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Endogeic Earthworm *Aporrectodea Caliginosa* as a Model Species for Studies of Modulation of Regeneration by Environmental Stressors

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Earthworms are commonly exposed to mechanical or chemical stimuli inducing body movements connected with expulsion of coelomocyte-containing coelomic fluid and/or the loss of body segments, thus the efficient regenerative processes are important for them. On the other hand, earthworms are commonly exposed to sudden changes of temperature or various kinds of soil pollutants. The aim of present studies was to test effects of thermal conditions and cadmium soil pollution on restoration of experimentally depleted coelomocytes or regeneration of amputated posterior segments in the adult individuals of endogeic earthworms *Aporrectodea caliginosa*. Coelomocyte depletion was performed at the start of experiments by electrostimulation-induced extrusion of coelomic fluid. Electrostimulated experimental worms and those with surgically amputated posterior segments as well as their untreated control counterparts were then maintained at various temperatures in the clean or cadmium-polluted soils. Four weeks after extrusion, numbers of coelomocytes in experimental worms were still significantly lower than those in their control counterparts kept in the same conditions, but restoration was more efficient in worms additionally stressed by transfer to higher temperature or exposure to Cd-polluted soil. Amputation of 10 posterior segments resulted in compensatory increase of body weights dependent on thermal conditions and lack of blastema formation. In contrast, regeneration blastema was slowly formed after amputation of 40 posterior segments in clean soil and in cadmium-polluted soil, and was detectable only 4-6 weeks after surgery. In conclusion, *A. caliginosa* is an interesting model species for comparative studies of mechanisms of effects of stress on regenerative processes in lumbricid worms.

Key words: earthworms, thermal stress, amoebocyte restoration, segment regeneration, cadmium accumulation

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INTRODUCTION

The annelids are an important group for investigations of evolution of regeneration abilities, as among them there are species incapable of any regeneration (e.g. leeches), and also species capable of regeneration an entire individual from a single mid-body segment. Polychaeta and Oligocheta species exhibit qualitative and quantitative variations in regeneration abilities, and their segmental body organization make interspecies comparisons relatively straightforward. The ability to regenerate posteriorly is almost universal, but anteriorly is less widespread (Bely et al., 2006; Bely and Sikes, 2010; Zoran, 2010).

Lumbricid species, including *Eisenia fetida* and *Lumbricus terrestris* posses marked capacities for regeneration of both posterior and anterior segments following surgical amputation, described already at the beginning of 20th century (for a review see Bely, 2006). We have shown recently that *Dendrobaena veneta* can regenerate amputated anterior body segments and also surgically extirpated cerebral ganglia ('brains') (Okrzesik et al., 2013). Regeneration of segments is crucial for earthworms as they are commonly subjected to sub-lethal predator attacks leading to loss of body parts.

Lumbricid earthworms also regenerate segments lost in response to noxious stimulation by self-fragmentation called autotomy (Zoran, 2010). The latter phenomenon may be connected with the microbial overload of coelomic cavity; microbes are encapsulated within multilayer "brown bodies" moving towards posterior segments of the body which became necrotic and undergo autotomy, followed by segments regeneration in favorable condition (Valembois et al., 1992, 1994; Wieczorek-Olchawa et al., 2003).

Earthworms may be subjected to other mechanical/chemical stimuli inducing spasmic body movements connected with expulsion of coelomic fluid containing cells, the coelomocytes, and soluble factors crucial for immunity, thus the regeneration of immune system is necessary. In some species (e.g. *Lumbricus* sp., *Aporrectodea* sp.) the coelomocytes consist of almost exclusively amoebocytes while in other species (e.g. *Eisenia* sp., *Dendrobaena* sp., *Allolobophora* sp.) consist of both amoebocytes and species-specific numbers of autofluorescent riboflavin-containing chloragocyte-derived eleocytes (Cholewa et al., 2006; Plytycz and Morgan, 2011). Depletion of coelomocytes is followed by the gradual restoration of their number and composition (Eyambe et al., 1991; Wieczorek-Olchawa et al., 2003; Klimek et al., 2012; Santocki et al., 2015).

