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Preliminary Report on Coelomocyte System During Ontogeny of the Earthworm *Dendrobaena veneta*

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Dendrobaena veneta belongs to the earthworm species which coelomic fluid contains two main cohorts of freely floating coelomocytes, namely amoebocytes and autofluorescent chloragocyte-derived eleocytes, with riboflavin being one of fluorophores stored in chloragosomal granules. The aim of the present studies was to follow the coelomocyte system during ontogeny of *D. veneta* reared at 17°C in the controlled laboratory conditions. In total, 90 worms of body weights from 0.1 g till 3.4 g, among them freshly hatched, juveniles, and clitellated adults, were subjected to electrostimulation-induced expulsion of coelomocyte-containing coelomic fluid. Expelled coelomocytes were analysed using a combination of cell counts, flow cytometric detection of eleocytes, and spectrofluorimetric measurements of riboflavin stored in coelomocytes. Flow cytometry revealed that the percentages of eleocytes slightly declined with worm body weights (r=0.16). A correlation between the body weights and the total number of coelomocytes (r=0.73) was more similar to that concerning amoebocyte numbers (r=0.68), than the number of eleocytes (r=0.52) and riboflavin content in coelomocyte lysates (r=0.66). Amount of riboflavin per eleocyte slightly declined with age/body weight (r=0.46). Characteristic spectra of riboflavin with a distinct emission peak at 525 nm were consistently obvious in coelomocyte lysates from juvenile and adult eartworms of body weight above 0.2 g, while these spectra were often absent and/or obscured by other fluorophore(s) with the emission peak at 380 nm in very small worms. The results of present experiments confirmed a general similarity of freely floating coelomocyte system in juvenile and adult worms while data concerning freshly hatched worms need further elucidation.

Key words: earthworms, ontogeny, coelomocytes, amoebocytes, eleocytes, riboflavin

INTRODUCTION

Like other annelid invertebrates, earthworms possess the metameric coelomic cavity filled with coelomic fluid containing various antimicrobial factors like lysozyme and antimicrobial peptides and free wandering cells named coelomocytes, all of them responsible for humoral and cellular earthworm immunity (Bilej et al., 2011). The earthworm coelomocytes consist of amoe-

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bocytes, being classical immunocytes (according to Ottaviani's nomenclature, 2011), plus a species-specific portion of eleocytes, being detached chloragocytes derived from the chloragogen tissue that surrounds the intestine. The chloragocytes/eleocytes, but not amoebocytes, exhibit autofluorescence, which predisposes them to flow cytometric analysis (Cholewa et al., 2006). This autofluorescence is restricted to chloragosomal vesticles (Plytycz et al., 2007) and comes from – among other sources – riboflavin (Koziol et al., 2006; Plytycz et al., 2006; Cygal et al., 2007; Plytycz and Morgan, 2011).

Dendrobaena veneta belongs to the species with a high number of eleocytes storing moderate amounts of riboflavin which is also accumulated in chloragocytes of the chloragogen tissue (Mazur et al., 2011; Plytycz and Morgan 2011; Rorat et al. 2013). The aim of the present research is to study of ontogeny of coelomocyte system in this species, from that in freshly hatched individuals, through juveniles to adults of moderate and large body weights.

MATERIALS AND METHODS

Earthworms

Adult *Dendrobaena veneta* (Oligochaeta; Lumbricidae), purchased from the commercial supplier (Ekargo, Słupsk), were reared in commercial soil (PPUH Biovita, Tenczynek) under controlled laboratory conditions (17°C; 12:12 LD). The worms were kept in plastic boxes with perforated lids and the moisture level was checked weekly. The worms were 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. Experiments were performed on 90 individuals of the wide range of body weights.

Coelomocyte extrusion

The earthworms were stimulated for 30 seconds with an electrical current (4.5 V or 12.3 V) to expel coelomic fluid with suspended coelomocytes through the dorsal pores. Briefly, the weighed earthworms were individually placed in Petri dishes containing 1-3 mL of extrusion fluid (phosphate-buffered saline, PBS, supplemented with 2.5 g/L ethylenediamine tetra-acetic acid, EDTA; Sigma-Aldrich); the extruded coelomocyte suspensions were used for spectrofluorimetric analysis of fluorophores or were fixed in 2% formalin (Sigma) and used for cell counts in haemocytometer and for flow cytometry.

