

RESEARCH REPORT

Searching for external sources of the riboflavin stored in earthworm eleocytes**P Sulik¹, M Klimek¹, P Talik¹, J Kruk², AJ Morgan³, B Plytycz¹**¹*Department of Evolutionary Immunobiology, Institute of Zoology, Jagiellonian University, Krakow, Poland*²*Faculty of Biochemistry, Biophysics, and Biotechnology, Jagiellonian University, Krakow, Poland*³*Cardiff School of Biosciences, Main Building, Cardiff University, Cardiff CF10 3US, Wales, UK**Accepted October 1, 2012***Abstract**

Riboflavin (vitamin B₂) is essential to maintain immune potency in animals and plants. So far it is accepted that animals cannot synthesise riboflavin; they rely on plant-sourced diets and intestinal bacteria for their supplies. A unique feature of earthworm 'hepatocyte-like' chloragocytes and chloragocyte-derived eleocytes floating in celomic cavity is the storage of riboflavin within intracellular granules. The hypothesis was that vegetarian food-deprivation or antibiotic/antifungal treatment inhibits riboflavin accumulation in eleocytes of *Eisenia andrei*. The 7-week starvation inhibited worm body weight gain and worm reproduction but had insignificant effects on celomocytes, both amoebocytes and eleocytes, and eleocyte riboflavin accumulation. The 1 week or 3 week antibiotic exposure had insignificant effects on worm coelomocytes and riboflavin content. Thus, a vegetarian diet and intestinal bacteria are not the exclusive or perhaps even the main sources of eleocyte riboflavin. The role of endosymbionts in earthworm flavonoid economy warrants targeted investigation. Moreover, the possibility of horizontal transfer of riboflavin biosynthesis genes from bacteria/fungi to earthworm genomes cannot be neglected.

Key Words: *Eisenia andrei*; food deprivation; antibiotic treatment; celomocytes; riboflavin**Introduction**

The earthworm immune system is very efficient (Bilej *et al.*, 2011). The immunocompetent cells (celomocytes) include amoebocytes, which are classical immunocytes according to nomenclature proposed by Ottaviani (2011), and species-specific chloragogen tissue-derived free chloragocytes (eleocytes). Eleocytes (detached chloragocytes), but not amoebocytes, exhibit autofluorescence (Cholewa *et al.*, 2006) that is evidently restricted to chloragosomal vesicles (Plytycz *et al.*, 2007).

Autofluorescent self-marking, and the simplicity of non-invasive retrieval of celomocyte suspensions, make eleocytes ideal subjects for flow cytometry analysis (*e.g.*, Cholewa *et al.*, 2006; Plytycz and Morgan, 2011). Spectrofluorimetry has revealed that riboflavin (vitamin B₂) is a prominent fluorophore contributing to eleocyte autofluorescence (Kozioł *et al.*, 2006; Cygal *et al.*, 2007). The percentage of autofluorescent eleocytes among celomocytes and

the amount of riboflavin stored within eleocytes are species-specific, with some species possessing a high proportion of fluid-suspended eleocytes whilst other species have very few such eleocytes in their celom (Plytycz *et al.*, 2006). Moreover, intrinsic edaphic variables (Plytycz and Morgan, 2011; Plytycz *et al.*, 2011), including metal contamination (*e.g.*, Kwadrans *et al.*, 2008; Homa *et al.*, 2010; Piotrowska *et al.*, 2010; Podolak *et al.*, 2011), can modulate eleocyte counts and riboflavin content. Lumbricid worms can expel celomocytes through their dorsal pores when irritated in natural environments (*e.g.*, by predators) or experimentally by physicochemical stimuli such as a mild electric shock (Roch, 1979), ultrasound (Hendawi *et al.*, 2004), or 5 % ethanol (Cooper *et al.*, 1995). Experimental manipulations do not affect worm viability, and their immune system gradually recovers after electro-stimulation (Olchawa *et al.*, 2003; Polanek *et al.*, 2011). Riboflavin is not stored exclusively in eleocytes. Recent observations indicate that the attached chloragocytes of lumbricid earthworms, whether they have high or very low numbers of chloragocyte-derived eleocytes floating freely in the celomic fluid, contain significant riboflavin levels (Mazur *et al.*, 2011). This finding

