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RESEARCH REPORT

Experimental induction of autotomy in two potential model lumbricid earthworms *Eisenia andrei* and *Aporrectodea caliginosa*

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Abstract

Mild electrostimulation of earthworms is commonly used for harvesting celomocyte-containing celomic fluid. We have noticed that frequent electrostimulations of worms can lead to the autotomy of posterior segments followed by their regeneration. Our present study aim was to develop an autotomy model in the eco-physiologically contrasting species, *Eisenia andrei* and *Aporrectodea caliginosa*, using direct current (DC), pulsating direct current (PDC), or immersion in the noxious anaesthetic MS-222. *A. caliginosa* was clearly more susceptible to autotomy than *E. andrei*, with both electro-stimulation regimes and MS-222 exposure inducing autotomy in the former species but only repeated PDC stimulations inducing segment loss in the latter. The observations lend credence to the hypothesis that autotomy in earthworms is caused by factors impinging upon the nervous system; they also indicate that repeated PDC stimulations could be an effective and reproducible means of facilitating segmental autotomy so that the fundamental cytological and molecular-genetic mechanisms underpinning the tissue loss event and subsequent regeneration can be studied in depth.

Key Words: autotomy; electrostimulation; lumbricid earthworms; regeneration; *Eisenia andrei; Aporrectodea caliginosa*

Introduction

Autotomy, literally meaning 'self-severing' or 'self-amputation', is a phenomenon involving the loss and often the subsequent regeneration of body parts such as appendages. It is manifest in a number of invertebrate (*e.g.,* arthropods and echinoderms) and vertebrate (*e.g*., salamanders and lizards) taxa where it usually serves as a self-defense mechanism enabling a potential prey organism to escape or confuse an attacking predator (Maginnis, 2006; Fleming *et al.*, 2007). Invertebrates lacking appendages, such as annelid worms, are also known to adaptively autotomize body segments, followed by their regeneration, as an anti-predatory strategy (Lesiuk and Drewers, 1999), as well as for the disposal of the products of antimicrobial and anti-eukaryotic parasite encapsulation (Field *et al.*, 2004), and as a response and mode of elimination of highly toxic levels of organic (Paris-Palacios *et al.*, 2010) and inorganic (Mendez-Fernandez *et al.*, 2013) residues.

Previously, we and others have used mild electrostimulation of lumbricid worms for the induction of convulsive body movements and concomitant extrusion of celomocyte-containing celomic fluid through body wall dorsal pores. This procedure, originally developed by Roch (1979), served as an effective non-invasive method for harvesting immunocompetent cells and soluble factors for *ex vivo* studies (e.g. Stankiewicz and Plytycz, 1998; Podolak-Machowska *et al.*, 2013; Rorat *et al.*, 2014). Using this procedure, we have observed that celomocyte depletion from *Eisenia spp.* during a regime of daily 1 min-duration electro-stimulations with current from a 4.5 V battery for 19 consecutive days was followed by a period of steady replenishment of the celomocyte counts without gross adverse effects on the worms (unpublished data). In contrast, we have found recently that multiply stimulations with the pulsating direct electric current from a 4.5 V cell phone electric charger leads to posterior segment autotomy in the eco-physiologically contrasting lumbricids *Eisenia andrei* (a litter-inhabiting 'epigeic' species) and *Aporrectodea caliginosa* (an 'edogeic' inhabitor of mineralized soil).