Flow cytometric measurement and analysis

Samples of coelomocytes were analysed with a FACScalibur flow cytometer (BD Biosciences). During analytical experiments, 5000 thresholded events per worm sample were collected and analysed on the basis of their forward scatter (FS) (for cell size) and sideward scatter (SS) (cell complexity) properties. Fluorescence FL1-H (emission 530 nm; excitation 488 nm) was recorded. The resulting files were analysed for percentages of autofluorescent granulated eleocytes using WinMDI 2.8 software (Joe Trotter, http://facs.scripps.edu), by producing dot plots of cell size versus FL1 autofluorescence.

Coelomocyte numbers

Total numbers of coelomocytes in each particular sample (CN) were counted in haemocytometer. Percentages of autofluorescence eleocytes (E%) were recorded by flow cytometry on density plots, and then numbers of eleocytes (EN) were calculated from the formula EN= (E%*CN)/100. Total number of amoebocytes (AN) were calculated as: AN=CN-EN.

Spectrofluorimetry and analysis

For spectrofluorimetry coelomocyte suspensions were either lysed with 2% Triton (Sigma-Aldrich) or centrifuged at 7.500 g for 30 min at 4°C. Spectrofluorometric measurements were performed on coelomocyte lysates or supernatans from centrifuged coelomocyte suspensions using a Perkin-Elmer LS50B spectrofluorimeter.

Emission spectra of riboflavin were recorded in the 380-680 nm range (lambda at 370 nm), while excitation spectra were recorded in the 300-500 nm range (lambda at 525 nm). The spectrofluorimetric signatures of unbound riboflavin were characterised by two maxima (at 370 nm and 450 nm) in the excitation spectrum, and a maximum at 525 nm in the emission spectrum. Arbitrary units (AU) of fluorescence were recorded using Microsoft Excel v. 97. The amount of riboflavin in the sample was proportional to the maximum at 525 nm in the emission spectrum.

Statistical analysis

Coelomocyte-connected parameters were calculated using Microsoft Excel version 97. The results are expressed as means and correlations between body weights of animals and eleocytes percentages / cell numbers.

RESULTS

Composition and numbers of coelomocytes

Flow cytometry and autofluorescent eleocytes

The results of flow cytometric analysis are included in Fig. 1. Histograms based on flow cytometric analysis of formalin-fixed coelomocytes revealed two distinct peaks of cells which differ in FL-1 fluorescence, i.e. non-fluorescent amoebocytes (A) and autofluorescent eleocytes (E) (Fig. 1a). Density plots of the cell size (FS) versus FL-1 allow quantification of amoebocytes (A), eleocytes (E) and cellular debris (D) (Fig. 1b). The percentage of eleocytes is relatively stable throughout the lifespan of *D. veneta* (X+SD=35+12.7%) showing very low tendency to decline with body weights (correlation coefficient r=0.16) (Fig. 1c).

Numbers of coelomocytes and body weight

The total number of coelomocytes correlates with body weights of specimens (correlation coefficient r = 0.73; Fig. 2a), what is more connected with body weight-dependent numbers of amoebocyte (r=0.68; Fig. 2b), than eleocytes (r=0.52) (Fig. 2c).

