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Autofluorescence in eleocytes of some earthworm species

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Abstract: Immunocompetent cells of earthworms, coelomocytes, comprise adherent amoebocytes and granular eleocytes (chloragocytes). Both cell populations can be expelled via dorsal pores of adult earthworms by exposure to an electric current (4.5 V) for 1 min. Analysis by phase contrast/fluorescence microscopy and flow cytometry demonstrated that eleocyte population of several species exhibits a strong autofluorescence. A high percentage (11-35%) of autofluorescent eleocytes was recorded in *Allolobophora chlorotica*, *Dendrodrilus rubidus*, *Eisenia fetida*, and *Octolasion* sp. (*O. cyaneum*, *O. tyrtaeum tyrtaeum* and *O. tyrtaeum lacteum*). In contrast, autofluorescent coelomocytes were exceptionally scarce (less than 1%) in representative *Aporrectodea* sp. (*A. caliginosa* and *A. longa*) and *Lumbricus* sp. (*L. castaneus, L. festivus, L. rubellus*, *L. terrestris*). Thus, this paper for the first time describes profound intrinsic fluorescence of eleocytes in some - but not all - earthworm species. The function (if any) and inter-species differences of the autofluorescent coelomocytes still remain elusive. (www.cm-uj.krakow.pl/FHC)

Key words: Amoebocytes - Eleocytes - Earthworms - Flow cytometry - Autofluorescence

Introduction

The earthworm coelomic cavity is filled with fluid containing free, wandering coelomocytes and their pigmented aggregates, called brown bodies, formed during encapsulation of invading bacteria and particulate waste products. The coelom communicates with the outer environment directly by dorsal pores and paired nephridial tubules through which metabolites are excreted. The dorsal pores represent also one of the important routes for the elimination of bacteria and "exhausted" coelomocytes. Under stress conditions, the coelomic fluid with its suspended cells can be rapidly expelled by increased intra-coelomic pressure [19]. The latter phenomenon has been exploited as a non-invasive method of sampling coelomic fluid and coelomocytes using electric current [12, 15], ethanol [4, 5] or ultrasound stimuli [10].

The classification of earthworm coelomocytes is largely based on differential staining, ultrastructure and granule composition, as well as behavioural traits such as adherence and chemotaxis [9]. The origin and relationships of the main populations of coelomocytes, namely amoebocytes and eleocytes [7] are not yet completely known. Perhaps amoebocytes derive from the mesenchymal lining of the coelom [9] while eleocytes originate by the detachment of chloragogen cells covering intestinal tract [1]. All these coelomic cells are involved in various aspects of cellular and humoral immunity: the former by phagocytosis [8], encapsulation [20], and cytotoxicity [17], and the latter by secretion of antimicrobial substances [6]. Chloragocytes/ eleocytes resemble the invertebrate liver cells in certain functions. For example, they are involved in the metabolism and storage of glycogen and lipids. They also transport nutrients via the circulation into the coelomic fluid as well as to various cells and organs [1, 9].

The most characteristic feature of eleocytes is the presence of distinct granules (chloragosomes) [1, 14]. During recent experiments we recorded a strong autofluorescence of eleocytes of *Dendrobaena veneta* [13], apparently absent in the amoebocytes. The number of autofluorescent eleocytes decreased in earthworms exposed to cadmium or copper and was restored after transfer to metal-free soil. This phenomenon yielded a new biomarker tool for the objective, rapid and reproducible quantification of the impact of various environmental toxicants on a cohort of immunocom-

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petent cells in a terrestrial receptor organism, the earthworm. However, during pilot studies on another earthworm species, *Lumbricus rubellus*, no autofluorescent coelomocytes were encountered. Therefore the main goal of the present study was to perform a rapid screening of 12 earthworm species to investigate the presence or absence of autofluorescent coelomocytes. As the number and composition of such cells may depend on exogenous (*i.e.* environmental) and endogenous (*i.e.* biotic, life-cycle) factors, all earthworms used for testing were collected within a short period of time in the same locality.