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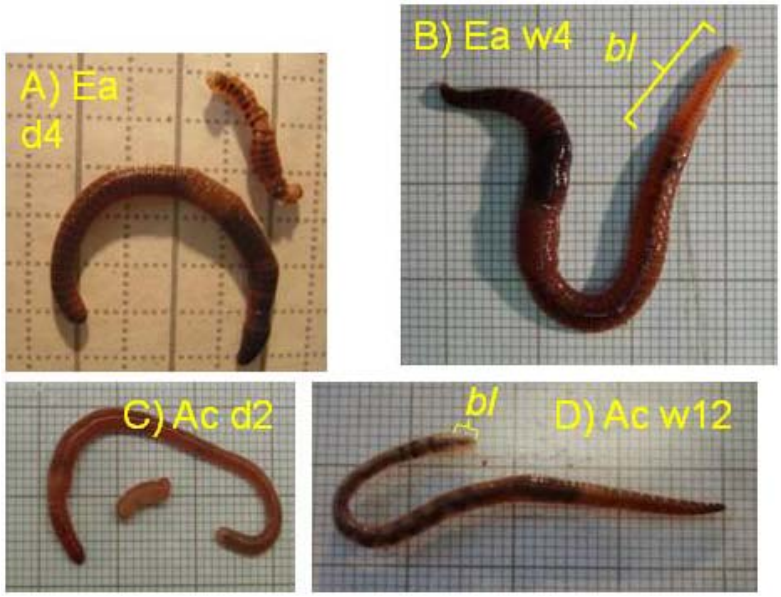

Fig. 1 Representative examples of fates of earthworms subjected to multiple electrostimulations with pulsating direct current (PDC) twice per day. (A) *Eisenia andrei* (Ea) soon after autotomy of several posterior segments on day 4 after 7 electrostimulations; (B) The same specimen of *E. andrei* 4 weeks later with regeneration blastema (*bl*); (D) *Aporrectodea caliginosa* (Ac) soon after autotomy of several posterior segments on day 2 after 4 electrostimulations, and (E) the same worm 12 weeks later with regeneration blastema (*bl*).

The primary aim of the present investigations was to develop and evaluate a reproducible model of autotomy in *E. andrei* and *A. caliginosa*, and to establish whether the susceptibility of the two species to autotomy was comparable. Apart from distinct ecological differences, these two earthworm species have other trait differences that are pertinent for our investigative purposes: *E. andrei* has a high proportion of free-floating, riboflavin-containing, eleocytes in addition to amebocytes in its celomic fluid, *A.caliginosa* does not (Cholewa *et al.*, 2006; Rorat *et al.*, 2014); *A. caliginosa*, but not *E. andrei*, is known to undergo facultative diapause under certain adverse environmental conditions (Bayley *et al.*, 2010), a physiological resting state that may be functionally linked to tissue regeneration in at least some earthworm species (Fragoso and Lozano, 1992); differential species sensitivity to toxicants (Kula and Larink, 1998), as exemplified by studies deploying cytological and DNA-damage biomarkers, respectively, indicating that *A. caliginosa* is more sensitive to sub-lethal concentrations of Zn (Spurgeon *et al.*, 2000) but less sensitive to sub-lethal concentrations of Cd (Fourie *et al.*, 2007) than *E. fetida*. Our study was motivated as a foundation for future studies to establish common lumbricid species as model organisms to probe the involvement of neurosecretions and the immune system in autotomy and regeneration. Analogous studies on *E. fetida* using a combination of low frequency electromagnetic and continuous light stimulation were recently initiated by Banovački and Matavulj (2013).

Table 1 Comparison of autotomized posterior segments of *Eisenia andrei* (*Ea*) and *Aporrectodea caliginosa* (*Ac*)

Species (worm numbers)	Body segments (range) mean±SD			
	Total numbers	autotomized/discarded		
		numbers	% numbers	% body mass
Ea (7)	(93 - 105) 99 ± 4.1	(14 - 50) 30 ± 14.3	(13 - 35) 27 ± 10.3	(7 - 29) 21 ± 11.6
Ac (8)	(117 - 140) 129 ± 8.3	(5 - 51) 25 ± 16.8	(5 - 13) 18 ± 12.8	(3 - 17) 12 ± 5.3