On the other hand, earthworms are subjected to annual cyclic changes (Kurek and Plytycz, 2003) and various environmental stressors like changes of ambient temperature (Cygal et al., 2007), soil pollution with heavy metals (e.g. Homa et al., 2003, 2007; Kwadrans et al., 2008; Plytycz et al., 2009, 2010, 2011a,b,c; Piotrowska et al., 2010; Podolak et al., 2011) or sewage sludge (Rorat et al., 2013), or nutritional status of animals (Polanek et al., 2011), and all these factors can modulate their immune system and regeneration capabilities.

The endogeic species, *Aporrectodea caliginosa*, common in forests, agriculture and gardens, through its burrowing and feeding activity is considered as a field-relevant species (Edwards and Bohlen, 1996). The aim of present studies was to find out if this species may be used as a convenient model for analyses of effects of thermal conditions or cadmium soil pollution on restoration of experimentally depleted coelomocytes and regeneration of amputated posterior segments.

MATERIALS AND METHODS

Earthworms

Adult earthworms *Aporrectodea caliginosa* (Oligochaeta; Lumbricidae) were sampled in the field in Klęczany (Southern Poland) and kept at the Department of Evolutionary Immunobiology, Institute of Zoology of the Jagiellonian University (Cracow, Poland). Earthworms were kept in plastic boxes with commercial soil (PPUH BIOVITA, Tenczynek) either at 8°C or 17°C, and fed *ad libitum* a mixed diet comprised of dried/boiled nettle (*Urtica dioica*) and dandelion (*Taraxacum officinale*) leaves, boiled/dried tea leaves, and powdered commercial mouse pellets.

Soil samples

Soil samples were prepared as described previously (Dutkiewicz et al., 2009). Air-dried, metal-free soil was spiked either with distilled water (con-

trol) or with cadmium chloride ($CdCl_2 * 2.5\ H_2O$; Sigma Aldrich, USA) at the nominal concentration of Cd equal 500 mg/kg.

Coelomocyte extrusion and counting

Coelomocyte extrusion was performed as described previously (DUTKIEWICZ et al., 2009). The weighed earthworms were individually placed in Petri dishes containing 3 ml of extrusion fluid (phosphate-buffered saline, PBS, supplemented with 2.5 g/l ethylenediamine tetra-acetic acid, EDTA; Sigma-Aldrich) and stimulated for 30 seconds with an electrical current from a battery (4.5V) to expel coelomic fluid with suspended coelomocytes through the dorsal pores. The coelomocyte suspensions extruded at the start and the end of experiments were used for cell counts in haemocytometer.

Posterior segments amputation

The weighed earthworms were anesthetized in carbonated drinking water by Soda Siphon (ISI, Stalgast, Warszawa, Poland) and 10 or 40 posterior segments were amputated with scalpel. Earthworms were weighed before and after amputation and then at weekly intervals.

Cadmium concentrations

Cadmium accumulation in whole earthworm bodies. Earthworms were left on wet filter paper in Petri dishes for 48 hours to depurate. Total cadmium concentrations in earthworms were determined after wet digestion of each individual separately in 5 ml suprapure concentrated HNO_3 and $HClO_4$ (7:1 v/v) (Sigma-Aldrich). Earthworms were prepared to digestion by liofilisation (freeze-drying) overnight and subsequent drying in laboratory dryer (50°C) to constant mass. Cadmium concentrations were measured by atomic absorption spectrometry (AAS) with a flame or graphite furnace nebuliser (Perkin-Elmer). Accuracy was verified by analysing four blanks and four replicates of standard certified material (Bovine liver, BRC®-185r, Sigma-Aldrich) with the samples.