Materials and methods

Earthworms. Adult (sexually mature) earthworms were collected from Bute Park, Cardiff, in November. In total, 12 species were obtained, namely *Allolobophora chlorotica* (Savigny, 1826); *Aporrectodea caliginosa* (Savigny, 1826); *Aporrectodea longa* (Ude, 1885); *Dendrodrilus rubidus* (Savigny, 1826); *Eisenia fetida* (Savigny, 1826); *Lumbricus castaneus* (Savigny, 1826); *Lumbricus festivus* (Savigny, 1826); *Lumbricus rubellus* (Hoffmeister, 1843); *Lumbricus terrestris* L. (1758); *Octolasion cyaneum* (Savigny, 1826); *Octolasion tyrtaeum lacteum* (Örley, 1881), and *Octolasion tyrtaeum tyrtaeum* (Savigny, 1826) [16]. All earthworms were maintained at 15˚C in the original soil and used for experiments within one week after collection. The numbers and body weights of individuals from each particular species are presented in Table 1.

Harvesting of coelomocytes. Earthworms were stimulated for 1 min with a 4.5 V electric current to expel coelomic fluid with coelomocytes through the dorsal pores according to a procedure modified after Roch [15]. Briefly, after weighing, washing and dry-blotting, the earthworms were placed individually in Petri dishes containing 1-4 mL (dependent on the earthworm body weight) of extrusion fluid (PBS supplemented with 2.5 g/L EDTA) to prevent cell aggregation [12]. Extruded coelomocytes were counted in a haemocytometer. Our preliminary experiments revealed negligible number of free coelomocytes retrieved by Pasteur pipette from the coelomic cavity through the incised body wall of animals soon after electric shock (data not shown). Cell viability was assessed using Trypan Blue exclusion test [11], after mixing equal volumes of coelomocyte suspensions and 0.4% Trypan Blue (Sigma) solution. Cell viability always exceeded 95%. As earthworms of various weights were used, the number of cells was calculated per unit of body mass (Table 1). Cells were examined by fluorescence microscopy (Olympus BH-2) and photographed (FUJIX Digital Camera HC-3002). Each earthworm was used only once and coelomocytes from each particular earthworm were analysed individually.

Flow cytometry. Unfixed freshly prepared coelomocytes were analysed with a FACScalibur flow cytometer (BD Biosciences). Initial range-finding experiments used Draq V (Biostatus, Leicestershire, UK), a cell-penetrating DNA-binding fluorochrome, to discriminate intact cells from debris in order to determine appropriate voltage gain settings. During analytical experiments, 10000 thresholded events per worm sample were collected, with forward and side scatter and FL-1H autofluorescence being recorded. The resulting FCS files were analysed, using WinMDI 2.8 software (Joe Trotter, http://facs .scripps.edu), by producing quadrants to sector density plots of side scatter versus FL1-H autofluorescence and thus to quantify the proportions of cells that were strongly autofluorescent. The calculation of coelomocytes per worm and worm mass allowed the number of autofluorescent cells to be estimated.

Statistical analysis. Results were expressed as means ± standard errors. Differences between means were determined by ANOVA and post hoc Tukey test (with the level of significance established at $p<0.05$) using Statistica version 4.0. Correlation coefficients were calculated using Microsoft Excel version 97.

Results

Coelomocytes

Coelomocytes extruded from coelomic cavity were counted in each individual earthworm and recalculated per its body weight. Mean values and standard errors are presented in Table 1.

Fluorescence microscopy

Fluorescence microscopy observations of coelomocytes from representative adult Lumbricidae revealed high proportions of strongly autofluorescent large granular cells (morphologically identified as eleocytes) in 6 investigated species, while in the other 6 species autofluorescent strongly granular cells were very seldom or absent. In all 12 investigated species the smaller, strongly adherent cells (amoebocytes) were devoid of autofluorescence (Fig. 1). To exemplify our findings, Fig. 1 demonstrates the presence of large eleocytes with strongly autofluorescent granules in *O. t. tyrtaeum* (Fig. 1a,b) and the absence of such cells in *L. rubellus* (Fig. 1c,d). In conclusion, autofluorescence seems to be confined to the species possessing in coelomic fluid a rich population of strongly granular eleocytes.