Materials and Methods

Earthworms

Experiments were performed on adult (*i.e.,* fully clitellate) *Eisenia andrei* and *Aporrectodea caliginosa*. *E. andrei*, originally bought from the commercial supplier EKARGO (Kepa Slupska, Poland), reared for several generations in the laboratory at the Institute of Zoology, Jagiellonian University, Kraków, Poland. Adult *A. caliginosa* were collected from the field (Kleczany, Southern Poland) and then cultured at the Jagiellonian University. Both species were routinely maintained at 17 - 23 ^{o}C, and fed with a mixed diet comprised of dried/boiled nettle (*Urtica dioica*) and dandelion leaves (*Taraxacum officinalis*), boiled/dried tea leaves, and powdered commercial mouse pellets. Within each species, individuals of similar body weights were used for each experiment.

Experimental scheme

In each experimental series weighed earthworms were individually immersed once or twice per day in 5 ml physiological saline and subjected to 60 sec-duration electrostimulations either: (i) with direct current (DC) from a battery (SONY 3R12; 4.5 V); or, (ii) with pulsating direct current (PDC) from the adapted cell phone chargers, *i.e.,* alternating current/direct current (Ac/Dc) adaptors either (input: 230 V, ~50Hz, 20 mA; output: 4.5 V, 400 mA) or (input: 230 V, ~50 Hz, 0.05 A; output: 4.8 V, ~350 mA). Both DC and PDC treatments induced extensive body movements connected with the expulsion of celomocyte-containing celomic fluid (Podolak-Machowska *et al.*, 2013). In some experiments, electrostimulation was preceded by 3 min anaesthesia in carbonated drinking water by Soda Siphon. In another set of experiments worms were immersed for 1 min in 5 ml 0.5 % MS-222 solution (Tricaine methanesulfonate; Ethyl 3-aminobenzoate methanosulfonate salt; Sigma-Aldrich; Fluca), that is a known strong irritant of worms and induces celomocyte extrusion (Podolak-Machowska *et al.*, 2013). In all experiments, worms were individually kept in boxes with moistened filter paper or soil and inspected daily for general condition and/or incidence of autotomy. In the latter case anterior and posterior parts of worms were weighed and segments were counted. Numbers of worms of both species used in particular experiments are given in respective Figures and Tables of the section Results and Discussion. Some autotomized worms were transferred to clean moist soil, fed *ad libitum* as per the laboratory cultures (see above), and observed for several weeks for regenerative blastema formation.

Immersion fluid from various sets of experiments was inspected for the presence/absence of celomocytes in hemocytometer. In the case of *E. andrei*, several 2 ml samples of celomocyte-containing fluids were lysed in 2 % Triton (Sigma-Aldrich) and used for measurements of riboflavin content using an LS50B Perkin-Elmer Spectrofluorimeter as described previously (Rorat *et al.*, 2014); this procedure was not undertaken with *A. caliginosa* because the celomic fluid of this species does not contain appreciable numbers of riboflavin-laden, free-floating, eleocytes.

Results and Discussion

Figure 1 shows representative examples of *E. andrei* and *A. caliginosa* soon after autotomy of posterior segments and, several weeks later, during blastema formation that evidently occurred earlier in the former species. The formation of a blastema indicates that autotomy potentially may be followed by regeneration of severed segments. It is pertinent that a detailed description of an analogous phenomenon in echinoderms was entitled "Autotomy as a prelude to regeneration in echinoderms" (Wilkie, 2001).

The total numbers of segments were relatively constant within intact *E. andrei* and *A. caliginosa* (99 ± 4.1 and 129 ± 8.3, respectively). During autotomy induced by PDC from a cell phone charger, *E. andrei* discarded a higher proportion of its segments than did *A. caliginosa* (27 ± 10.3 % versus 18 ± 12.8 %, respectively), which corresponded to body mass losses of 21 ± 11.6 % versus 12 ± 5.3 %, respectively (Table 1). These observations imply the existence of species-specific morpho-regional differences in the susceptibility to autotomy in these earthworm species.