Cadmium concentration in soil samples. Total cadmium concentrations in soil were determined after wet digestion of each soil sample in 10 ml suprapure concentrated HNO_3 and $HClO_4$ (7:1 v/v) (Sigma-Aldrich). Before digestion, soils were dried overnight in laboratory dryer (105°C) to constant mass. Cadmium concentrations were measured by atomic absorption spectrometry (AAS) with a flame or graphite furnace nebuliser (Perkin-Elmer). Accuracy was verified by analysing four blanks and four replicates of standard certified material (CRM025-050, Sandy Loam 8, RT Corp.) with the samples.

EXPERIMENTAL SCHEMES

Restoration of depleted coelomocyte

Effects of thermal conditions on coelomocyte restoration. In total 48 worms of similar body weights (X±SD=0.45±0.026 g) were selected, 24 from them kept at 8°C and 24 from them kept at 17°C. At each temperature, 12 worms were left untreated (controls – C), and 12 were subjected to electrostimulation-induced coelomocyte depletion (experimental electrostimulated – E). They were transferred to boxes with new soil, 6 worms per box. One series of boxes (C, E) was left in the original temperature (either 8°C or 17°C), while the second series (C, E) was transferred from 8°C to 25°C (8-25°C), or from 17°C to 8°C (17-8°C). After 4 weeks coelomocytes of all worms were extruded and counted. Comparisons were performed between coelomocytes from C and E worms within each thermal regime.

Effects of cadmium soil pollution on coelomocyte restoration. In total 24 worms of similar body weights (X±SD=0.42±0.046 g) were selected from those kept at 17°C. Half of them (12 worms) were left untreated (C, control worms) and remaining 12 worms were subjected to electrostimulation-induced coelomocyte depletion (E, electrostimulated experimental worms). The worms were placed into boxes with the fresh clean soil (c) or cadmium-polluted soil (p), 6 worms in each box, forming Cc, Ec, Cp, Ep groups. Coelomocytes of all worms were extruded 4 weeks after start of experiments and the worm bodies were used for analyses of Cd accumulation in worms from clean and polluted soil samples.

Regeneration of amputated posterior segments

Effects of thermal condition on worms with amputated 10 posterior segments. In total 24 worms of similar body weights (X±SD =0.42±0.051 g) were selected from those kept at 17°C. Haft of them (12 worms) were left untreated (control – C) while remaining 12 worms were subjected to amputation of 10 posterior segments (A – amputated). Worms were transferred to 4 boxes with a new soil, 6 worms per box. One series was transferred to 8°C (groups C17-8°C, A17-8°C), while the second series was transferred to 22°C (C17-22°C, A17-22°C). All earthworms were weighed and observed for regeneration blastema formation during 8 consecutive weeks.

Regeneration of 40 posterior segments and effects of cadmium soil pollution. In total 24 worms of similar body weights (X±SD =0.42±0.062 g) were selected from those kept at 17°C and transferred to 22°C. Twelve worms were left untreated (controls – C), and 12 were subjected to amputation of 40 posterior body segments and weighed again (A – amputated). Worms were transferred to boxes with new soil, 6 worms per box. One series was left in the fresh unpolluted soil (clean, c) forming Cc and Ac groups, while the second series was transferred to the cadmium-polluted (p) soil forming Cp and Ap groups. All earthworms were weighed and observed for regeneration blastema formation during 8 consecutive weeks.

Statistical analysis

Recorded parameter values for earthworm body weights and their coelomocytes were expressed both as direct means and/or as percentages of the values for the appropriate control groups. Data were expressed as X±SE and analysed Mann-Whitney's U test or by ANOVA with post hoc Tukey's test; $P<0.05$ was established as the level of significance.

