

MINIREVIEW

Unexpected results and open questions from experiments on regeneration in lumbricid worms**B Plytycz¹, J Bigaj¹, A Falniowski², AJ Morgan³**¹*Department of Evolutionary Immunology, Institute of Zoology, Jagiellonian University, Krakow, Poland*²*Department of Malacology, Institute of Zoology, Jagiellonian University, Krakow, Poland*³*Cardiff School of Biosciences, Main Building, Cardiff University, Cardiff CF10 3US, Wales, UK**Accepted September 20, 2016***Abstract**

Lumbricid worms are commonly subjected to noxious stimuli leading to expulsion of celomic fluid or to loss of body segments; therefore regeneration of lost segments and restoration of the depleted cellular and soluble components of celomic fluid are of fundamental importance for these animals. Series of experiments was performed on regeneration abilities in well-defined epigeic species *Eisenia andrei*, *E. fetida* and *Dendrobaena veneta*, and endogeic *Aporrectodea caliginosa*. Efficient regeneration of the lost anterior or posterior segments was consistently observed in *Eisenia* sp. In a sharp contrast, *D. veneta* regenerated amputated anterior segments or extirpated suprapharyngeal ganglia ('brains') while regeneration of posterior segments was never recorded so far in this species. In *A. caliginosa* a loss of posterior segments was followed either by compensatory body growth or by formation of regeneration blastema. In all species regeneration was cold-inhibited while was resistant to cadmium soil pollution. The efficiency of regenerative processes in *E. andrei* and *E. fetida* might be connected with quality and quantity of some components of their celomic fluid; lysenin is unique for these species and riboflavin is much more abundant in *Eisenia* sp. than in other lumbricids investigated so far.

Key Words: earthworms; autotomy; regeneration; celomocytes; amebocytes; riboflavin**Introduction**

Lumbricid worms are commonly subjected to sub-lethal predator attacks leading to loss of body segments. Body segments may be lost also by self-amputation called autotomy (Zoran, 2010). Earthworms may be subjected to mechanical/chemical stimuli inducing spasmic body movements connected with expulsion of celomic fluid containing the celomocytes and soluble factors crucial for immunity (Bilej *et al.*, 2011; Ottaviani, 2011). Therefore regeneration of lost segments or restorations of the extruded cellular and soluble components of celomic fluid are of fundamental importance for these animals. Segmental body organization coupled with easy noninvasive celomic fluid retrieval make them attractive models for qualitative and quantitative interspecies comparisons of regeneration capabilities (Bely *et al.*, 2006; Bely and Sikes, 2010; Zoran, 2010).

We have investigated the effects of some endogenous and exogenous factors on the restoration of certain depleted components of celomic fluid as well as the regeneration of the lost body segments in three epigeic lumbricid species, *Dendrobaena veneta* (Dv), *Eisenia andrei* (Ea), and *E. fetida* (Ef) delimited by barcoding of the mitochondrial cytochrome C oxidase subunit I (COI) gene and supplemented in some specimens by MUG/MUG-like fluorophore (4-methylumbelliferyl β -D-glucuronide) characteristics (Fig. 1), by the methods described previously (Rorat *et al.*, 2014). We have examined two distinct cohorts of *E. fetida*: one that expresses (EfM+) and one that does not express (EfM-) a MUG-like fluorophore. These three composting species are easy to maintain and culture under laboratory conditions. In addition, we investigated a fourth species, the endogeic *Aporrectodea caliginosa* (Ac), which normally inhabits mineralized soil and is not so easy to maintain.

Depletion of celomic fluid was achieved by electrostimulation-induced expulsion (*e.g.*, Rorat *et al.*, 2014); loss of posterior segments was achieved either by surgical amputation or experimentally