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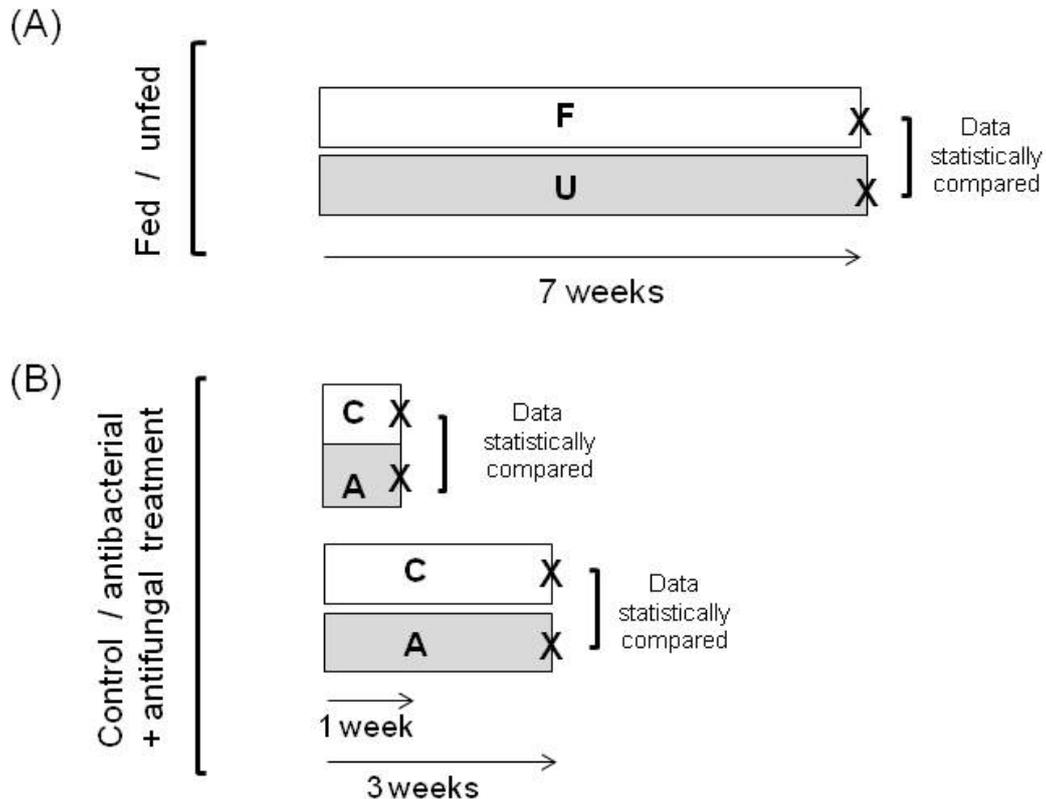


Fig. 1 Schematic representation of two independent experiments designed to investigate the effects of (A) food deprivation and (B) antibiotic/antifungal treatment, respectively, on riboflavin content in the eleocytes of *Eisenia andrei*. In Experiment A, worms were either fed ad libitum (F treatment) or unfed (U treatment). In Experiment B, worms were exposed either to water-soaked filter paper (controls: C treatments) or to filter paper soaked with antibiotic/antifungal cocktail Cefuroxime/Fluconazole (A treatments). At the end of 7-week (Experiment A) or 1-week and 3-week (Experiment B) experimental periods, celomic fluid was expelled by electro-stimulation (X-es) and analysed.

indicates that riboflavin storage plays an important role in earthworm biology.

Riboflavin is synthesized by plants and many microorganisms (Bacher *et al.*, 2000). Animals lack the riboflavin biosynthesis machinery (Fassbinder *et al.*, 2000). Consequently, the main sources of the vitamin for earthworms are highly likely to be a plant-based detritivorous diet, intestinal microflora, and from other endosymbionts. For example, there is direct evidence for insects (Nakabachi and Ishikawa, 1999) and nematodes (Taylor *et al.*, 2012) being provided with riboflavin by their endosymbiotic bacteria.

The main aim of the present work was to find the primary source of the riboflavin accumulated in the eleocytes of the epigeic, composting, earthworm species *Eisenia andrei*. In the first series of experiments the worms were food-deprived (U - unfed) while in the second series they were treated with an antibiotic/antifungal cocktail (A). The working hypothesis was that eleocyte riboflavin accumulation would be decreased in celomocyte lysates by worm starvation or by reducing the gut flora, respectively. It turned out, however, that the effects of such treatments were statistically

insignificant, and therefore other putative sources of riboflavin accumulated in eleocytes are discussed.

