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Riboflavin content in autofluorescent earthworm coelomocytes is species-specific

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Abstract: We have recently shown that a large proproportion of earthworm coelomocytes exhibit strong autofluorescence in some species (*Dendrobaena veneta, Allolobophora chlorotica, Dendrodrilus rubidus, Eisenia fetida, and Octolasion spp.*), while autofluorescent coelomocytes are very scarce in representatives of *Lumbricus spp.* and *Aporrectodea spp.* Riboflavin (vitamin B2) was identified as a major fluorophore in *Eisenia fetida* coelomocytes. The main aim of the present experiments was to quantify riboflavin content in autofluorescent coelomocytes (eleocytes) from several earthworm species through a combination of flow cytometric and spectrofluorometric measurements. Spectrofluorometry of coelomocyte lysates showed that riboflavin was non-detectable in the coelomocytes of *Aporrectodea spp.* and *Lumbricus spp.*, but was a prominent constituent of lysates from species with autofluorescent eleocytes. In the latter case, riboflavin content was the highest in *E. fetida*, followed by *Octolasion spp.* > *A. chlorotica* > *D. rubidus*. The riboflavin content of coelomocytes correlates positively with eleocyte autofluorescence intensity measured by flow cytometry and visible with fluorescence microscopy.

Key words: Earthworms - Coelomocytes - Autofluorescence - Riboflavin - Lipofuscin - Flow cytometry - Spectrofluorometry

Introduction

Coelomocytes are immunocompetent cells suspended and circulating in the coelomic fluid constituting the hydrostatic skeleton of oligochaete annelids, including earthworms. These cells can be rapidly expelled under stressful conditions through inter-segmental dorsal pores in the body wall by increased coelomic pressure [16]. The phenomenon has been exploited as a non-invasive method of sampling coelomic fluid and coelomocytes using electric current [14], ethanol [5], or ultrasound stimuli [8].

Two main cohorts or lineages of coelomocytes, namely amoebocytes and eleocytes, are present in various proportions in different earthworm species [10]. There are indications that amoebocytes derive from the mesenchymal lining of the coelom [7], whilst eleocytes (chloragocytes) differentiate from the chloragogen cells that cover the coelomic surfaces of the alimentary tract and major blood vessels [1, 13]. Analysis by phase contrast/fluorescence microscopy and flow cytometry has demonstrated a high percentage (>10%) of autofluorescent eleocytes in 6 out of 12 investigated earthworm species, namely in *Dendrobaena veneta* [11], *Allolobophora chlorotica, Dendrodrilus rubidus, Eisenia fetida*, and *Octolasion spp.* (*O. cyaneum, O. tyrtaeum tyrtaeum* and *O. tyrtaeum lacteum*). In contrast, less than 1% of autofluorescent coelomocytes was recorded in representatives of *Aporrectodea spp.* (*A. caliginosa* and *A. longa*) and *Lumbricus spp.* (*L. castaneus, L. festivus, L. rubellus, L. terrestris*) [4].

In a recent paper, we have shown by spectrofluorometry (excitation and emission spectra, and fluorescence lifetime) and HPLC analysis of coelomocyte suspensions and supernatants that riboflavin is the

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Abbreviations: Ach - *Allolobophora chlorotica*; Dr - *Dendrodrilus rubidus*; Ef - *Eisenia fetida*; Lt - *Lumbricus terrestris*; Oct - *Octolasion sp.*; RF - riboflavin; a.u. - arbitrary units

main fluorophore responsible for fluorescence in *Eisenia fetida* eleocytes [9]. This is a significant observation in the context of expanding present understanding of the mechanisms of cellular immunity in earthworms [6], because a number of studies on mammalian systems have confirmed a potentiating role for riboflavin in immune responses. For example, riboflavin (vitamin B2) increases neutrophil migration [17], count, and activation [12] in mice and cattle. The vitamin also displays a potent ability, probably through its reducing properties, to incapacitate the human malaria parasite *Plasmodium falciparum* [2].

Short wavelength fluorescence of unknown origin (excitation at 320 nm and emission at 370 nm) was recently detected by Albani *et al.* [3] in the coelomic fluid of *E. andrei*, while a similar fluorescence signal was absent from samples derived from the closely related and ecologically similar species, *E. fetida*. Thus, comparative coelomic fluid fluorescence measurements might differentiate morphologically similar or undistinguishable earthworm species.