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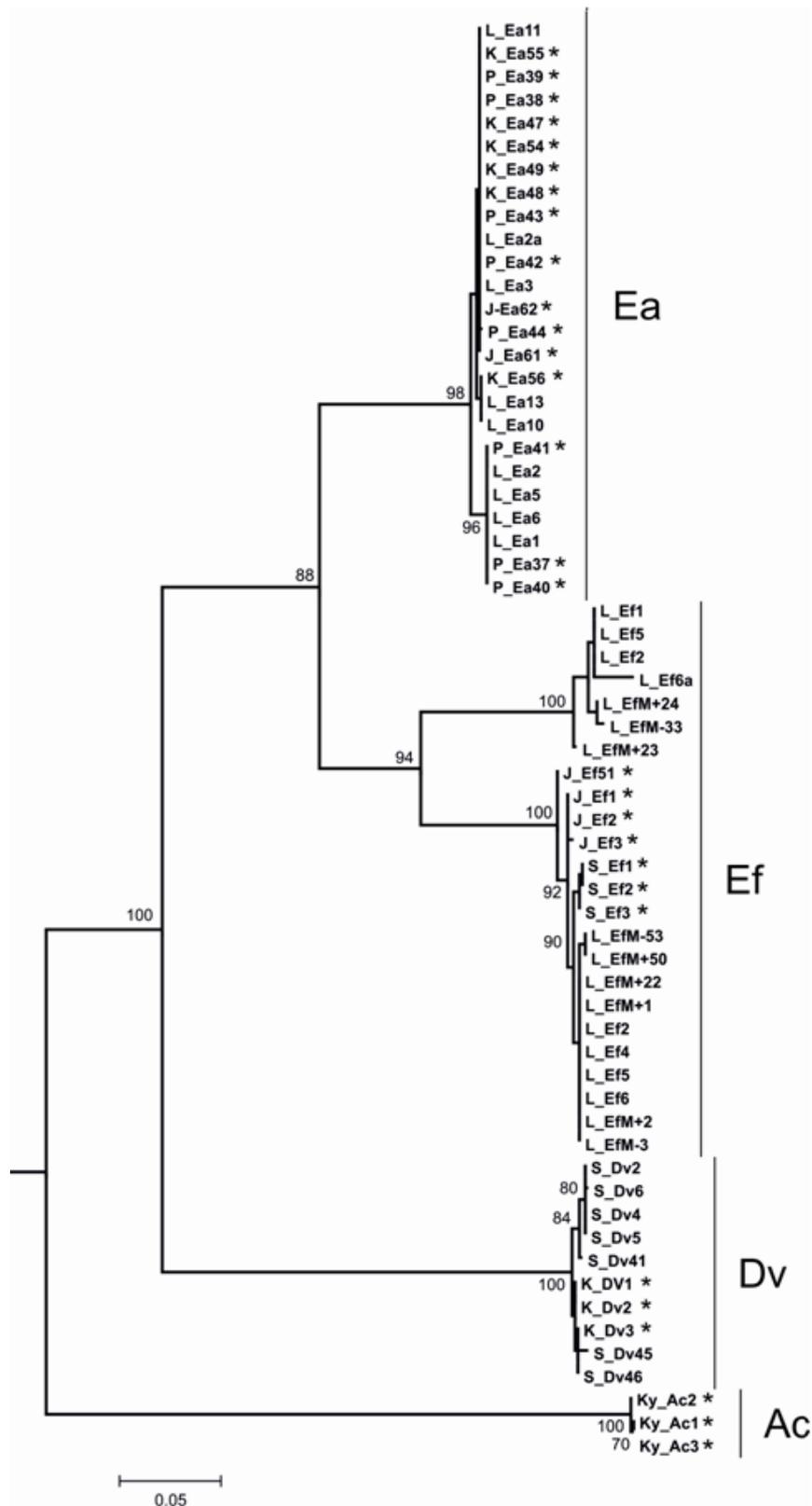


Fig. 1 Phylogenetic tree based on sequences of COI gene with clades corresponding to specimens of *Eisenia andrei* (Ea), *Eisenia fetida* (Ef) (some of them identified as EfM+ or EfM-), *Dendrobaena veneta* (Dv), and *Aporrectodea caliginosa* (Ac) delivered from laboratory cultures from France (Lille - L); Hungary (Pecs - P), and Poland (Kluczborok - K; Slupsk - S; Jasiencyk - J; Kleczany - Ky). Maximum likelihood tree, computed under Tamura 3-parameter with invariable sites, bootstrap supports (10,000 replicates) given if > 50 %. COI sequences 654 bp long were analysed; most of them are already published (Santocki *et al.*, 2016a: GenBank Accession numbers KM823553-KM823574; Swiderska *et al.*, 2016: GenBank Accession numbers KX671534-KX671544); new sequences marked by asterisks are deposited in GenBank (Accession numbers KX781363-KX781391).

Table 1 Celomocyte restoration characteristics and regeneration of anterior and posterior segments in four lumbricid species: *Eisenia andrei* (Ea); *E. fetida* (EfM+, EfM-); *Dendrobaena veneta* (Dv); *A. caliginosa* (Ac)

Investigated factors		Lumbricid species					
		Ea	Ef		Dv	Ac	
			EfM+	EfM-			
Lost body segments	Anterior regeneration (Y/?)	Y	Y	Y	Y	?	
	Posterior regeneration with blastema formation (Y/N)	Y	Y	Y	N	N/Y	
Restoration of celomic fluid components	Celomocytes	Amebocytes	+++ f	+++ f	+++ f	+++ f	+++ f
		Autofluorescent eleocytes	+++ s	+++ s	+++ s	+++ s	---
	Fluorophores	Riboflavin	++++ m	++++ m	++++ m	+ m	-+
		MUG	++++ f	++ f	---	---	---
	Hemolytic factors	Lysenin family proteins	+++ f/s	+++ f/s	+++ f/s	---	---

Presence/absence (+/-) of investigated factors and their restoration/regeneration abilities/rates considered as present/lacking (Y/N), and fast (f), moderate (m) or slow (s). Note that restoration of cellular and soluble components of celomic fluid and segment regeneration in *Eisenia* spp. are relatively resistant to soil pollution, and regenerative processes in all species are inhibited at low ambient temperatures (for detail see text).

induced self-amputation (autotomy) (see Galuszka *et al.*, 2015; Kocinski *et al.*, 2016); anterior brain-containing segments were either amputated or only cerebral ganglia were surgically extirpated (Okrzęsik *et al.*, 2013; Molnar *et al.*, 2015).

Here we review the main results from the published and unpublished experiments. A summary of our findings is presented in Table 1.