Figure 2A and Table 2 show that among worms stimulated once per day with PDC from the cell phone charger, all 12 *E. andrei* individuals exhibited autotomy between the 4th and 22nd days after the start of stimulation regime, while all 9 *A. caliginosa* in the treatment group autotomized significantly earlier at between the 2nd and 5th days. Median autotomy times post-initiation of once-daily PDC stimulations were 8 days (*E. andrei*) and 4 days (*A. caliginosa*). Thus, the median number of PDC stimuli required to induce autotomy was 8 (*E. andrei*) and 4 (*A. caliginosa*).

Figure 2B and Table 2 show that a twice-daily regime of PDC stimulations induced autotomy more efficiently than once-daily stimulation. Twice-daily PDC resulted in all 18 *E. andrei* individuals in the treatment group autotomizing their posterior segments between the 2nd and 11th days post-initiation, whilst we observed autotomy in one *A. caliginosa* individual as early as after the second electrostimulation on day 1, with all 14 *A. caliginosa* individuals in the treatment group exhibiting autotomy by day 5. Thus, median autotomy time was reduced from 8 days to 4.5 days (*E. andrei*), and from 4 days to 3 days (*A. caliginosa*), with twice-daily compared with once-daily PDC stimulations. Nevertheless, the median number of electro-stimuli required to induce autotomy was similar whether the stimuli were given once- or twice-daily: 8 stimuli (once-daily) versus 7-8 stimuli (twice-daily) in *E. andrei*, and 4 stimuli versus 5 - 6 in *A. caliginosa*, the period being consistently longer in *E. andrei* (Table 2). [Note that in the twice-daily treatments, it was not possible to resolve the number of stimuli required to induce autotomy to a