The main aims of the present study were to measure and compare the riboflavin contents of coelomocytes from a number of ecophysiologically different earthworm species to establish whether there is a correlation with the previously observed inter-species differences in eleocyte autofluorescence.

Materials and methods

Earthworms. Clitellate (sexually mature) earthworms (*Allolobophora chlorotica, Aporrectodea caliginosa, Aporrectodea longa, Dendrodrilus rubidus, Eisenia fetida, Lumbricus castaneus, Lumbricus festivus, Lumbricus rubellus, Lumbricus terrestris,* and *Octolasion spp.*) [15] (8-12 individual worms per species) were collected from an uncontaminated site at Pontcanna Fields, Cardiff, in November/December. All earthworms were maintained in a constant temperature, fixed light regime (12:12 dark:light) room at 19°C in their field soil and used for experiments within one week after collection.

Harvesting of coelomocytes. Earthworms were stimulated for 1 minute with a 4.5V electric current to expel coelomic fluid with coelomocytes through the dorsal pores according to a procedure modified after Roch [14]. 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 [10]. Extruded coelomocytes were counted in a haemocytometer and adjusted to 10^6 cells per sample. Cells were examined in transmission bright field and by fluorescence microscopy (Olympus BH-2, objective ×20) and photographed (Fujix Digital Camera HC-3002) or used for flow cytometry and/or spectrofluorometry. Each earthworm was used only once and coelomocytes from each particular earthworm were analysed individually.

Flow cytometry. Coelomocytes were analysed with a FACScalibur flow cytometer (BD Biosciences). During analytical experiments, 10000 thresholded events per worm sample were collected, with side scatter (for cell complexity/granularity) and forward scatter (for FL-1H fluorescence; excitation 488 nm; emission 530 nm) being recorded. The resulting 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 FL1-H histograms. The proportions of strongly autofluorescent cells (arbitrarily assessed as those located to the right from the value 200 on the FL1-H axis) were quantified, and co-ordinates of the main peak of autofluorescent cells were assessed. Mean values concerning the percentage of autofluorescent cells and autofluorescence intensity in each particular species were compared.

Spectrofluorometric measurements. The spectrofluorometric measurements were performed on coelomocyte suspensions and/or lysates with 2% Triton (Sigma). Riboflavin (Sigma) solution with 2% Triton was used for preparation of the standard curve. Measurements were performed using F-4500 Fluorescence Spectrophotometer and U-2800 Spectrophotometer (Digilab Hitachi). Excitation spectra were recorded between 300-520 nm (emission at 522 nm), while emission spectra were recorded between 380-700 nm using excitation at 370 nm, 360 nm, or 350 nm.

Statistical analysis. Results were expressed as means ± standard deviations. Differences between means were determined by the Mann-Whitney U test (with the level of significance established at $p<0.05$), using Microsoft Excel v. 97.

Results

Fluorescence microscopy

The present results confirmed earlier findings [4] that the coelomocytes from *Lumbricus* and *Aporrectodea* species were seldom autofluorescent but, in contrast, that the granular eleocytes (but not amoebocytes) of certain other species exhibited autofluorescence, albeit displaying species-specific intensity levels. Autofluorescence was relatively strong in eleocytes from *E. fetida* (e.g. Fig. 1a top) and *Octolasion spp.*, but was weak in the eleocytes of *A. chlorotica* (e.g. Fig. 1a bottom) and *D. rubidus*.

Flow cytometry

Microscopic observations were supported by flow cytometry. Figure 1b shows examples of representative histograms (left panel) and density plots (right panel) from a representative series of experiments performed on cells investigated on the same day under the same conditions. Coelomocytes from *E. fetida*, *A. chlorotica*, *D. rubidus*, and *Octolasion spp.* were each bimodally distributed, with the cohort of cells to the right of each FL1-H histogram representing autofluorescent eleocytes; a corresponding peak was absent in *L. terrestris*. It is noteworthy that the positions of the peaks representing autofluorescent eleocytes differ in the four species possessing them: the autofluorescence peak in *E. fetida* is strongly biased toward the right on FL1-H axis (indicating the strongest autofluorescence intensity) (Fig. 1b top), whilst the autofluorescence peak has a distinct left bias in the three remaining species (*e.g.* in *A. chlorotica*, Fig. 1b bottom).