Restoration of some components of celomic fluid after experimental expulsion

The investigated composting species, Ea, Ef, and Dv, belong to lumbricids possessing two main populations of cells floating in celomic fluid, *i.e.*, the immunocompetent amebocytes and autofluorescent chloragocyte-derived granular eleocytes (Fig. 2a), the latter containing various nutritive substances, *e.g.*, riboflavin (see Plytycz and Morgan, 2011). In contrast, Ac is an example of lumbricid species possessing in celomic fluid almost exclusively the amebocytes. Riboflavin is stored in high or moderate quantities in eleocytes of *Eisenia* sp. and Dv, respectively (Rorat *et al.*, 2014; Santocki *et al.*, 2014, 2016a), but is also present in the attached chloragocytes residing on the celomic surface of the alimentary canal of all investigated lumbricid species (Mazur *et al.*, 2013).

Controlled expulsion of celomic fluid through dorsal pores of the earthworms may be induced by various irritants like 5 % ethanol (Cooper *et al.*,

1995), ultrasounds (Hendawi *et al.*, 2004) or mild electric current (Roch, 1979), the latter procedure modified and used in present studies (see Galuszka *et al.*, 2015; Kociński *et al.*, 2016). Drastic depletion of morphotic elements is followed by several week-lasting recovery process, originally described in *Lumbricus terrestris* devoid of eleocytes (Eyambe *et al.*, 1991). In Ac, another lumbricid species devoid of chloragocyte-derived eleocytes, restoration of amebocytes was even faster in Cd-polluted soil than in the clean soil; in this species restoration was temperature-dependent, being slower at 6 °C than at 25 °C (Galuszka *et al.*, 2015).

In the composting species investigated here restoration of amebocytes was consistently faster than that of eleocytes (Klimek *et al.*, 2012; Santocki *et al.*, 2016a, b), as exemplified in Ea on Figures 2b, c. A few weeks after electrostimulation-induced depletion, the number of eleocytes was still very low, and they were qualitatively different than their counterparts from the untreated worms, as they were much less prone to degranulation than the old eleocytes from untreated worms (Santocki *et al.*, 2016a, b). Restoration of riboflavin content was faster than the increase of eleocyte numbers (Fig. 2c); newly formed eleocytes store more riboflavin in their granules than mature eleocytes (Klimek *et al.*, 2012, Santocki *et al.*, 2016a, b); moreover, riboflavin present in fluid may derive also from the attached chloragocytes of chloragogenous tissue.