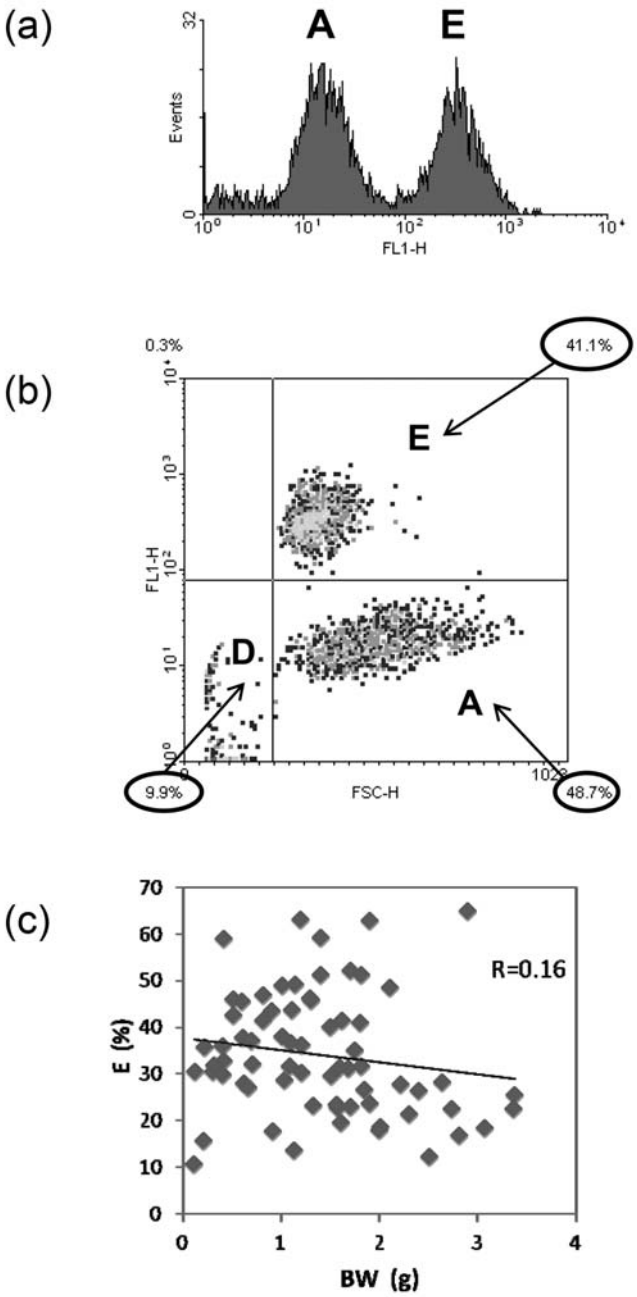

Fig. 1. Flow cytometric analysis of formalin-fixed coelomocytes extruded from a representative sample of coelomocytes of *D. veneta*. (a) The histogram showing groups of amoebocytes (A) and highly fluorescent eleocytes (E). (b) The density plot of coelomocyte size (X-axis) versus intensity of FL-1 fluorescence (Y-axis) showing the percentages of eleocytes (E), amoebocytes (A) and cellular debris (D); (c) Percentage of eleocytes (E) in specimens of various body weights of *D. veneta*. Correlation coefficient r=0.16.