RESULTS

Cadmium concentration in soil and accumulation in whole earthworm bodies

After 4-week exposure to the commercial clean or cadmium polluted soil, cadmium accumulation in *A. caliginosa* whole bodies was 10 mg/kg d.w. and 475 mg/g d.w., respectively, while Cd concentration in dried soil was 0.225 mg/kg d.w. in the clean soil, and 968 mg/kg d.w. of soil soaked with cadmium at nominal concentration equal 500 mg/kg (Table 1). This apparent discrepancy may be caused by addition of cadmium chloride to air-dried commercial soil with high organic matter content.

Coelomocyte suspensions

Coelomocyte suspensions extruded at the start and at the end of experiments contained almost exclusively the amoebocytes.

Effects of thermal condition on restoration of depleted amoebocytes

Effects of thermal conditions on the rate of restoration of coelomocytes after experimental depletion are illustrated on Fig. 1. Four weeks after electrostimulation the numbers of amoebocytes

TABLE 1. Cadmium concentration at the end of 4-week experiments in soil samples either unpolluted (control) or Cd-polluted with cadmium chloride at nominal concentration 500 mg/kg^{-1} air-dried soil, and cadmium accumulation in the whole bodies of earthworms *Aporrectodea caliginosa* maintained 4 weeks either in control soil or Cd-polluted soil

		Cd concentrations/accumulations mg/kg means±SD (n=4–6)
soil	Control	0.225±0.0188
	Cd-polluted	968±128.6
earthworms	in control soil	10±7.2
	in Cd-polluted soil	475±193.8

in experimental groups (E) were still lower than those in their counterparts living in the same thermal conditions (C groups), but the difference between the E and C group was insignificant in worms transferred from 8°C to 25°C (88% versus 100%). In worms kept constantly at 8°C or 17°C, or shocked by transfer from 17°C to 8°C, the percentages of amoebocytes in worms rebuilding amoebocyte systems were equal only 42%, 49%, and 61% of respective controls, being significantly below values in the groups of untreated worms from the same thermal conditions (Fig. 1).

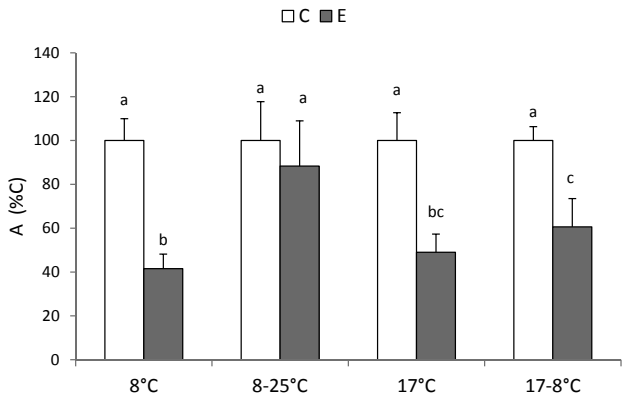

Fig. 1. Effects of thermal conditions on restoration of amoebocytes in *Aporrectodea caliginosa* during 4 weeks after electrostimulation-induced depletion of amoebocyte-containing coelomic fluid. Numbers of amoebocytes at the end of experiments in electrostimulated groups (E) are expressed as percentages of amoebocyte numbers in the respective untreated control worms (C, considered as 100%) kept at the same thermal regimes. Groups of E and C worms were kept either at constant temperatures (8°C or 17°) or transferred at the start of experiments to higher or lower temperatures (8-25°C or 17-8°C). Results expressed as means ± SE (n=6). Various letters at means significantly different (P<0.05).

Effects of cadmium accumulation in worms on restoration of depleted coelomocytes

At 17°C, numbers of amoebocytes in worms electrostimulated at the start of experiments and kept for 4 weeks either in clean or Cd-polluted soil were equal 39% and 70%, respectively, of those retrieved from the untreated worms kept at the same conditions (Fig. 2), the differences being statistically significant. Thus restoration of depleted amoebocytes was more advanced in Cd-polluted soil than in the unpolluted soil sample.