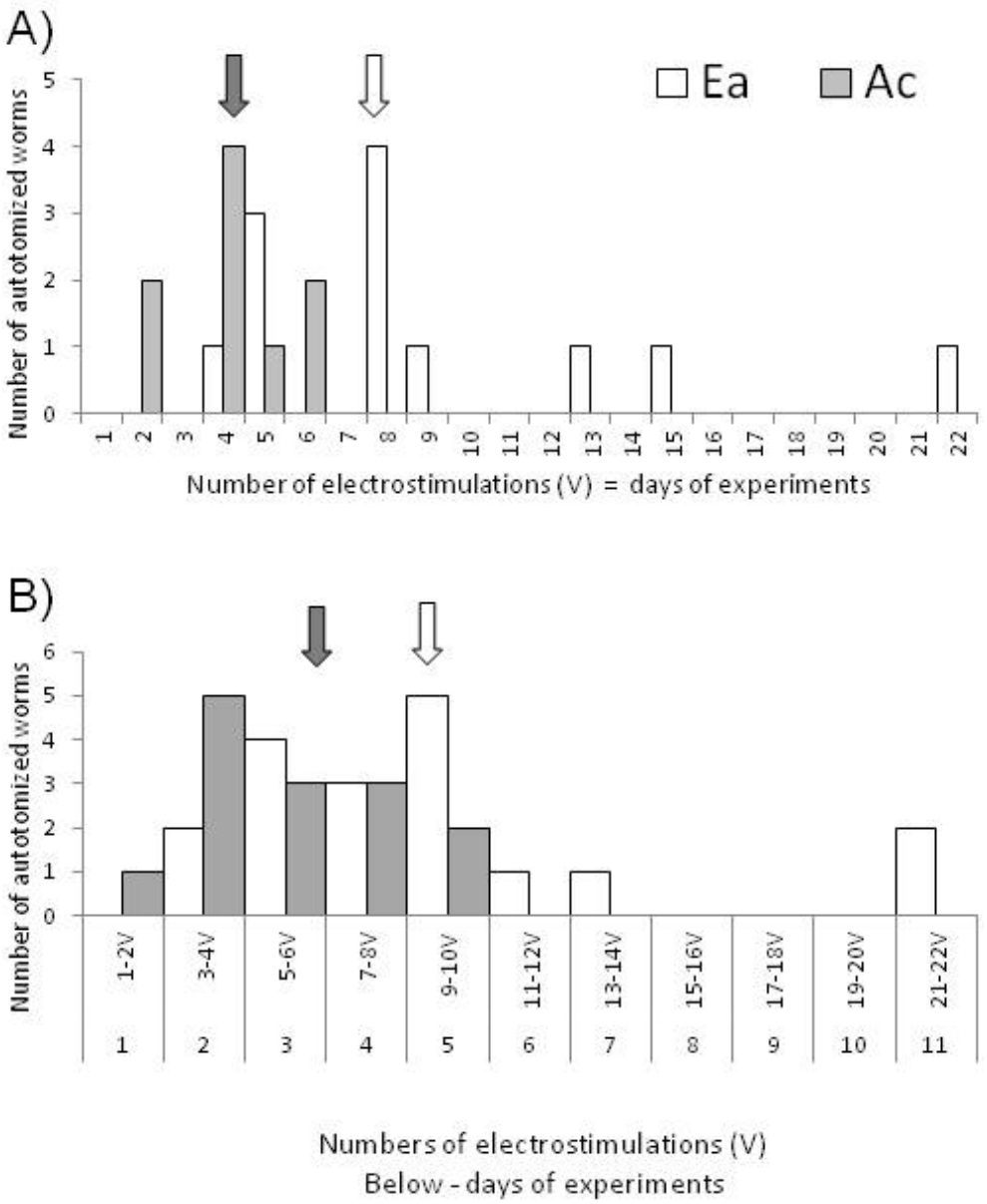

Fig. 2 Incidences of autotomy after multiple electrostimulations (V) with pulsating direct current (PDC) applied: (A) once per day or (B) twice per day in *Eisenia andrei* (Ea) or *Aporrectodea caliginosa* (Ac). Days of autotomy by the median worm of each species are marked by open and gray arrows (Ea and Ac, respectively).

Table 2 Autotomy of posterior segments of *Eisenia andrei* (Ea) and *Aporrectodea caliginosa* (Ac) induced by multiply electrostimulation with PDC from the cell phone charger, performed once (1x) or twice per day (2x). Start of experiments is considered as day 1

worms	times per day PDC treatments	worm numbers autotomising / Total	days of autotomy		numbers of electrostimulations before autotomy	
			range	median	range	median
Ea	1x	12/12	4-22	8	4-22	8
	2x	18/18	2-11	4.5	3-22	7-8
Ac	1x	9/9	2-5	4	2-4	4
	2x	14/14	1-5	3	2-10	5-6