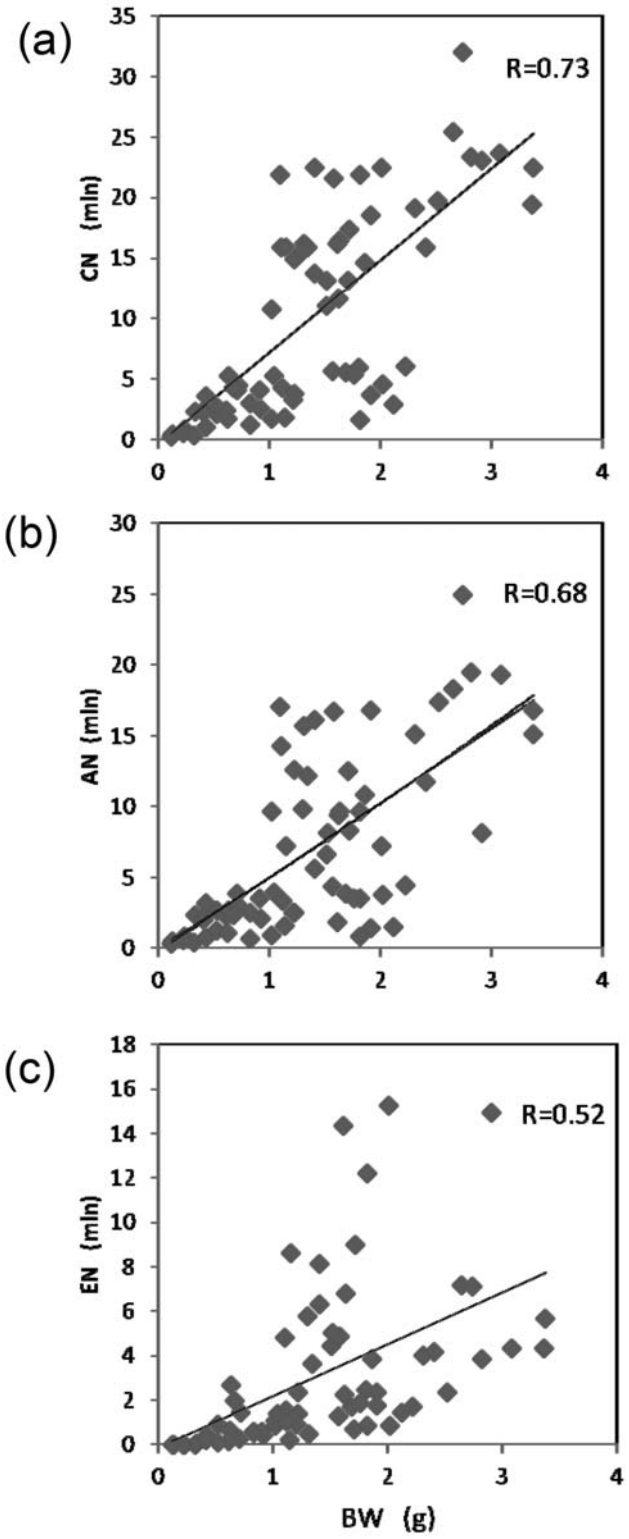

Fig. 2. Coelomocyte numbers (in millions) in specimens of various body weights (BW) of Dendrobaena veneta. (a) Total coelomocyte numbers (CN), among them (b) amoebocyte numbers (AN) and (c) eleocyte numbers (EN); r – correlation coefficients.

Riboflavin content

Fluorescence spectra of coelomocyte supernatants and lysates

Figure 3 shows the representative fluorescence spectra obtained on the coelomocyte supernatant and coelomocyte lysate obtained from the same sample of coelomic fluid derived either from the adult specimen of *D. veneta* (Fig. 3a, b) or the freshly hatched worms (Fig. 3 c, d). In the adult specimen (Fig. 3a-b), excitation spectra of supernatants and lysates were almost identical, exhibiting two distinct peaks at about 370 nm and 450 nm (Fig. 3a). In a sharp contrast, the emission spectra of coelomocyte supernatants from adult worms were different than those of lysates. The supernatant spectra exhibited only one riboflavin-derived peak at about 520-530 nm. In contrast, the riboflavin-derived peak in Triton-lysates was preceded by additional emission peak of fluorescence of the unknown origin (X fluorophore, according to Cygal et al., 2007) with a maximum of about 420 nm (Fig. 3b).

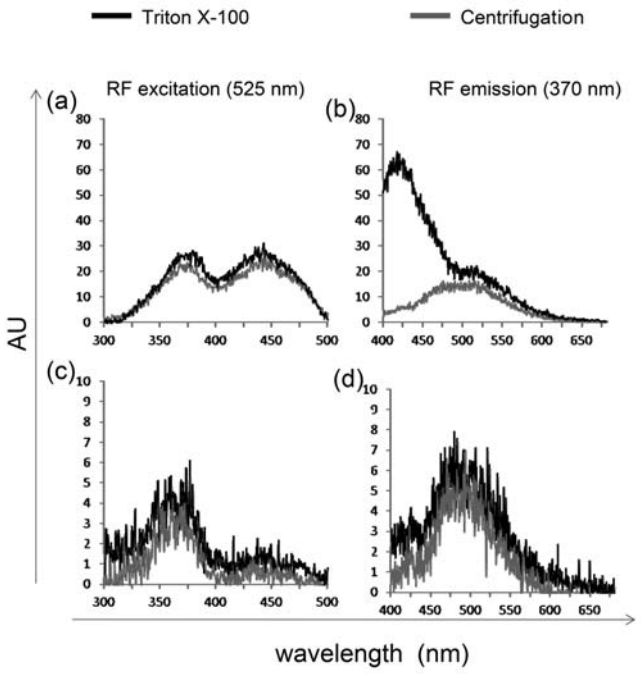

Fig. 3. Fluorescence excitation (lambda = 525 nm) (a, c) and emission (lambda = 370 nm) (b, d) spectra derived from lysates (black lines) and supernatants (grey lines) of coelomocyte samples extruded from the large (a-b) and small (c-d) specimen of *D. veneta* (2.3 g and 0.12 g b.w., respectively).

Fig. 3c, d show that excitation and emission spectra of coelomocyte samples from the newly hatched *D. veneta* worm were different than those from the older specimen (compare with Fig. 3a, b). In the representative newly hatched specimen of *D. veneta* (0.12 g body weight), excitation spectra of both supernatants and lysates were almost identical, exhibiting a distinct peak at about 370 nm, while the second one at about 450 nm being only slightly marked (Fig. 3c). Also the emission spectra from this sample were similar in coelomocyte supernatants and lysates, with a distinct peak at 480 nm (Fig. 3d).