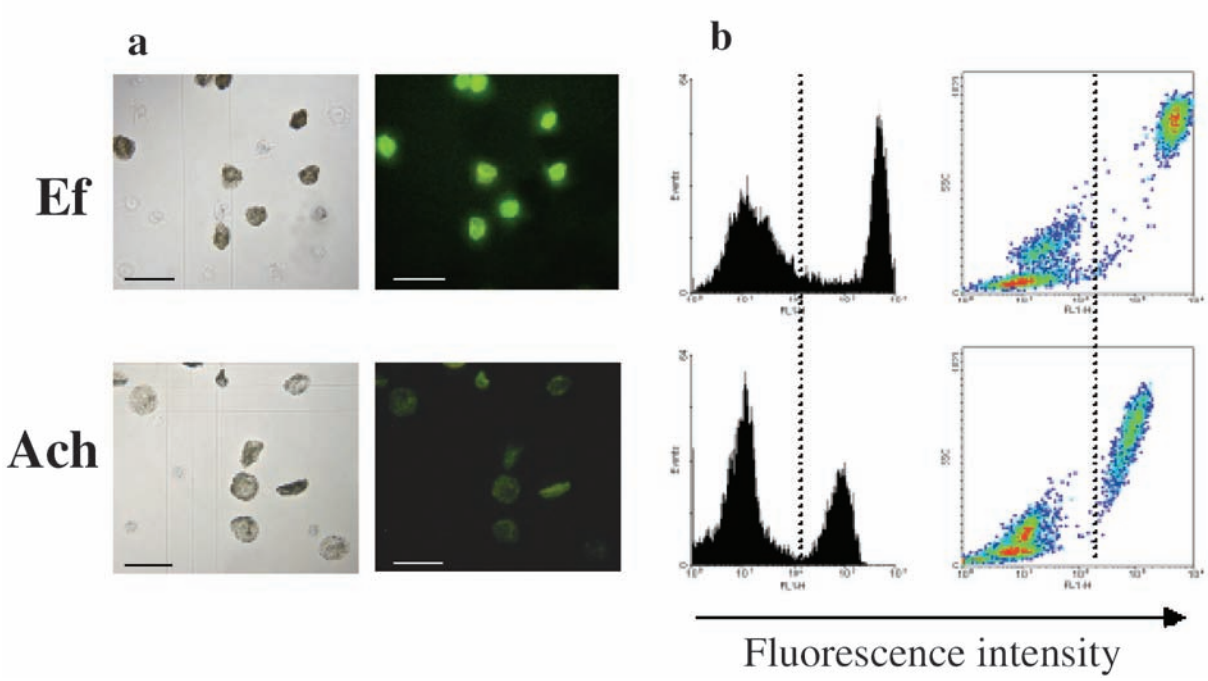

Fig. 1. Representative examples of autofluorescence intensity in *Eisenia fetida* (Ef, top) and *Allolobophora chlorotica* (Ach, bottom) coelomocytes. **a** - microscopy in haemocytometer; left - bright field, right - fluorescence (illumination of the same intensity) in both specimens. Only large granular cells (eleocytes) are fluorescent. Scale bars - 50 μm. **b** - flow cytometry; left - FL1-H histograms, right - density plots (FL1-H against cell complexity SSC). The distinct right peaks (at FL1-H above 200, i.e. right to the dotted vertical lines) are related to autofluorescent eleocytes.

Percentage of autofluorescent cells and quantitative analysis of autofluorescence intensity

Statistical analysis was restricted to the group of species with distinct populations of autofluorescent eleocytes (more then 20%) arbitrarily assessed as the cells in quadrants located to the right to 200 on FL1-H axis. The mean percentages of autofluorescent eleocytes in *E. fetida* and *A. chlorotica* (44% and 38%, respectively) were higher than those in *D. rubidus* and *Octolasion spp.* (25% and 24%, respectively) (Fig. 2a). Fluorescence intensity was highest in *E. fetida*, lower in *Octolasion spp.*, and very low in *A. chlorotica* and *D. rubidus* (mean X values on FL1-H being 3367, 670, 485, and 1309, respectively) (Fig. 2b).

Spectrofluorometry

Spectrofluorometric analyses were performed on coelomocyte lysates in 2% Triton because they yielded much more consistent results then those obtained on cell suspensions or supernatants (data not shown).