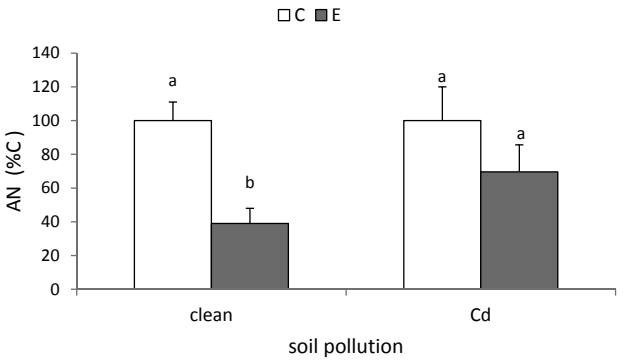

Fig. 2. Effects of cadmium accumulation in worm bodies on numbers of amoebocytes in *Aporrectodea caliginosa* either intact (C) or after electrostimulation-induced depletion of amoebocyte-containing coelomic fluid (E). Numbers of amoebocytes in the E groups are expressed as percentages of those in the respective C groups (considered as 100%). Means ± SE (n=6). Various letters at means statistically significantly different (P<0.05).

Effects of thermal conditions on worms with amputated 10 posterior segments

Worms from A groups with amputated 10 posterior segments lost appr. 15% of their initial body weight and that of untreated controls. Both the untreated worms and those with amputated 10 posterior segments were transferred from 17°C to lower or higher temperature (8°C or 22°C) and kept there for 8 weeks (Fig. 3). At 22°C the control worms gradually increased body weights till 160% of the initial value, and their counterparts with 10 amputated segments reached body weights similar to those in untreated worms at the same temperature already 3 weeks after amputation. In worms transferred to 8°C, the body weights were gradually decreased in the untreated worms reaching 80% of initial value at the end of experiments. Body weights of their counterparts with amputated 10 segments kept in cold slightly increased and reached the body weights of their controls at 5-6 weeks after amputation. Then body weights of E17-6°C worms were even higher than those of C17-8°C worms. Regeneration blastemas were absent in the A worms in both thermal regimes during the whole observation periods (Fig. 3).

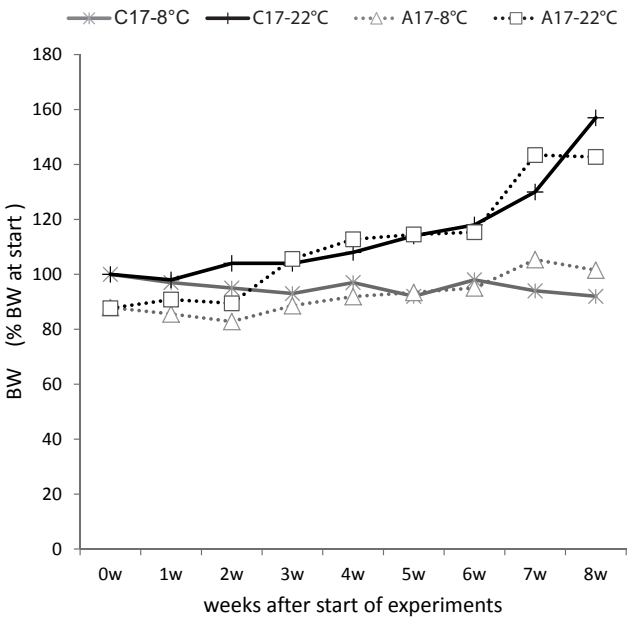

Fig. 3. Effects of thermal conditions on body weights in *Aporrectodea caliginosa* worms either untreated (C groups) or with amputated 10 posterior segments (A groups). At the start of experiments worms were transferred from 17°C either to 8°C or 22°C, thus four groups were created, 6 worms each (C17-8°C, A17-8°C, C17-22°C, A17-22°C). Changes of body weights (BW) are expressed as percentages of the initial body weights at the start of experiments. Means from 6 worms in each group. Open symbols at means in the A groups (squares and triangles) indicate absence of regeneration blastemas.