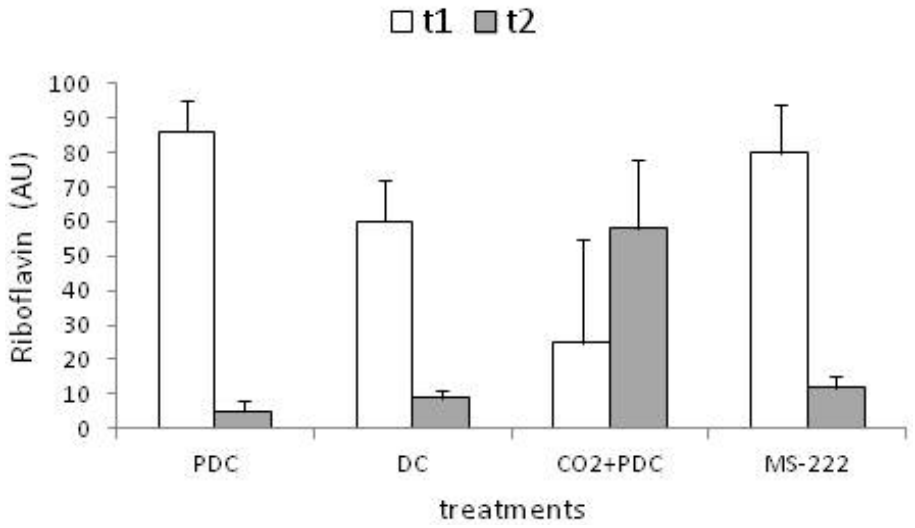

Fig. 3 Riboflavin (in arbitrary units AU) derived from celomocytes extruded to immersion fluid by *Eisenia andrei* worms stimulated for the first time (t1) and then 8 h later (t2) by pulsating direct current (PDC) from the phone charger or by direct current (DC) from the battery or PDC after anesthesia in CO_2-water (CO_2+PDC) or by immersion in 0.5 % MS-222. X±SE, n = 4.

single number]. The differential responses of the two eco-physiologically contrasting earthworm species to electrostimulation was not surprising in light of the fact that species-specific sensitivity to electric current has been observed in fish. For example, PDC treatment caused mortality of fingerlings of Chinook salmon *Oncorhynchus tschawytscha* (Collins *et al.*, 1954) while rainbow trout (*Salmo gairdneri*) were resistant to similar treatment (Maxfield *et al.*, 1971).

The aim of our subsequent experiments was to address the question of whether the autotomy of posterior segments in *E. andrei* and *A. caliginosa* is caused primarily by electric stimulation or by a massive loss of coelomic fluid containing coelomocytes and soluble factors. Consequently, a comparison was made of the results of 5-day experiments involving two stimulations per day, performed on 4 groups of worms: 1) electrostimulated with PDC; 2) electrostimulated with DC; 3) electrostimulated with PDC after anesthesia in CO_2-water (Lubics *et al.*, 2002); 4) immersed in MS-222 without electrostimulation. After worm removal from immersion fluids, microscopic inspection revealed in all samples the presence of expelled celomocytes, among them amebocytes and eleocytes in the case of *E. andrei*, but amebocytes only in *A. caliginosa*.

The observed species-related differences in cellular constitution of the immersion fluids was consistent with the fact that *A. caliginosa* is a lumbricid exclusively containing free-floating amebocytes in its celomic fluid, while *E. andrei* is a lumbricid known to contain both major celomocyte types (*i.e.*, amebocytes and autofluorescent eleocytes) (Cholewa *et al.*, 2006; Rorat *et al.*, 2014). Autofluorescence of *E. andrei* eleocytes derives mainly from yellow riboflavin (Koziol *et al.*, 2006; Plytycz and Morgan, 2011; Rorat *et al.*, 2014); this is macroscopically evident because the extrusion fluids acquire a subtle yellowish hue, and was subjected to quantitative spectrofluorimetric analysis (Fig. 3). The amount of riboflavin tended to be higher in samples after PDC compared with DC electrostimulation, albeit the difference was statistically insignificant. Attempts to suppress the inadvertent extrusion of celomocytes into immersion fluid by mild anesthesia in carbonated water (*e.g.*, Lubics *et al.*, 2002) were only partly successful because small measureable amounts of riboflavin were still detectable in immersion fluid.

Table 3 Results of 5-day attempts to induce autotomy of posterior segments of *Eisenia andrei* (Ea) and *Aporrectodea caliginosa* (Ac) by electrostimulation with PDC from cell phone charger, or with DC from battery, or PDC after CO_2 anesthesia (CO_2+PDC), or by immersion in MS-222, all treatments performed twice per day

Worms	Treatments	worm numbers autotomising/total
Ea	PDC	14/18
	DC	0/4
	CO_2+PDC	1 /4
	MS-222	0/4
Ac	PDC	13/13
	DC	1/3
	CO_2+PDC	3/3
	MS-222	2/7