Fluorescence spectra of coelomocyte lysates from adult and newly hatched specimens

Fig. 4 shows fluorescence spectra of coelomocyte lysates derived from 3 adult clitellate worms, with body weights from 1.02 g to 1.96 g (Fig. 4a,b) and 3 newly hatched worms, body weights 0.14 g to 0.16 g (Fig. 4c, d). The riboflavin-specific shapes of excitation and emission spectra are generally similar in adult worms while particular individuals differ in the high of emission peaks at 420 nm and 525 nm, the latter being proportional to riboflavin content in the samples (Fig. 4b). In a sharp contrast, coelomocyte lysates from very small specimens differ in shapes, one of them (derived from the worm "p") being already similar to that in adult worms while two others having riboflavin-specific shape absent and/or obscured by other fluorophore(s) with a peak of emission at 480 nm (Fig. 4d).

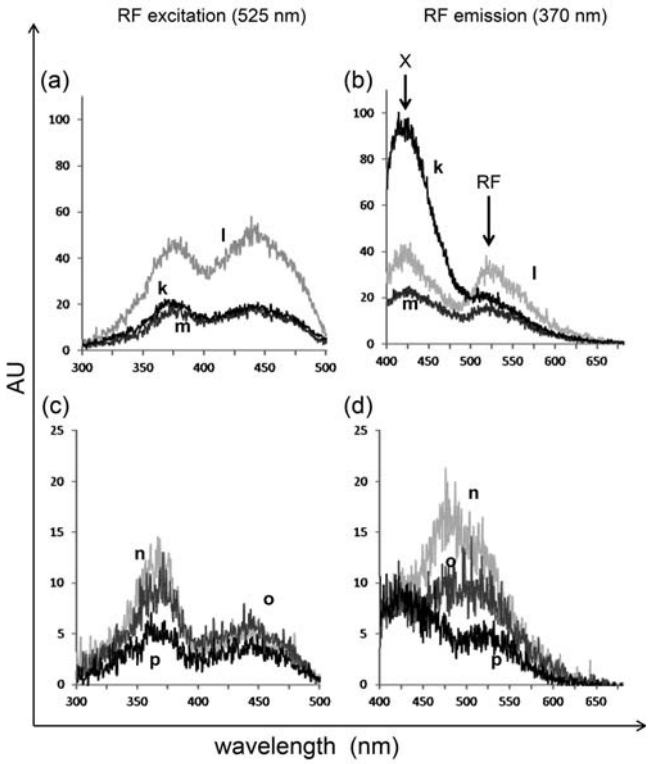

Fig. 4. Fluorescence excitation (lambda = 525 nm) (a, c) and emission (lambda = 370 nm) (b, d) spectra derived from coelomocyte lysates from samples extruded either from (a-b) three large (k: 1.96 g; l: 1.02 g; m: 1.6 g b.w.) or (c-d) three small (n: 0.16 g; o: 0.14 g; p: 0.14 g b.w.) specimens of *D. veneta*.

Riboflavin content

Total amount of riboflavin retrieved from coelomic cavity of electrostimulated earthworms increases with body weights of animals (r=0.63; Fig. 5a), while the amount of riboflavin calculated per eleocyte decreases with body weight of earthworms (r=0.46) (Fig. 5b).

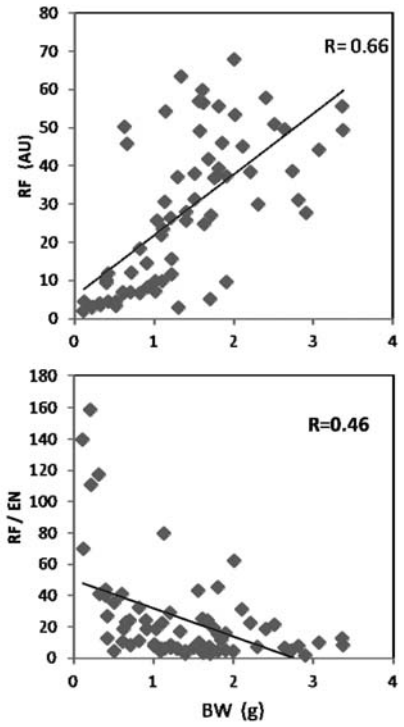

Fig. 5. Riboflavin (RF) content in coelomic cavity of specimens of various body weights (BW) of *D. veneta*. (a) Total riboflavin content (in arbitrary units; AU) in coelomocyte-containing coelomic fluid samples extruded from electrostimulated *D. veneta*. (b) Riboflavin content per eleocyte numbers (RF/EN). r – correlation coefficients.