Flow cytometry

Flow cytometry confirmed microscopical observations; both techniques revealed a significant number of autofluorescent coelomocytes in 6 out of 12 investigative species (Fig. 1e). Flow cytometric analysis of FL1-H autofluorescence against side scatter (indicative of cell complexity or granularity) revealed that autofluorescence was mainly restricted to the cohort of highly granular cells (eleocytes). The proportions of cells which were strongly autofluorescent, was species-specific.

Quantitative analysis

The percentage of autofluorescent cells established by flow cytometry was species-specific, ranging from negligible (<1%) to 35% (Table 1, Fig. 2a). The 12 Lumbricidae species sampled differed significantly in body weight, namely from 0.17±0.02 g (*D. rubidus*) to 3.96±0.27 g (*L. terrestris*) (Fig. 2b). The number of coelomocytes expelled from the coelom of earthworms ranged from $0.3\pm0.1 \times 10^6$ (*L. castaneus*) to $3.4\pm0.5 \times$

Table 1. Autofluorescent cells in coelomocytes of 12 earthworm species.

Earthworms			Coelomocytes				
			Total		Autofluorescent		
Species	N	BW [g]	TC [$\times 10^6$]	TC/BW [$\times 10^6$/g]	% AFC	AFC [$\times 10^6$]	AFC/BW [$\times 10^6$/g]
Allolobophora chlorotica	7	0.22±0.01	1.3±0.1	5.7±0.7	30±4	0.39±0.06	1.7±0.3
Aporrectodea caliginosa	5	0.91±0.12	0.4±0.1	0.6±0.2	1.0±0.3	0.004±0.002	0.006±0.003
Aporrectodea longa	5	1.90±0.16	1.6±0.5	0.9±0.3	0.2±0.1	0.003±0.002	0.002±0.001
Dendrodrilus rubidus	7	0.17±0.02	1.1±0.1	5.5±1.0	21.0±6.5	0.23±0.08	1.2±0.4
Eisenia fetida	5	0.59±0.04	3.0±0.3	5.3±0.5	22±1	0.65±0.08	1.1±0.1
Lumbricus castaneus	5	0.19±0.01	0.3±0.1	1.7±0.4	0.5±0.2	0.002±0.001	0.009±0.004
Lumbricus festivus	5	0.97±0.06	2.0±0.3	2.2±0.2	0.05±0.03	0.0010±0.0006	0.0011±0.0007
Lumbricus rubellus	5	0.59±0.04	0.8±0.1	1.4±0.1	0.5±0.3	0.004±0.002	0.007±0.004
Lumbricus terrestris	5	3.96±0.27	1.9±0.3	0.5±0.1	0.5±0.3	0.009±0.005	0.002±0.001
Octolasion cyaneum	3	1.54±0.03	1.5±0.1	0.9±0.1	22±8	0.33±0.12	0.2±0.1
Octolasion tyrtaeum lacteum	5	0.40±0.02	1.3±0.3	3.0±0.7	11±3	0.15±0.05	0.3±0.1
Octolasion tyrtaeum tyrtaeum	5	0.70±0.07	3.4±0.5	4.7±0.5	35±8	1.17±0.31	1.6±0.4

N – number of individuals; BW – body weight; TC – coelomocytes; TC/BW – coelomocytes per g body weight; %AFC – % autofluorescent coelomocytes; AFC – number of autofluorescent coelomocytes per earthworm; AFC/BW – number of autofluorescent cells/g body weight; means ± SE.

