metal exposure.

Similar to our study, the induction of overexpression of the *mt* gene was recently shown in *E. andrei* earthworms after exposure to copper, which is a widely used fungicide, especially in organic farming (Mincarelli et al., 2019). Moreover, an induction of *mt* expression was also

noticed in other earthworms including *Dendrobaena octaedra* and *Lumbricus rubellus* exposed to Cu (Mustonen et al., 2014; Höckner et al., 2015). The population of *Dendrobaena octaedra* exposed to Cu-Ni contaminated soil was shown to have higher *mt* expression levels in comparison to earthworms not exposed to metals. What is even more interesting, during artificial exposure experiment, exposure affected the *mt* expression, but only in the earthworms collected from the clean, unpolluted soil, showing that there is a delay in the *mt* response of earthworms which were not exposed to metals in the past. On the contrary, earthworms collected from the Cu-Ni smelting site showed not only higher but also constant levels of *mt* expression (Mustonen et al., 2014). Such results reflect on the role of the *mt* expression and the homeostasis of several metals and protection against oxidant damage.

4.2. Superoxide dismutase and glutathione S-transferase

Glutathione S-transferase (GST) is an enzyme known to take part in the metabolism of lipophilic organic contaminants and in cellular protection against oxidative stress. A study by Mkhinini et al. (2019) showed an induction of *gst* expression after the exposure of *E. andrei* to treated wastewater (TWW) containing several trace metals. However, such effects were observed in earthworms exposed to TWW for more than 20 days. Our study showed no effect of cadmium exposure to the expression of this gene in a 7-day period, which could suggest that the *gst* mechanism is induced after prolonged, chronic exposure to heavy metals, but it is not an immediate response to the presence of contamination. In another study, the antioxidant gene expression of *E. fetida* was explored after worm exposure to various concentrations of hexabromocyclododecane. The results showed a significant up-regulation of the *sod* gene but no significant differences in the expression rate of *gst*, which further suggests its involvement in protection against the oxidative damage occurs during a prolonged period of time (Shi et al., 2018). Moreover, a recent study of Maity et al. (2018) focused on the oxidative stress response of *E. fetida* and *Eutyphoeus waltoni* exposed to Cd, the overexpression of the *gst* gene increased significantly post-exposure and was dose-dependent. However, in *E. fetida*, after 2 days post-exposure, the expression of *gst* remained the same as in control animals, which also suggest its late-onset. In a transcriptomic study by Chai et al. (2019), focused on cadmium toxicity in *E. fetida*, a significant up-regulation od *sod* genes was also observed in earthworms exposed to 10, 30, 60 mg kg^{-1} of Cd^{2+} in dry soil, respectively.

4.3. Lysenin

Lysenin is a protein involved in immunity processes produced by coelomocytes and chloragocytes (Munguira et al., 2017). The presence of lysenin and lysenin-related proteins was documented in *E. andrei* and *E. fetida* and their putative hybrids (Swiderska et al., 2016). In the present study, the expression rate of *lys* gene was significantly down-regulated post Cd exposure in all tested animals (*Ea, Ef, Ha*). A similar decrease in *lys* expression was noticed by Rorat et al. (2017) in *E. andrei* and *Brulle* et al. (2011) in *E. fetida*. However, the mechanisms behind such response are still unknown. In a study by Wang et al. (2010) the expression of *lys* was also shown to diminish after earthworms were exposed to *E. coli*. Besides, Wu et al. (2013), discovered that lysenin-related protein was significantly up-regulated post-exposure to PAH phenanthrene. Hence, lysenin expression in earthworms can have a complex response to exposure to different contaminants. Overall, the results imply the existence of a complex regulatory network that needs to be further explored in future studies.

4.4. Hybrids response to environmental stressors

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

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

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

5. Conclusions

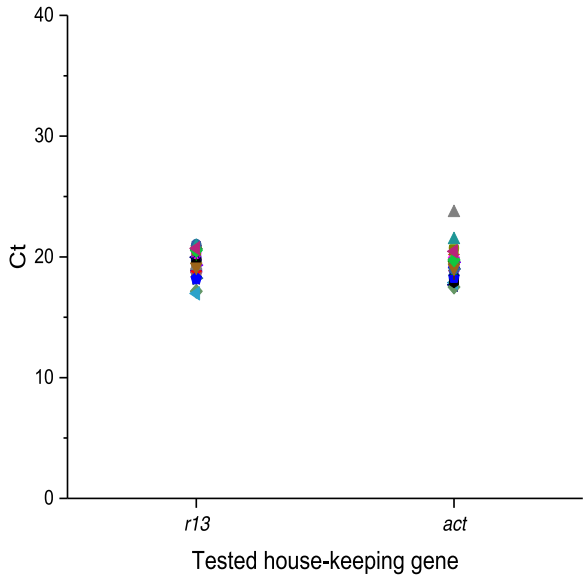
The insights gained in the presented study have improved our understanding of metal resistance of *E. fetida*, *E. andrei* and their hybrids. The results indicate that all of them are very efficient cadmium bioaccumulators with similar mechanisms of Cd detoxication. Consistently, upregulation of *mt* and *sod* expression was fastest in hybrids, slower in *Ef* and slowest in *Ea*. Hypothetically *Ef* and *Ea* differ in their mechanisms controlling expression of stress-related genes and immunity-connected genes thus further studies of these genes and their interactions in hybrids are necessary.

CRediT authorship contribution statement

Marta Jaskulak: Writing - original draft, Visualization , Methodology, Investigation **Agnieszka Rorat**: Writing - original draft, Visualization , Methodology, Investigation. **Ligia Kurianska-Piatek**: Investigation. **Sebastian Hofman**: Investigation, Conceptualization, **Janusz Bigaj**: Investigation. **Franck Vandenbulcke**: Writing - review & editing, Supervision. **Barbara Plytycz**: Conceptualization, Investigation, Methodology, Writing - review & editing, Writing - original draft, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial



Appendix Figure 1. Validated reference genes - Expression rate of two tested HKG: actin (*act*) and ribo13 (*r13*) All results are expressed as means, n = 50.

interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Investigations are financially supported by the National Centre of Science (Poland) (2016/23/B/NZ8/00748) and by the Jagiellonian University (Poland) (K/ZDS/005405).

Appendix A

Appendix Fig. A1.

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