Riboflavin solutions in PBS with 2% Triton were used to obtain the control excitation spectrum at 522 nm (with two accompanying peaks at 370 nm and 450 nm) (Fig. 3a) and the emission spectrum with a peak at 522 nm (Fig. 3b), and for constructing standard curves (at 360 nm and 370 nm) (Fig. 4a). Qualitatively similar excitation spectra were obtained from coelomocyte lysates from the species with high number of autofluorescent cells. Figure 3c presents comparisons of the emission spectra of the representative samples of 1×10^6 coelomocytes from the four earthworm species. The peaks at 522 nm are evident in each of them, albeit of different height in the order *E. fetida* >> *A. chlorotica* > *Octolasion spp.* >> *D. rubidus*. Figure 3c also illustrates the existence of an appreciable intra-species variability in *E. fetida* (compare Ef1 and Ef2). These observations on *E. fetida* from Cardiff fully correspond with those obtained previously on Polish populations [9]. They indicate that riboflavin accumulation in eleocytes is not simply a site-specific biochemical phenomenon reflecting local dietary constituents. Figure 3d shows the details of fluorescence in three individuals of *D. rubidus*, with riboflavin peaks being evident but very low in comparison with those in *E. fetida*. Emission peaks at 522 nm were negligible to non-detectable in coelomocyte lysates from *Lumbricus spp.* and *Aporrectodea spp.*, species deficient in autofluorescent cells.

Calculation of riboflavin content in autofluorescent cells

The emission value at 522 nm in each particular sample was converted to amount of riboflavin (in ng)

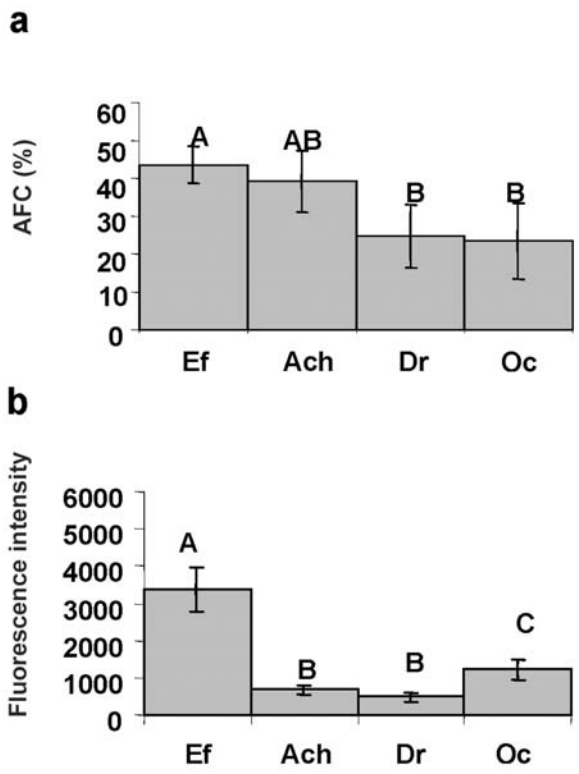

Fig. 2. The results of flow cytometry analysis of coelomocytes from four earthworm species: *Eisenia fetida* (Ef), *Allolobophora chlorotica* (Ach), *Dendrodrillus rubidus* (Dr), and *Octolasion spp.* (Oct). a - percentages of autofluorescent cells; b - intensity of autofluorescence. Means ± SD. Different capital letters are located at means statistically different according to Mann-Whitney U test, at $p<0.05$.