Fates of worms with amputated 40 posterior segments and effects of cadmium-polluted soil

Amputation of the 40 posterior segments caused lost of appr. 38% of the initial body weights (Fig. 4). In the clean soil, the body weights of the untreated worms increased and reached 162% of the initial value after 8 weeks, while body weights of worms with amputated posterior segments were relatively stable, fluctuating around 62% of the initial values but since the 4th week after amputation the regeneration blastemas were formed (insert in Fig. 4).

In the cadmium polluted soil, body weighs of the control worms were stable during the 8 weeks, while in worms with amputated 40 posterior segments they gradually increased from appr. 62% till 81% of the initial body weights during the 8 postoperative weeks. Moreover, since the 6th postoperative week regeneration blastemas were formed (Fig. 4).

DISCUSSION

The results of present experiments fully confirmed that coelomic fluid of *A. caliginosa* contains only one distinct cohort of freely floating coelomocytes, i.e. the amoebocytes (Cholewa et al., 2006; Dutkiewicz et al., 2009), being functional equivalents of vertebrate macrophages (Ottaviani, 2011), playing a prominent role in earthworm immunity (Bilej et al., 2011). It turned out that in this species the content of amoebocytes after experimental depletion is restored very slowly, as four weeks after electrostimulations their numbers were still below those in the untreated counterparts both at the low and high temperature. However, restoration was faster in worms transferred from cold to warmth, and also from clean to cadmium-polluted soil. Amoebocytes derive from the mesenchymal lining of the coelom and are mitotically active also after detachement (Parry, 1975; Homa et al., 2008). Perhaps transfer to higher temperature or to cadmium-polluted soil induced massive proliferation and/or changes in composition of soil microbiota and, in turn, proliferation of earthworm amoebocytes involved in earthworm immunity.

Restoration of experimentally depleted amoebocytes was described previously in *Lumbricus terrestris* as a long-lasting process (Eyambe et al., 1991). Then it turned out that this process was much more complex in *Dendrobaena veneta* (Klimek et al., 2012), *Eisenia andrei* and *E. fetida* (Santocki et al., 2015). Coelomic fluid of *D. veneta* and *Eisenia sp.* contains two distinct cohorts of coelomocytes, i.e. amoebocytes and chloragocyte-derived granular eleocytes detached from chloragogenous tissue. Amoebocyte counts in *D. veneta* and *Eisenia* sp. returned to the control level much faster than eleocytes of the same individuals (Klimek et al., 2012; Santocki et al., 2015), and faster than amoebocytes in *L. terrestris* (Eyambe et al., 1991) and in *A. caliginosa* from present experiments. Putatively restoration of amoebocytes may be augmented by eleocyte-derived riboflavin stored in species-specific amounts in granules of eleocytes (Plytycz et al., 2006; Plytycz and Morgan, 2011). Riboflavin (vitamin B2) plays an important role in immunity (Powers, 2003), including regeneration (Samuel et al., 2012; Plytycz and Morgan, 2015). Riboflavin can act as a potent chemoattractant for immune cells (Mazur