Table 4 Celomocytes/riboflavin loss and posterior segments autotomy in *Eisenia andrei* and *Aporrectodea caliginosa* earthworms induced by repeated stimulations with PDC from cell phone charger or DC from battery without or with CO_2 anesthesia (CO_2+PDC) or by immersion in MS-222

	Eisenia andrei		***Aporrectodea caliginosa***	
Repeated treatments	**Loss of celomocytes and riboflavin**	**Autotomy**	**Loss of celomocytes**	**Autotomy**
PDC	+	+	+	+
DC	+	-	+	+/-
CO_2+PDC	+/-	+/-	+	+
MS-222	+	-	+	+/-

MS-222 is an effective anesthetic for ectothermic vertebrates such as fish (Neiffer and Stamper, 2011) and amphibians (*e.g.*, Jozkowicz and Plytycz, 1998). However, MS-22 is noxious to earthworms (Podolak-Machowska *et al.*, 2014), and in the present experiments was found to induce the 'leakage' riboflavin from *E. fetida* at levels comparable to those observed after PDC electrostimulation. Second treatments of the same worms performed 8 h later resulted in the detection of negligible amounts of riboflavin in all but the carbonated-water/PDC treatment group (Fig. 3). These findings indicate that worms lose most of their celomocytes during the first PDC, DC, and MS-222 stimulations, and that carbonated-water anesthesia did not fully inhibit the effect.

The 5-day-experiments confirmed that *E. andrei* is less prone to autotomy than *A. caliginosa* (Table 3). All *E. andrei* and *A. caliginosa* autotomized after repeated electro-stimulation with PDC. However, none of 4 *E. andrei* autotomized tail segments during 10 successive (*i.e.*, twice daily) DC treatments, while 1 of 3 *A. caliginosa* did; none of 4 *E. andrei* immersed in MS-222 autotomized tail segments while 2 of 7 *A. caliginosa* did; each of the 3 *A. caliginosa* immersed in carbonated-water before PDC electrostimulation discarded tail segments, while only 1of 4 *E. andrei* did (Table 3).

As summarized in Table 4, *E. andrei* discarded posterior segments only after repeated electrostimulation with PDC. In contrast, *A. caliginosa* did after a variety of repeated stressors, both those involving electrostimulation alone, MS-222 alone, and electrostimulation in combination with CO_2 anesthesia. Thus, in this endogeic lumbricid species, autotomy can be induced by physical and chemical factors capable of extruding celomocytes, although the loss of immune-competent cells may be a spurious concomitant effect of the treatments rather than in any direct way causative. It is conceivable that electrostimulation and MS-222 treatment both cause autotomy by impinging on the nervous system.

The mechanism(s) connecting the stimuli inducing autotomy in lumbricid earthworms and the morphological manifestations of the phenomenon are as yet unclear. However, Lesiuk and Drewers (1999), who developed an apparatus for focal body compression of the freshwater oligochaete *Lumbriculus veriegatus* to induce autotomy, observed that body fragmentation in this species could be blocked by nicotine pre-treatment. This suggests that nicotinic cholinergic receptors are involved in the autotomy reflex, and offers sustenance to the notion that the nervous system is fundamentally implicated in earthworm autotomy. Extrapolating from *L. variegatus* to terrestrial oligochaetes should perhaps be done with caution because spontaneous self-fragmentation is a prominent asexual reproduction strategy characteristic of *Lumbriculus*. Nevertheless, the simple PDC method that we have described appears to reliably induce autotomy of posterior segments in *E. andrei* and *A. caliginosa*. This may serve as a convenient model for studies on cytological and molecular-genetic mechanisms underpinning autotomy and subsequent tissue/organ regeneration in these soil-dwelling metazoans. Moreover, it will be interesting to compare the details of segment restoration in surgically-amputated and autotomized earthworms.

Acknowledgments

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