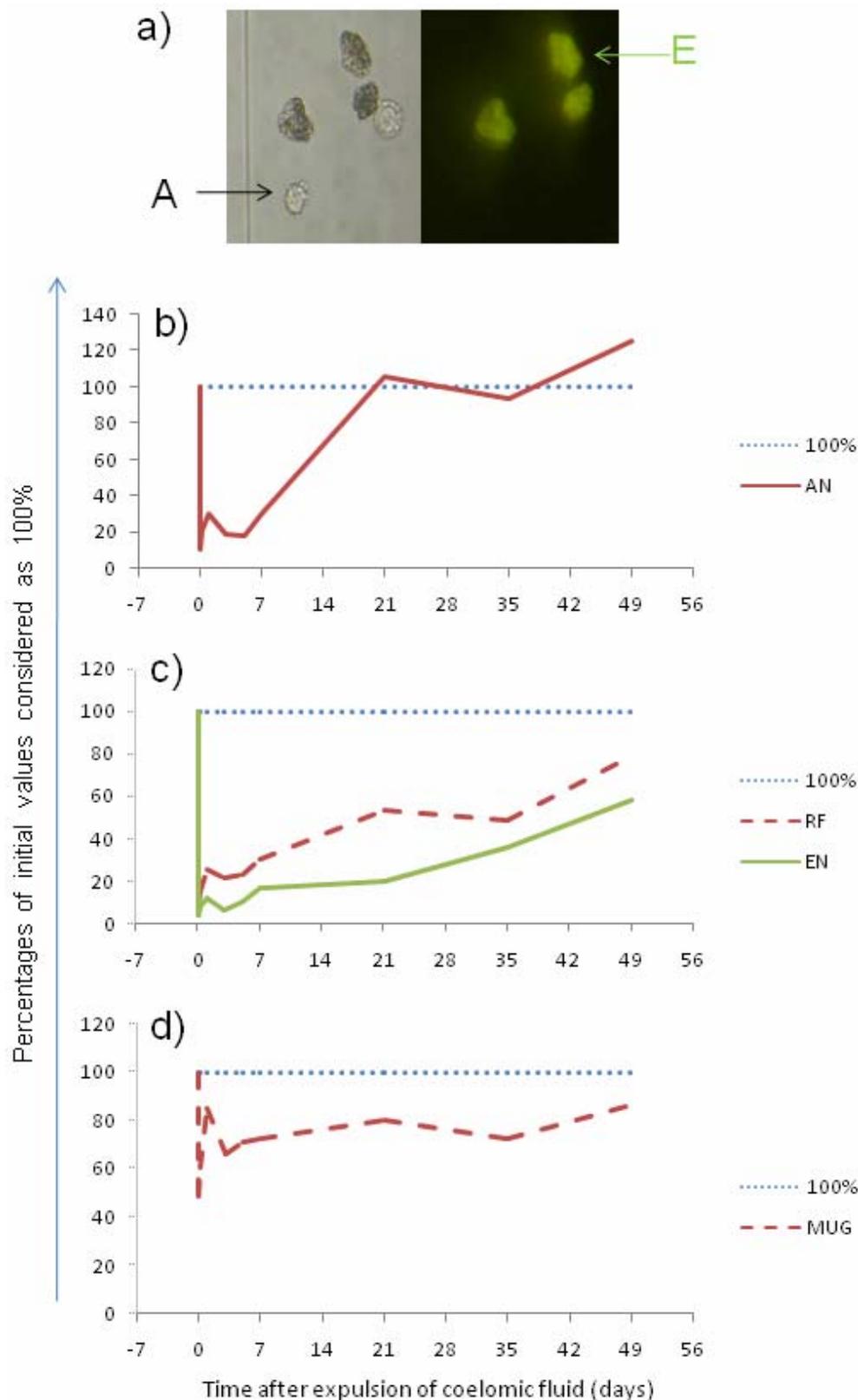


Fig. 2 Some components of celomic fluid of *Eisenia andrei*. a) group of 5 celomocytes in fluorescent microscope (left: bright field; right: blue light), among them 2 amebocytes (A) and 3 autofluorescent eleocytes (E); b-d) Restoration of the components of celomic fluid after experimental depletion induced at time 0 by electrostimulation (4.5V, 30 sec), expressed as percentages of initial values (C) considered as 100 % (modified according to Santocki *et al.*, 2016b): b) numbers of amebocytes (AN); c) numbers of eleocytes (EN) and riboflavin (RF) content; d) content of MUG fluorophore.

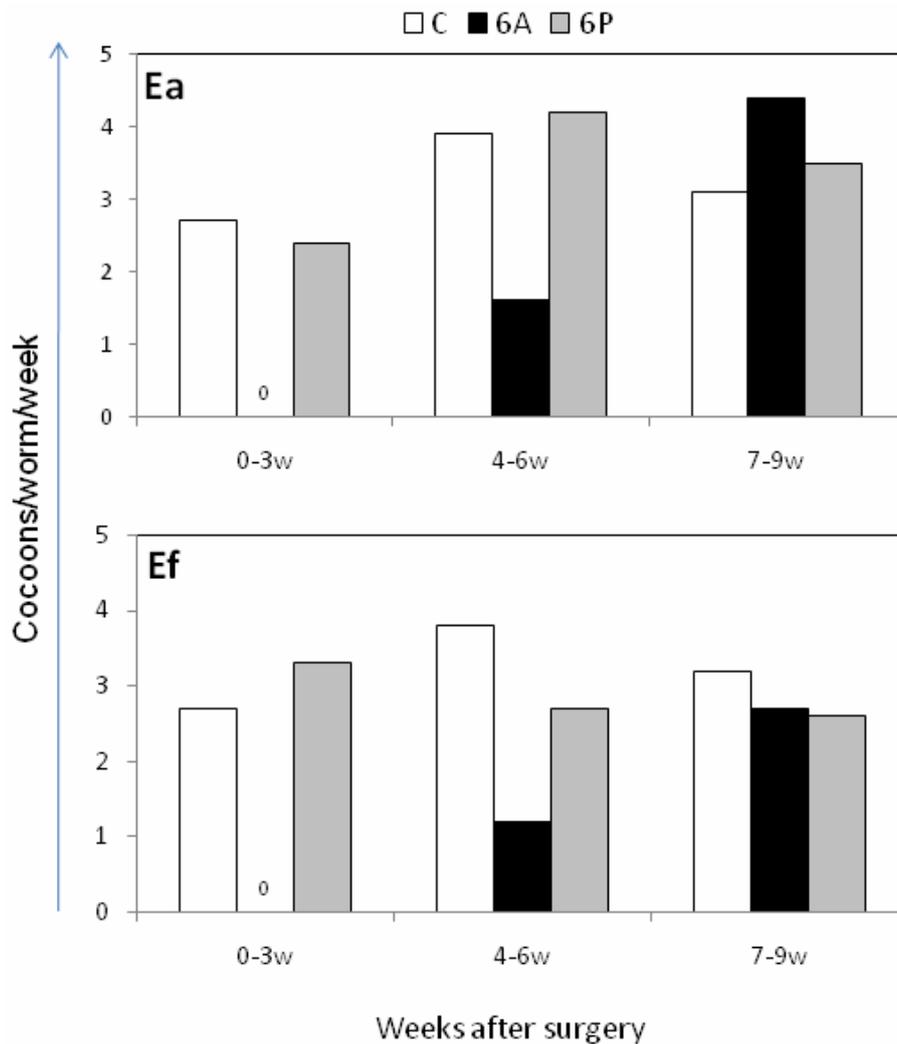


Fig. 3 Effects of anterior or posterior segments amputation on reproduction of composting worms. Cocoons production in *Eisenia andrei* (Ea) and *E. fetida* (Ef) either intact control worms (C) or those subjected at time 0 to surgical amputation of either 6 anterior segments (6A) or 6 posterior segments (6P), 8 worms in each group. Note that in groups (6A) of both species cocoons were absent during the first 3 weeks and their production started between 4th and 6th week after surgery, while in both species cocoon production was unaffected in worms with amputated posterior segments (6P). Numbers of cocoons in boxes containing 8 worms from either (C), (6A), or (6P) groups of Ea or Ef, calculated per worm and per week.