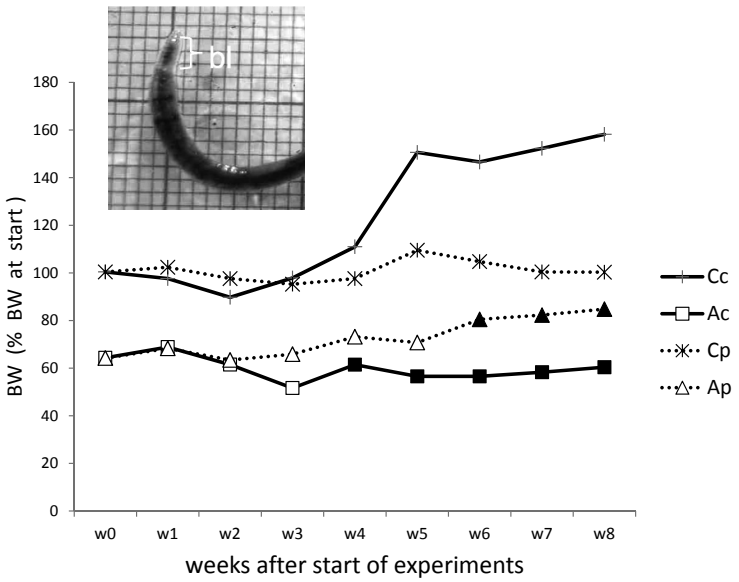

Fig. 4. Effects of cadmium soil pollution on body weights of *Aporrectodea caliginosa* worms either untreated (C groups) or with amputated 40 posterior segments (A groups). At the start of experiments worms kept at 22°C were transferred either to the fresh unpolluted soil (Cc, Ac groups) or to cadmium polluted soil (Cp, Ap groups). Changes of body weights are expressed as percentages of initial body weights at the start of experiments. Means from 6 worms in each group. Open symbols in the A groups (squares and triangles) indicate absence of regeneration blastemas, while solid symbols (squares and triangles) indicate the presence of regeneration blastemas. Insert: regeneration blastema (bl) in the representative worm from Ac group, 6 weeks after start of experiments.

et al., 2011) putatively facilitating the formation of the multicellular brown bodies encapsulating parasites (Wieczorek-Olchawa et al., 2003), and augments regeneration of amputated earthworm body segments as evidenced by the blocking the inhibitory effects of the antibiotics on blastema formation (Samuel et al., 2012).

In *A. caliginosa* from present experiments, blastema was formed slowly after amputation of 40 posterior segments as it become visible only 4 and 6 weeks after surgery in the clean or cadmium-polluted soil, respectively, while loss of only 10 segments was followed by the compensatory body weight increase without blastemas detectable during 8 postoperative weeks. This is in a sharp contrast to an efficient blastema formation in *Eisenia andrei*, which occurs quickly after amputation either low or high numbers of segments (in preparation). Putatively this discrepancy is connected with the lack of riboflavin-storing eleocytes in the former species and their abundance in the latter (Rorat et al., 2014; Santocki et al., 2015).

In the present experiments, exposure of *A. caliginosa* to cadmium-polluted soil was performed according to the same protocol as that used previously (Dutkiewicz et al., 2009). Cadmium accumulation in worm bodies was similar to the previous one, and caused inhibition of body weight gain without profound effects on coelomocytes (Dutkiewicz et al., 2009). However, cadmium accumulation in worm bodies from present experiments induced faster restoration of depleted coelomocytes and an increase of body weights of worms with 40 amputated segments in comparison with respective groups of worms kept in a clean soil. Perhaps induction of expression of stress proteins, like metalloproteins and HSPs (Homa et al., 2005, 2010, 2015) is responsible for enhancement of various vital processes, including regeneration.

In conclusion, restoration of experimentally depleted amoebocytes in *A. caliginosa* is relatively slow. Experimental amputation of a few posterior segments is followed by a compensatory increase of body weight, while amputation of massive parts

of posterior segments induces regeneration blastema formation. Both processes are in a sharp contrast to those in *E. andrei*. Worms from the latter species rapidly restore depleted amoebocytes (Santocki et al., 2015; Plytycz et al., in preparation) and are able to a fast formation of regeneration blastemas in a case of the loss of both small and large parts of the body (in preparation). In the both species regenerative processes are modified by environmental stressors. The interspecies differences are putatively connected with the presence of large amounts of riboflavin in eleocytes freely floating in coelomic fluid in *E. andrei*, that are absent in *A. caliginosa*. This hypothesis is worth of experimental verification.

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