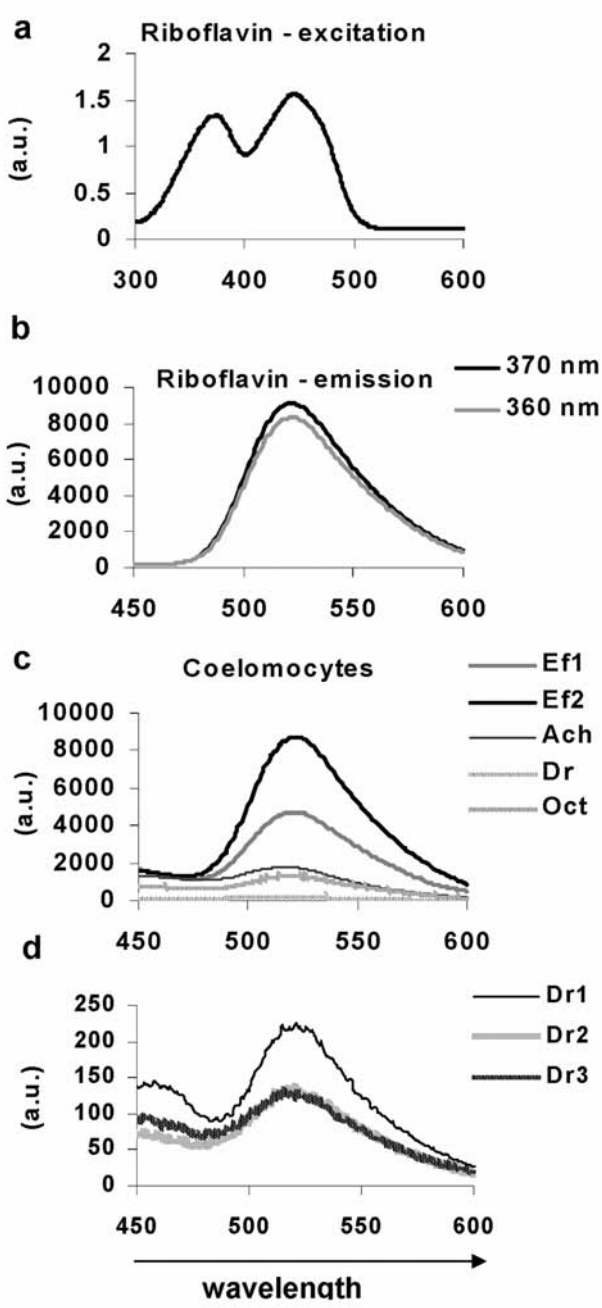

Fig. 3. Spectrofluorometry of riboflavin (Sigma) standard solution (a, **b**) and representative samples of 1×10^6 earthworm coelomocyte lysates (c, d) in PBS with 2% Triton. **a** - riboflavin excitation spectrum monitored at emission wavelength of 522 nm; **b** - riboflavin emission spectrum at 360 nm and 370 nm; **c** - emission spectra (at 370 nm) of samples of *Eisenia fetida* (Ef1, Ef2), *Allolobophora chlorotica* (Ach), *Dendrodrilus rubidus* (Dr), and **Octolasion spp.** (Oct) coelomocytes; **d** - emission spectra (at 370 nm) of samples of 3 individuals of *Dendrodrillus rubidus* (Dr1, Dr2, Dr3) coelomocytes.

according to the standard curve (Fig. 4a). The mean values of riboflavin content per 1×10^6 coelomocytes are displayed in Fig. 4b. As fluorophores responsible for coelomocyte autofluorescence are accumulated mainly (if not exclusively) in eleocytes, thus we have to recalculate the amount of riboflavin in each particular lysate of 10^6 coelomocytes to that in eleocytes only. Cytometric analysis gave us information about the percentages of eleocytes in each sample (means seen in Fig. 2a), thus we could calculate the number of eleocytes per 10^6 coelomocytes, and then the amount of riboflavin per one eleocyte in each particular sample (in pg). The mean amount of riboflavin (in pg) in one eleocyte for each species are displayed in Fig. 4c. A striking similarity is evident between Figs 2b and Fig. 4c, indicating a correspondence between species-specific fluorescence intensity and the amount of riboflavin per autofluorescent cell.

Discussion

Microscopy, flow cytometry, and spectrofluorometry from previous [4, 9] and present experiments are suggestive of inter-species differences in the quantity and/or quality of eleocyte fluorophores. A striking correlation between species-specific fluorescence intensity and the amount of riboflavin per autofluorescent cell indicates that riboflavin is the main source of autofluorescence in the earthworm species with autofluores-