Restoration of components of celomic fluid was more efficient in juvenile than in adult worms (Santocki *et al.*, 2016a). Contrary to our working hypothesis, the kinetics of restoration of amebocytes, eleocytes and riboflavin content in juvenile Ea was not affected in worms maintained in soil polluted with cadmium at concentrations inhibiting worm growths and maturation (Takacs *et al.*, 2016, submitted). Also in adult Ea worms, similar exposure to cadmium polluted soil inhibited cocoon production with negligible effects on restoration of the depleted components of celomic fluid (Rorat *et al.*, in preparation). Putatively metallothioneins are responsible for protection of cadmium exposed celomocytes (Homa *et al.*, 2015; Rorat *et al.*, in preparation).

The MUG fluorophore is a molecular marker of *E. andrei* (Albani *et al.*, 2003), but MUG-like fluorophore is also present in some specimens of *E. fetida* (EfM+) (Rorat *et al.*, 2014; Santocki *et al.*, 2016a; Swiderska *et al.*, 2016). The initial contents of MUG/MUG-like fluorophores in celomic fluid of Ea was less depleted after electrostimulation and restored relatively quickly (Fig. 2d); much faster than restoration of riboflavin (compare Figs 2c, d) (Santocki *et al.*, 2016b; Swiderska *et al.*, 2016). The biological function and cellular source/s of MUG/MUG-like molecules is presently unknown. Are MUG-positive *E. fetida* earthworms the interspecific hybrids between Ea and EfM-? Experiments aimed in answering this are in progress.

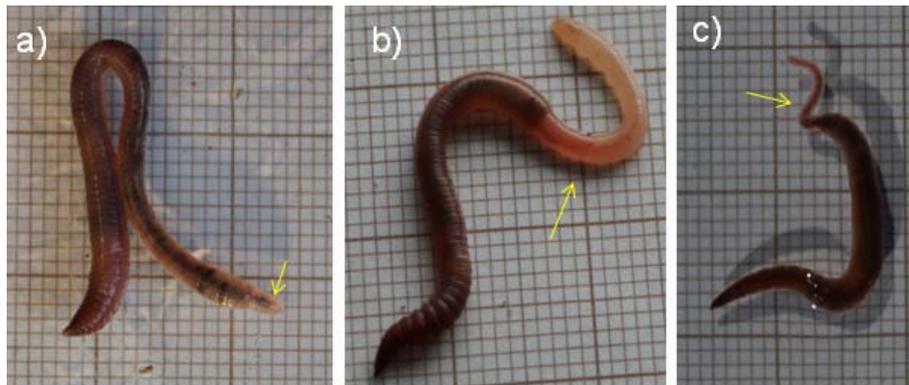


Fig. 4 Various forms of regeneration of amputated or autotomized posterior segments in *Aporrectodea caliginosa* (Ac): a) delayed formation of blastema, 3 months after amputation; b) very unusual non-pigmented blastema several months after autotomy; c) worm amputated and kept in cold for 3 weeks without rudiments of blastema, and then formation of tiny blastema within 2 weeks after transfer to room temperature.

Lysenin, *i.e.*, hemolytic sphingomyelin-binding pore-forming protein (Roch, 1979; Cooper *et al.*, 2002; De Colibus *et al.*, 2012; Bokori-Brown *et al.*, 2016) and lysenin-related proteins were detected in all investigated specimens of *Eisenia* sp., both Ea and Ef (the latter both EfM+ and EfM-) but not in *D. veneta* (Swiderska *et al.*, 2016). After experimental expulsion of celomic fluid, content of lysenin was restored faster in cellular fraction containing lysenin-storing eleocytes than in soluble fraction of celomic fluid (Swiderska *et al.*, 2016). However, lysenin is still a puzzling component of defense system of *Eisenia* sp., as the presence of its main target, sphingomyelin, seems to be restricted mainly to vertebrate cells membranes (Kobayashi *et al.*, 2000; Shakor *et al.*, 2003). In contrast, both the *Eisenia* earthworms (McLaughlin 1971) and the main invaders of their celomic cavity, *i.e.*, bacteria, fungi, and small invertebrates, are devoid of sphingomyelin in the cell membranes (Hori and Sugita, 1993), thus in the earthworms its antimicrobial function may be performed by sphingomyelin-independent mechanisms (Cooper *et al.*, 2002; Bruhn *et al.*, 2006), *e.g.*, due to its opsonizing properties (Hayashi *et al.*, 2013). Putatively another function of lysenin in *Eisenia* sp. earthworms might be induction of avoidance behavior of the potential vertebrate predators (like birds, hedgehogs, or moles) after irritating their mucosa with lysenin-containing celomic fluid. Hypothetically, we cannot exclude that lysenin might be involved in wound healing and/or regenerative processes.

Regeneration of extirpated brains or amputated anterior brain-containing segments

Surgical extirpation of suprapharyngeal 'cerebral' ganglia ('brains') or amputation of brain containing anterior segments of Dv caused an immediate inhibition of reproduction that was recovered in tandem with restoration of brain integrity, including active neurosecretory cells (Okrzesik *et al.*, 2013; Molnar *et al.*, 2015).