DISCUSSION

The results of present experiments fully confirmed that coelomic fluid of *D. veneta* contains two distinct cohorts of freely floating coelomocytes, i.e. amoebocytes and eleocytes (Klimek et al. 2012; Rorat et al. 2014), and added the new information that the autofluorescent eleocytes are present already in newly hatched worms and their percentage stays at a relatively stable level throughout the whole lifespan of earthworms of this species, being slightly declining in worms of growing body weights. The results confirmed that riboflavin is one of fluorophores responsible for eleocyte autofluorescence in adult and juvenile worms while riboflavin presence in eleocytes of freshly hatched worms needs further elucidation. The total number of coelomocytes increases with body weight, thus putatively also with age of earthworms and corresponds better with the number of amoebocytes, being the invertebrate counterparts of the vertebrate macrophages (Ottaviani 2011), than with the number of eleocytes, being mature chloragocytes detached from chloragogenous tissue.

Eleocytes, but not amoebocytes, exhibit autofluorescence confined to their intracellular granules called the chloragosomes (Plytycz et al., 2007) and derived from riboflavin (vitamin B2) (Koziol et al., 2006; Sulik et al., 2012) and other fluorophores (Cygal et al., 2007; Rorat et al., 2014). Riboflavin (vitamin B2) plays an important role in immunity of animals (Verdrengh and Tarkowski, 2005), plants (Zhang et al., 2009), and bacteria (Atkinson et al., 2009). In a case of earthworm coelomocytes riboflavin can act as a potent chemoattractant (Mazur et al., 2011) putatively facilitating the formation of the multicellular brown bodies encapsulating parasites (Wieczorek-Olchawa et al., 2003). Recently it turned out that that riboflavin augments regeneration of amputated earthworm body segments and it blocks the inhibitory effects of the antibiotics on blastema formation (Johnson et al., 2012). Earthworms may be subjected to mechanical/chemical stimuli and/or sub-lethal predator attacks leading to the extrusion of coelomocytes and/or loss of body parts, thus regeneration of cells, tissues and organs has adaptive value.

A loss of coelomocytes through dorsal pores may be elicited by various factors which induce convulsive body movements, like some chemicals including ethanol treatment (Eyambe et al., 1991; Cooper *et al.*, 1995), ultrasounds (Hendawi et al., 2004), or electrostiomulation (Roch, 1979) the latter method commonly used for the controlled coelomocyte retrievel for experimental purposes (e.g. Plytycz and Morgan 2012). The number and composition of coelomocytes may be modified also by other environmental factors, e.g. ambient temperature (Cygal et al., 2007), soil pollution (e.g. Plytycz et al., 2011), or nutritional status of animals (Polanek et al., 2011).

The indiscriminate loss of a significant proportion of the free-floating coelomocytes is followed by their slow gradual restoration (Eyambe et al., 1991; Olchawa et al., 2003; Klimek et al., 2012). Klimek et al. (2012) reported that the amoebocyte count in *D. veneta* returned to control levels within 4 weeks after experimental extrusion in otherwise intact worms, while the recovery of eleocyte counts lags significantly behind that of amoebocytes, perhaps due to the different life cycles of the two cell types. Parry (1975) found that amoebocytes are mitotically active while eleocytes, as detached mature chloragocytes, show higher replenishment inertia. The distinctiveness of amoebocytes is also reflected in their functional capacities, for example, the selective uptake of metallo-nanoparticles (Hayashi and Engelman, 2013). Thus, it is tempting to conclude that amoebocytes play more prominent roles than eleocytes in immunity (Bilej et al. 2011).

It was also evidenced that riboflavin content was restored much more rapidly after electrostimulated coelomocyte depletion, than restoration of numbers of freely floating eleocytes (Klimek et al., 2012). This could entail the trafficking of riboflavin from relatively immature attached chloragocytes to more mature chloragocytes that are about to be released into the coelom to become eleocytes. An alternative hypothesis is that bacterial and fungal gut endosymbionts, the main source of riboflavin for earthworms according to Sulik et al. (2012), are somehow regulated by factors involved in maturation and/or restoration of the immune system. Therefore data concerning riboflavin content in freshly hatched and juvenile worms need further elucidation.

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