10^6 (*O. t. tyrtaeum*) (Fig. 2c). Flow cytometry facilitated the calculation of the total number of fluorescent cells per earthworm, and per unit body weight (Table 1). The low percentages (<1%) and negligible numbers of autofluorescent cells were recorded in three representatives of *Lumbricus* sp. (*L. castaneus*, *L. festivus*, *L. rubellus*, *L. terrestris*) and in two representatives of *Aporrectodea* sp. (*A. caliginosa* and *A. longa*). The high percentages and the high numbers of autofluorescent cells (both when expressed as a portion of total pool of coelomocytes and recalculated per unit body weight) were recorded in *Allolobophora chlorotica, Dendrodrilus rubidus, Eisenia fetida, and Octolasion sp.* (*O. cyaneum, O. tyrtaeum tyrtaeum, O. tyrtaeum lacteum*) (Figs 2c,d).

Correlations

The number of coelomocytes per gram of body weight was the lowest in the largest species, *L. terrestris* ($0.5\pm0.1 \times 10^6$/g), but the highest in *A. chlorotica* ($5.7\pm0.7 \times 10^6$/g), a medium sized species (0.22±0.01 g) (Fig. 2d). This indicates the absence of a simple correlation between the body size/weight and the number of coelomocytes inhabiting the coelomic cavity (correlation coefficient r = 0.18, Fig. 3a), a phenomenon recorded previously during an earlier, more restricted, comparative study on the coelomocytes of *L. terrestris*, *E. fetida*, *D. veneta* and *A. chlorotica* [12].

There was a high positive correlation between the number of autofluorescent coelomocytes and the total number of coelomocytes (r = 0.77, Fig. 3b), but the correlation coefficient was even higher after recalculation to take account of inter-species differences in body weight (r = 0.93, Fig. 3c).

Discussion

We are unaware on any papers dealing in detail with the intrinsic fluorescence of earthworm coelomocytes, although some authors mentioned the presence of fluorescent granules in the cells forming the chloragogen tissue around the intestine [14] or in some free-floating coelomocytes [8]. Moreover, the coelomic fluid of *Eisenia andrei* displays specific fluorescence absent in that of *E. fetida* [2].

In the present paper, a population of autofluorescent large granular eleocytes among coelomocytes expelled with coelomic fluid during electric shock was convincingly detected by two complementary methods, fluorescence microscopy and flow cytometry. A remarkable divergence was detected in different earthworm species, as cells with autofluorescent granules (eleocytes) were common in representatives of the genera *Allolobophora, Dendrodrilus, Eisenia* and *Octolasion* while such granular cells were very seldom in coelomic exudates from *Aporrectodea* sp. and *Lumbricus* sp.