Materials and Methods

Earthworms

Adult *Eisenia andrei* (Oligochaeta; Lumbricidae), field-sampled in manure and compost heap in the Sadecki Mountains (southern Poland), were kept under controlled conditions (16 ± 1 °C; 12:12 LD) in the laboratory. The worms were kept in plastic boxes with perforated lids and the moisture level was checked weekly. Groups of worms with similar individual body weights (c. 0.5 -0.6 g) were used.

Experimental design (Fig. 1)

Worms were subjected to celomocyte extrusion at the end of 7-week experiments on effects of starvation or after 1-week or 3-week experiments on effects of antibacterial/antifungal factors.

Food deprivation (Fig. 1A)

Worms with similar body weights (appr. 0.6 g) (2 boxes, 8 worms per box) were maintained on

fresh commercial soil (PPUH BIOVITA, Tenczynek). In the first box worms were fed ('F'), *i.e.*, provided *ad libitum* with a mixed diet comprised of dried/boiled nettle (*Urtica dioica*) and dandelion (*Taraxacum officinale*) leaves, and in the second the worms were deprived of food (*i.e.*, unfed, 'U'). After 7 weeks on the contrasting dietary regimes, all of the worms were weighed, their celomocyte-containing celomic fluid was extruded and analysed, and the egg capsules (cocoons) in each box were counted. The results were compared and statistically analysed.

Antibacterial and antifungal treatment (Fig. 1B)

Earthworms were exposed dermally to solution of antibacterial/antifungal agents since such a procedure was very efficient in studies on effects of heavy metal solutions on worm celomocytes (*e.g.*, see Olchawa *et al.* 2006; Plytycz *et al.* 2011). Worms of similar body weight (appr. 0.5 g), 10 worms per group, were kept individually in plastic tubes on a substrate comprised entirely of daily-exchanged shredded soft filter paper (5.7 g per tubes) soaked either with water (*i.e.*, controls: 'C' groups) or with an antibiotic/antifungal cocktail ('A' groups). The antibiotic used was Cefuroxime (Zinacef 750 inj., GLAXO), a second generation cephalosporin. The antifungal agent was Fluconazole (Diflucan 2 mg/ml inj., Pfizer), a triazole antifungal drug. These agents were diluted and combined to give concentrations of 10,000 mg kg⁻¹ of Cefuroxime and 200 mg kg⁻¹ of Fluconazole per in the filter paper. After one week and 3 weeks, 5 individual worms from each of the treatments ('C' and 'A', respectively) were retrieved, weighed, and their celomocytes extruded and analysed. Comparisons and statistical analysis were performed between 'C'/'A' groups after 1 week, and 3 weeks, separately.

Celomocyte extrusion

The earthworms were stimulated for 1 min. with an electric current (4.5 V) to expel celomic fluid with suspended celomocytes through the dorsal pores. Briefly, the weighed earthworms were individually placed in Petri dishes containing 3 ml of extrusion fluid (phosphate-buffered saline, PBS, supplemented with 2.5 g/l ethylenediamine tetraacetic acid, EDTA), and 2 ml samples of the extruded celomocyte suspensions were used for spectrofluorimetric analysis; 1 ml was fixed in 2 % formalin and used for cell counting in hemocytometer and flow cytometry.

Soil-derived bacteria

Soil samples were prepared according to modified procedure used previously (Wieczorek-Olchawa *et al.*, 2003). In short, sample of air dried commercial soil (2 g) used in present experiments was shaken in 10 ml of sterile PBS on a laboratory shaker type 358 s (Elpan, Poland). After 1 h sedimentation, 1 ml of supernatant was added to 9 ml sterile bacterial broth (Biomed, Warszawa, Poland). After overnight incubation at room temperature, suspension of soil-derived bacteria was used for testing the efficiency of antibacterial/antifungal agents.