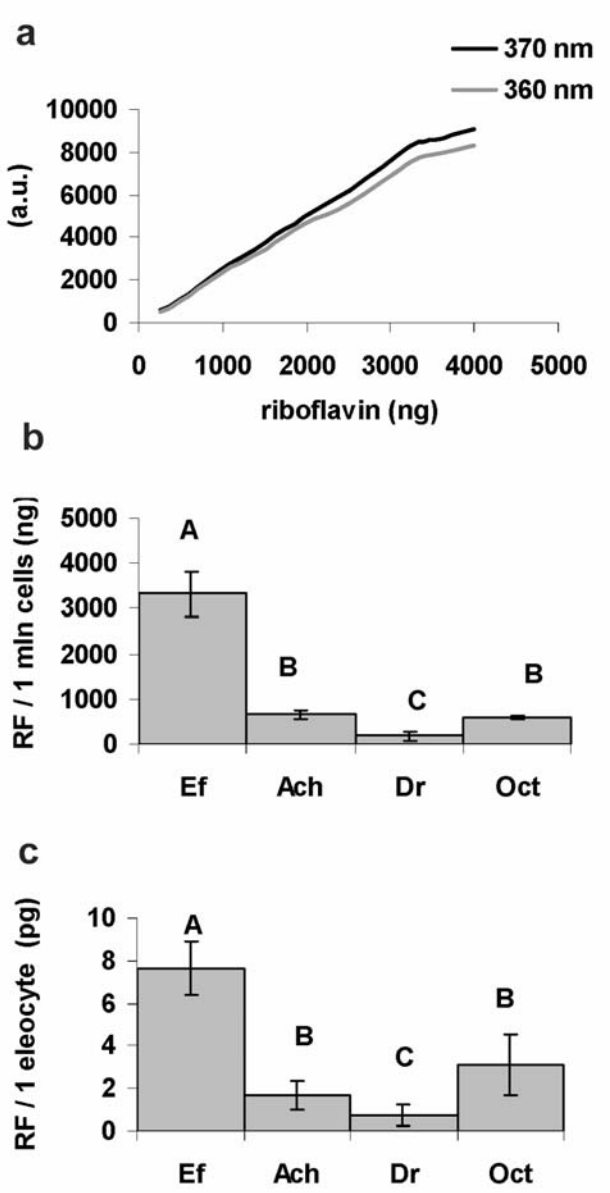

Fig. 4. Riboflavin content in coelomocytes from the four earthworm species: *Eisenia fetida* (Ef), *Allolobophora chlorotica* (Ach), *Dendrodrilus rubidus* (Dr), and *Octolasion spp.* (Oct). **a** - riboflavin standard curves at 370 nm and 360 nm; **b** - amount of riboflavin (in ng) in the samples containing 1×10^6 coelomocytes; **c** - amount of riboflavin (in pg) per single autofluorescent coelomocyte (eleocyte). Means ± SD. Different capital letters are located at means statistically different according to Mann-Whitney U test, at $p<0.05$.

cent eleocytes. The amount of riboflavin shows both inter-specific differences in the order *E. fetida* >> *A. chlorotica* > *Octolasion spp.* >> *D. rubidus* and pronounced intra-species variability. Species-specific and individual differences in riboflavin (vitamin B2) content may reflect species-specific nutritional preferences and/or vitamin B2 availability in the local food resources. We cannot exclude that riboflavin also originates from the lysosome-mediated autophagy of mitochondria in ageing chloragocytes/eleocytes. It is premature to ascribe a biological role (if any) to the vitamin B2 accumulation in earthworm eleocytes; we may assume that these cells function as vitamin stores/suppliers to the organism and/or some vitamins intensify eleocyte function during immune responses.

HPLC analysis of *E. fetida* coelomocytes revealed two peaks of fluorescence, one main peak corresponding to that of the riboflavin standard, and a minor unidentified peak with a shorter retention time [9]. The latter implies the presence of a secondary distinct fluorophore(s) that may contribute to eleocyte autofluorescence in this, and perhaps other, earthworm species. Two peaks in the fluorescence emission spectra (at 460 nm and 520 nm) were observed by Peeters-Joris [13] in homogenates of *Allolobophora caliginosa* chloragocytes with 365 nm excitation. We surmise that the long-wavelength peak in chloragocytes of *A. caliginosa* [13] corresponds with the riboflavins detected in *E. fetida* and *A. chlorotica* eleocytes in our present experiments, thus consolidating the hypothesis that free-floating eleocytes originate through the sloughing of attached, mature chloragocytes into the coelomic fluid that bathes them [1].

In conclusion, riboflavin (detectable by spectrofluorometry at 360-370 nm wavelength excitation) is the predominant molecular contributor to the autofluorescence displayed by the eleocytes of certain earthworm species, albeit its amount is species-specific: *E. fetida* > *Octolasion spp.* > *A. chlorotica* > *D. rubidus*.

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