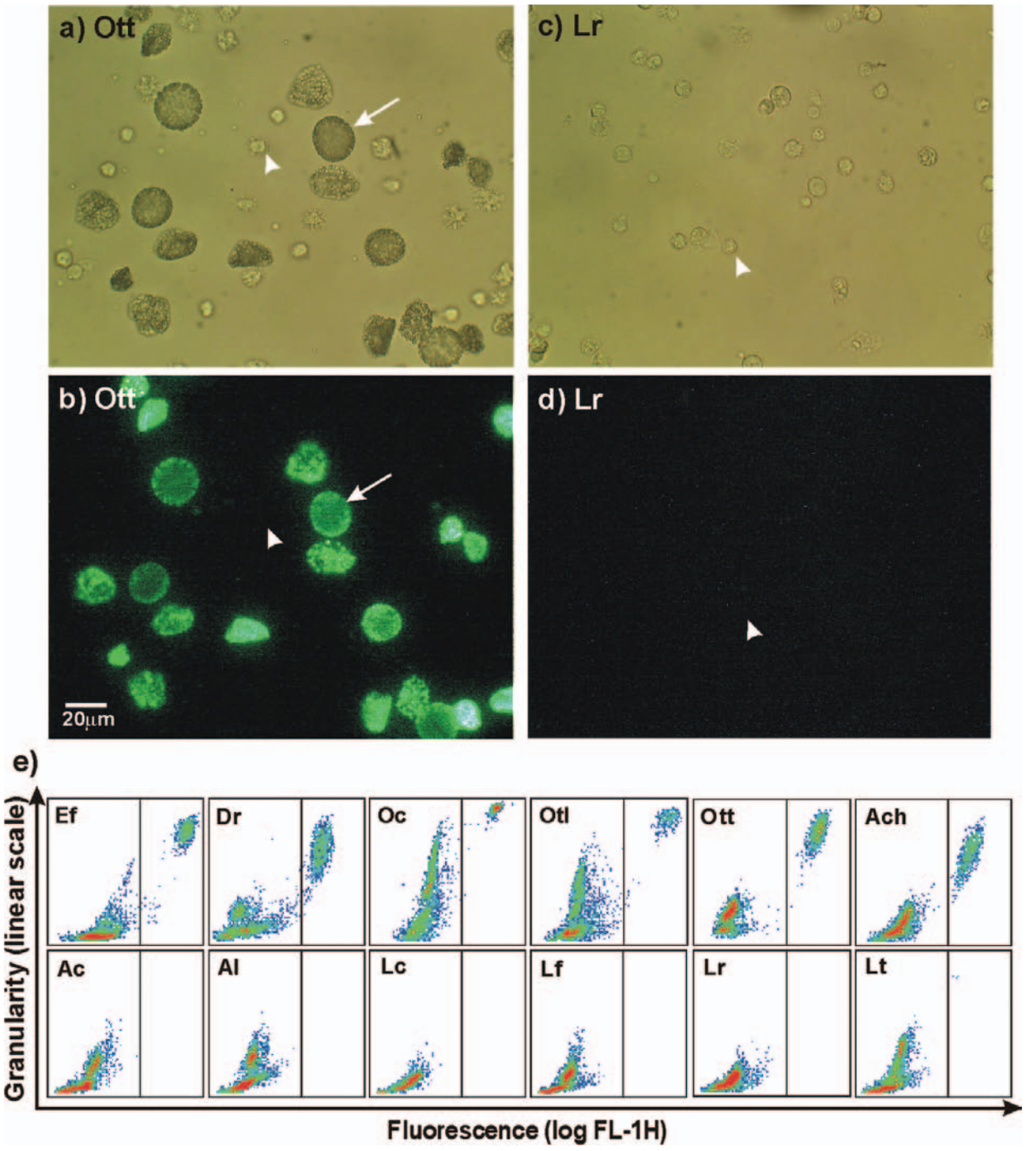

Fig. 1. Coelomocytes of *Octolasion tyrtaeum tyrtaeum* (**a, b**) and *Lumbricus rubellus* (**c, d**). Phase-contrast (upper row), and fluorescence (lower row). Autofluorescent eleocytes are indicated by arrows and non-autofluorescent amebocytes by arrowheads. Scale bar = 20 µm. **e.** Representative examples of flow cytometric density plots of coelomocytes from 12 earthworm species (family - Lumbricidae). Note: Autofluorescent coelomocytes (if present) are in the right part of each plot. Abbreviations: Ach – *Allolobophora chlorotica*; Ac – *Aporrectodea caliginosa*; Al – *Aporrectodea longa*; Dr – *Dendrodrilus rubidus*; Ef – *Eisenia fetida*; Lc – *Lumbricus castaneus*; Lf – *Lumbricus festivus*; Lr – *Lumbricus rubellus*; Lt – *Lumbricus terrestris*; Oc – *Octolasion cyaneum*; Otl – *Octolasion tyrtaeum lacteum*; Ott – *Octolasion tyrtaeum tyrtaeum*.