Restoration of reproduction, thus also regeneration of vital brain centers, was markedly more efficient in worms with intact immune system than in those subjected to electrostimulation-induced loss of amebocytes, eleocytes, and soluble components of immune system. And vice versa, restoration of components of celomic fluid was faster in intact worms than in their counterparts engaged in regenerating their extirpated brains (Molnar *et al.*, 2015). Such experimental model is unquestionably amenable to further detailed examinations of interactions between earthworm neuroendocrine and immune systems (Plytycz and Morgan, 2015).

Since a reappearance of temporarily inhibited cocoon production is a convenient biomarker of regeneration of the brain morphology and functions, the present studies included monitoring reproductive activity of unmanipulated (control) adult specimens of Ea and Ef, the latter either EfM+ and EfM-, and their counterparts with surgically amputated of either six anterior (brain-containing) or six posterior segments. As in Dv, cocoon production was hardly affected by amputation of posterior segments, while was completely inhibited for several weeks in all brainless worms, and then was gradually restored (Fig. 3). Thus, amputation of anterior segments or brain extirpation/regeneration is an attractive model for studies of neurohormonal-immune interactions in the composting species. However, to date we observe that such invasive treatment causes mortality in Ac worms.

Species-specific reactions to loss of posterior segments

In the present studies either surgical amputation of posterior segments was performed or their controlled self-amputation (autotomy) was induced. Autotomy of body segments followed by their regeneration is an adaptive strategy of lumbricid worms as anti-predator strategy and the disposal of encapsulated parasites or toxic products (Lesiuk and Drewers, 1999). Experimental controlled autotomy of posterior segments may be

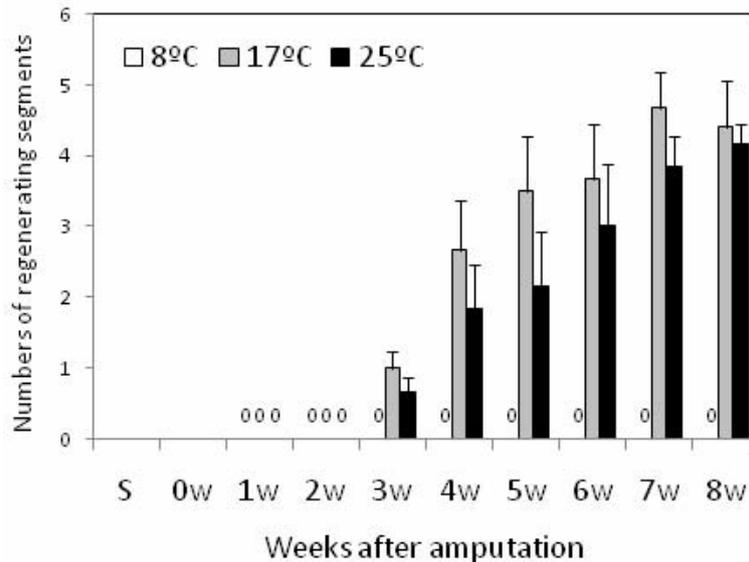
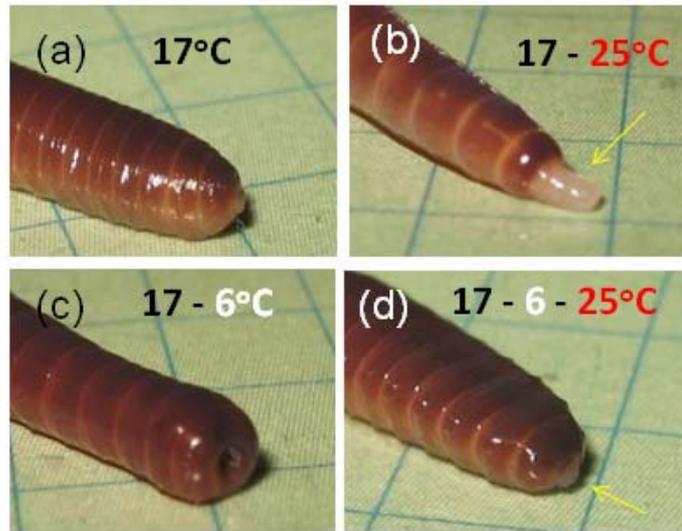


Fig. 5 Effects of temperature on regeneration of amputated posterior segments in *E.andrei* from Kluczbork (a) or Pecs (b). Top: Photos of blastema formation (yellow arrows) 17 days after amputation of 10 posterior segments in worms a) kept at 17 °C; b) transferred after amputation to 25 °C; c) transferred after amputation to 6 °C; d) transferred after amputation for 10 days to 6 °C and then to 25 °C (photos by Majos P). Note the lack of blastema in worms kept in cold. Bottom: numbers of regenerating segments (SN) in earthworms adapted to either 8 °C, 17 °C, or 25 °C, estimated at the start of experiments (S) on week 0 (0w) and then during 8 subsequent weeks after surgery; means \pm SE (n = 4 worms per group); 0 - no blastema.

induced in worms subjected to multiply electrostimulation. The pulsating direct electric current from a 4.5 V cell phone electric charger was necessary to induce it in *E. andrei*, while *A. caliginosa* were more prone for autotomy that was evoked not only by pulsating direct current but also by direct current from battery or immersion in noxious MS-222 solution. Autotomized segments in *Ea* were quickly replaced by newly formed once, while segment regeneration, if any, was significantly delayed in *Ac* (Kocinski *et al.*, 2016). Such reproducible models of autotomy may serve for

studies on molecular-genetic mechanisms underpinning autotomy and subsequent regeneration.