Statistical analysis revealed that autofluorescent eleocytes are common in the earthworm species with a high number of coelomocytes per body weight. We can speculate that such an "excess" of coelomocytes may originate from the detachment of some chloragogen cells forming the intestinal lining. Another possibility is

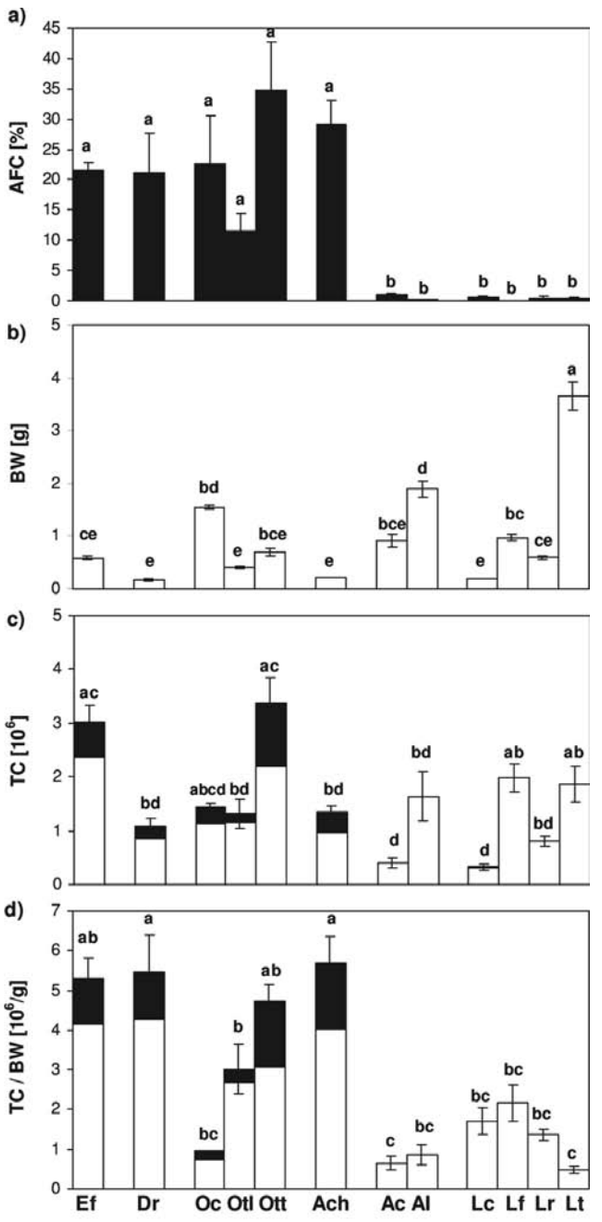

Fig. 2. Characteristics of coelomocytes in 12 earthworm species. **a.** Percentage of autofluorescent coelomocytes (AFC). **b.** Earthworm body weight (BW). **c.** Total number of coelomocytes (TC). **d.** Coelomocyte number per g body weight (TC/BW). Black columns (a, c and d) denote the proportion of autofluorescent cells (AFC). Within each panel, columns with different lower-case letters are statistically different at p≤0.05 determined by ANOVA and *post hoc* Tukey tests.