In *A. caliginosa*, reaction to amputation of posterior segments was very variable, either absent or significantly delayed (Fig. 4). Amputation of low numbers (e.g., 6 - 10) of posterior segments in *Ac* did not induce blastema formation but was followed by the compensatory body growths, while amputation of 40 segments induced significantly delayed regeneration blastema formation (Fig. 4a), that was not affected by cadmium soil pollution

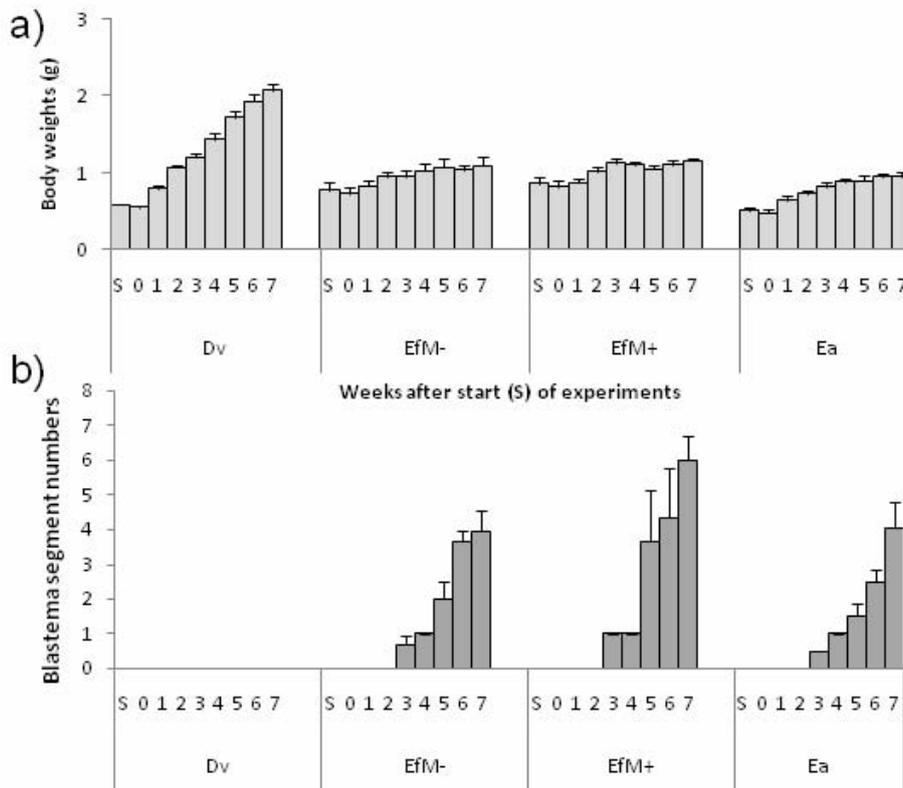
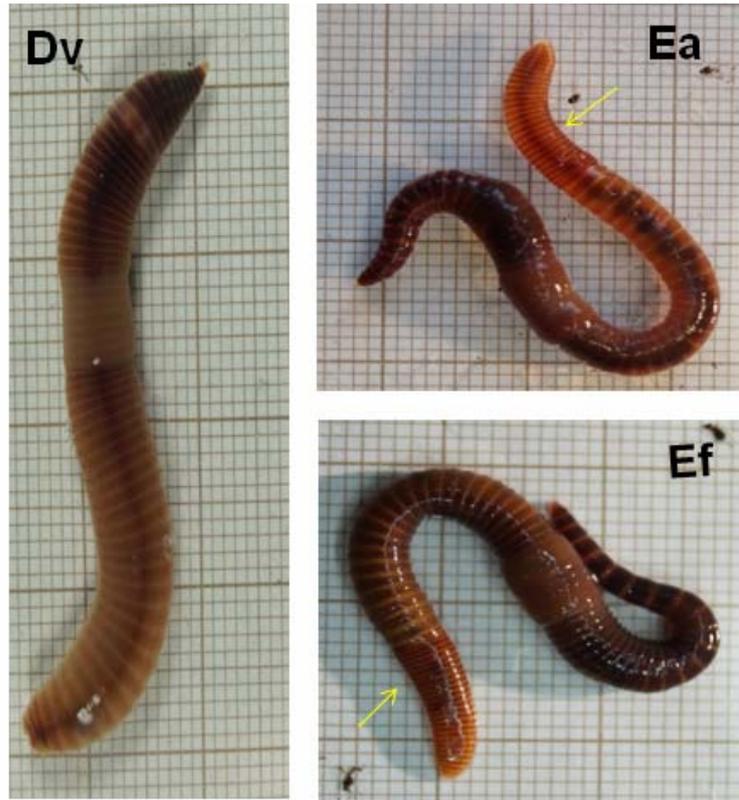


Fig. 6 Effects of amputation of posterior segments in *Dendrobaena veneta* (Dv), *Eisenia fetida* (Ef, either Ef- or Ef+), and *Eisenia andrei* (Ea) kept at 23 °C. Bottom part: Changes of a) body weights (BW) and b) numbers of regenerating segments estimated at the start of experiments (S) on week 0 (0w) and then during 7 subsequent weeks after surgical amputation of 10 posterior segments; means \pm SE (n = 3 worms per group); 0 - no blastema. Top part: Photos of Dv, Ea, and Ef taken 3 months after amputation of posterior segments at 25th segment after clitellum. Note the lack of regenerating segments in Dv, and their presence in Ea and Ef (yellow arrows).