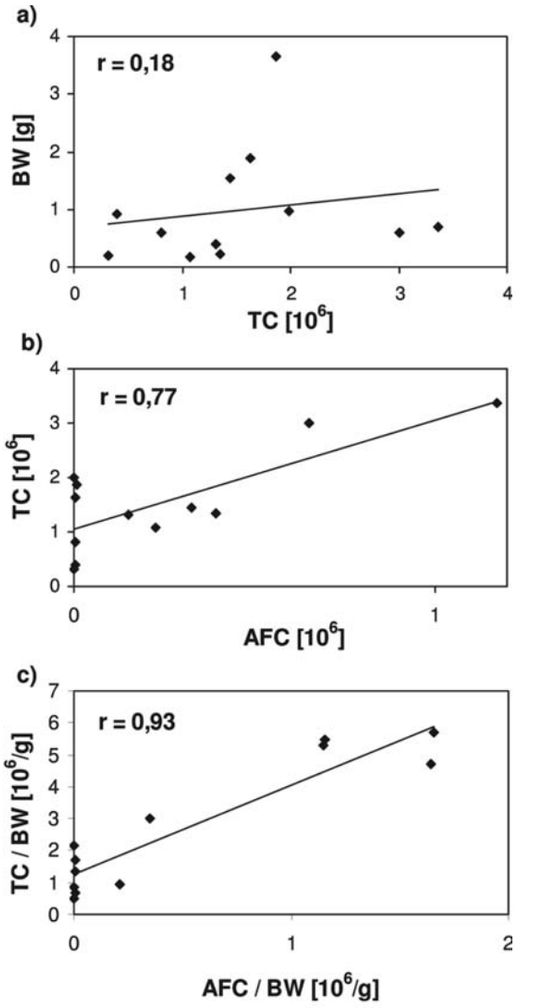

Fig. 3. Correlations between the species-specific (**a**) total number of coelomocytes (TC) and body weight (BW); (**b**) number of autofluorescent coelomocytes (AFC) and total number of coelomocytes (TC); (**c**) number of autofluorescent coelomocytes per body weight (AFC/BW) and number of coelomocytes per g body weight (TC/BW); r – correlation coefficient.

that free-floating amoebocytes become fluorescent during clearance of chloragosomes shed from the degraded chloragogen cells; experiments on verification of such hypothesis are in progress. It is also conceivable that the coelomocytes from the species equipped with an "excess" of these cells are capable of performing distinct function(s), such as the synthesis/storage of fluorescent material, including flavins [3], porphyrins [22], and/or lipofuscins [18, 21].

The synthesis of lipofuscin and melanin during formation of brown bodies by *Eisenia fetida andrei* coelomocytes was convincingly demonstrated by Valembois *et al.* [21]. These brown pigments result from the insolubilization of oxidised organic substrates and represent the last stage of catabolism and segregation of unwanted materials. Thus lipofuscin is a good candidate for a source of autofluorescence in earthworm chloragocytes/eleocytes. Our recent spectrofluorometric analysis

unequivocally revealed participation of flavins (perhaps originating from mitochondria degraded by autophagy) in fluorescence of earthworm coelomocytes and coelomic fluid, especially pronounced in *Eisenia fetida* (in preparation). Interestingly, according to Albani *et al.* [2], the cell-free coelomic fluid of this species is devoid of specific fluorescence characteristic for that in *E. andrei*, derived from fluorophores different than flavins [2]. We may assume that the pool of fluorophores synthesized and/or stored in eleocytes may be different from those accumulated in the coelomic fluid, perhaps derived from variety of cells apart from coelomocytes.

At present we cannot eliminate the possibility that species with low autofluorescent cell content in their electrically stimulated coelomic exudates have some means of retaining their population of large, granular, immune cells. If this is the case, then two inferences may be made. First, the observed inter-species differences relate less to the composition of the community of the cells suspended in earthworm coelomic fluid, but more to inter-species differences in the behaviour and fate of the cells. Second, eleocytes are indeed derived from chloragocytes. The second supposition is strongly supported by our preliminary observations of chloragocytes from intestinal lining of *Dendrobaena veneta* and *Lumbricus terrestris.* The chloragogenous tissue of *D. veneta* contains an abundant population of autofluorescent cells what corresponds with high percentage of autofluorescent eleocytes among free coelomocytes of this species. In contrast, autofluorescent cells are scarce in the chloragogenous tissue of *L. terrestris* what corresponds with a scarcity of free autofluorescent eleocytes in the coelomic fluid. At present we cannot exclude a possibility that the number and autofluorescence of chloragocytes/eleocytes is related to nutrition compounds of the particular species and may vary in the annual cycle.

Although the precise function and inter-species differences of the autofluorescent coelomocytes still remain elusive, changes in autofluorescence have been identified in *Dendrobaena veneta* in response to environmental stressors, such as metal pollution [13], a phenomenon that may well be used as a new, fast, simple and reproducible tool in ecotoxicology.

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