(Galuszka *et al.*, 2015). In one instance we observed a long regeneration non-pigmented 'tail' in Ac (Fig. 4b). In several small specimens of Ac amputated and kept for 3 months at 6 °C blastemas were absent but very thin "fragile" blastemas appeared soon after the worm transfer to 20 °C (Fig. 4c).

In *E. andrei*, amputation of body segments induced blastema formation that was rapid at 17 °C and 25 °C but was completely inhibited at low temperature (8 °C) (Fig. 5, bottom). As exemplified on top of Figure 5, amputation of 10 posterior segments in Ea kept at 17 °C was followed by regeneration blastema formation that was faster in worms transferred to higher temperature (25 °C), while was absent in worms transferred to cold (8 °C), but that was quickly rescued after worm transfer to 25 °C (Fig. 5, top). At high temperature, blastema formation was unaffected in Ea kept in cadmium polluted soil (Takacs *et al.*, submitted) putatively due to protective role of metallothioneins (Rorat *et al.*, in preparation). Similar cold-inhibition and cadmium resistance of blastema formation was observed in *A. caliginosa* (Galuszka *et al.*, 2015).

Unexpectedly, we have so far been unable to observe blastema formation and regenerating posterior segments in *D. veneta*. Figure 6 (bottom) shows that regeneration of the amputated ten posterior segments was consistently present and similar in Ea and Ef, the latter both Ef+ and Ef-, while was consistently absent in *D. veneta*. In this experiment initial body weights of worms were similar, but body weight gain was much faster in Dv than in the both *Eisenia* sp., thus it might be supposed that loss of a few segments only was neglected due to the fast body growth. However, when specimens of *Eisenia* sp. and *D. veneta* were amputated at the 25th segment after clitellum, 4 months after surgery regenerated segments were clearly visible in Ea and Ef but not in Dv (Fig. 6, top).

In conclusion, both autotomy and surgical amputation of posterior segments in Ea and Ef are followed by formation of regeneration blastema that is cold-inhibited but efficient at high temperatures. Formation of regeneration blastema is either absent or significantly delayed or modified in Ac, and consistently absent in Dv. In other words, blastema-involved epimorphic type of regeneration is common in *Eisenia* sp., while *D. veneta* perform morphallactic type of regeneration. Therefore comparative studies on these closely related and ecophysiologically similar species may serve as convenient models to delineate differences and similarities in the regenerative capabilities at the genetic, molecular and cellular levels (Alvarado and Tsonis 2006).

Summary and concluding remarks

A summary of our main findings is presented in Table 1. Anterior regeneration is present in *Eisenia* sp. and *D. veneta*, but head amputation was lethal for Ac investigated so far. Posterior regeneration with blastema formation was consistently present in *Eisenia* sp., consistently absent in *D. veneta*, and absent or attenuated in Ac. Among depleted celomic

fluid components, restoration of amebocytes was efficient in all species. Autofluorescent eleocytes were present and restored slowly in *Eisenia* sp, and *D. veneta*; Riboflavin content was very high in *Eisenia* sp. and lower in Dv, but in both species regenerated in a moderate rate. MUG/MUG-like fluorophore was present and restored fast in all Ea and some specimens of Ef (EfM+). Hemolytic proteins of lysenin family are restricted to *Eisenia* sp.

Why *D. veneta*, with a very efficient brain regeneration capability has a weak posterior segment blastema formation is an open question. Is this connected with lower riboflavin content in Dv eleocytes relative to the same cell type in Ea and Ef species? What is a source of the abundant riboflavin stores in *Eisenia* spp.? Is this connected with horizontal gene transfer as suggested earlier (Sulik *et al.*, 2012)?

Evidently, restoration of brain-containing anterior segments is of highest priority for all epigeic earthworms often exposed to predator attacks, but studies on endogeic Ac shall be continued. Loss of posterior segments may be followed by compensatory body growth and/or regeneration blastema formation. Blastema formation is most efficient in *Eisenia* sp., possessing in celomic cavity huge amounts of riboflavin and lysenin. Participation of riboflavin in regeneration is well documented (Johnson *et al.*, 2012).

Participation of factors derived from celomic cavity of *Eisenia* sp. in wound healing is extensively investigated (Cooper *et al.*, 2004; Grdisa *et al.*, 2004; Matausijc-Pils *et al.*, 2010). At the place of wounds secreted lysenin with opsonizing properties could cover the damaged endogenous cells and aid the phagocytes to engulf them (Hayashi *et al.*, 2013). Since wound healing consists of parallel processes of regeneration and scar formation (White *et al.*, EOLLS), some celomic factors of *Eisenia* species (e.g., riboflavin and/or lysenin) may act in favor of regenerative blastema formation. In other lumbricid species some external and/or endogenous factors might be necessary to elicit the latent regeneration abilities (Bely and Sikes, 2010).

Acknowledgements

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