Effects of Cefuroxime and Fluconazole on soil-derived bacteria

Ten µl of bacteria suspensions were added to 90 µl of bacterial broth in 96-well flat-bottomed plates (Falcon), supplemented either with Cefuroxime (C) (final concentration 10,000 mg/l), or Fluconazole (F) (final concentration 200 mg/l), or Cefuroxime/Fluconazole cocktail (CF) at the same concentrations, or with an equivalent volume of PBS (controls); wells filled with broth supplemented with C, F, C/F, PBS, but without addition of soil-derived bacteria served as controls of sterility. 8 wells were used per each treatment. Plates were incubated for 20 h at room temperature. The viability of bacteria in the various treatment groups was assessed by MTT reduction test, according to modified method described by Wieczorek-Olchawa *et al.* (2003). The yellow MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; Sigma) is reduced to blue formazan by dehydrogenases of living bacteria. In practice, 10 µl of MTT (from 5 mg/ml working solution) was added to each well and incubated for 15 min in darkness. Absorbance was measured at 570 nm on an ExpertPlus (AsysHitech GmbH, Austria) spectrometer.

Flow cytometric measurement and analysis

Samples of celomocytes were analysed with a FACScalibur flow cytometer (BD Biosciences). During analytical experiments, 10,000 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 using WinMDI 2.8 software (Joe Trotter, <http://facs.scripps.edu>), by producing dot plots of cell size versus FL1 autofluorescence.

Spectrofluorimetry and analysis

Spectrofluorometric measurements were performed on 2 ml riboflavin (Sigma-Aldrich) solution as a standard and on celomocyte-suspension lysates (lysed with 2 % Triton; Sigma-Aldrich) using an LS50B Perkin-Elmer Spectrofluorometer. Emission spectra of riboflavin were recorded in the 460 - 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 are characterised by two maxima (at 370 nm and 450 nm) in the excitation spectrum, and a maximum at 525 nm in the emission spectrum. Riboflavin standard curve was prepared using serial dilution of pure riboflavin (Sigma-Aldrich). The emission value at 525 nm in each particular celomocyte lysates was converted to amount of riboflavin (in µg) according to the standard curve, as described previously (Plytycz *et al.*, 2006).

To verify species identification, in randomly selected samples spectra of fluorophore specific for *E. andrei* were recorded according to the Albani *et al.* (2003) protocol; emission spectra were recorded in the 340 - 480 nm range (lambda at 320 nm), while excitation spectra were recorded in the 260 - 360 nm

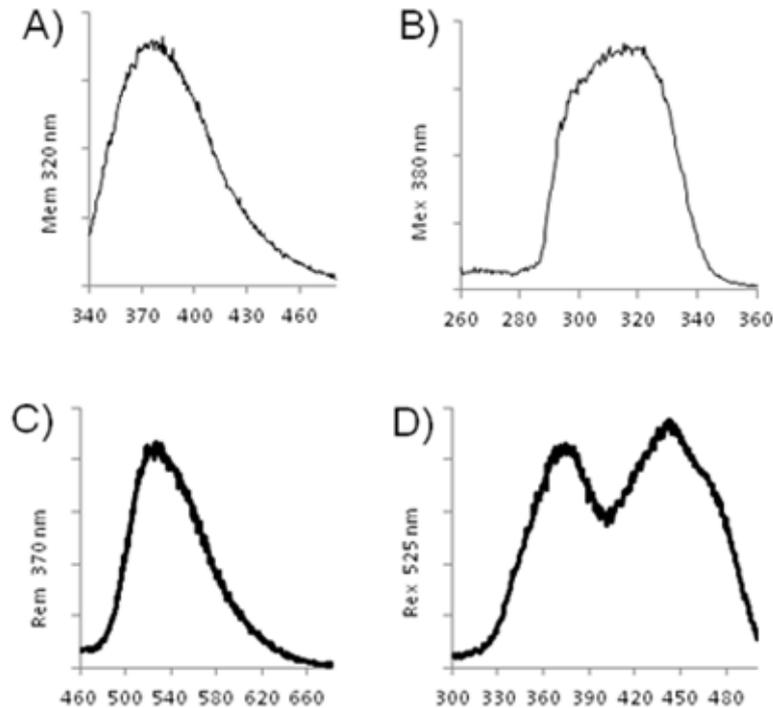


Fig. 2 Fluorescence spectra of celomocyte lysates from *Eisenia andrei*. Top row: spectra of fluorophore specific for *E. andrei*: A - emission (Mem lambda at 320 nm; peak at 370 - 380 nm); B - excitation (Mex lambda at 380 nm; peak at 314 - 320 nm). Bottom row: spectra of riboflavin: C - emission (Rem lambda at 370 nm; peak at 520 - 525 nm); D - excitation (Rex lambda at 525 nm; peaks at 370 nm and 450 nm).

range (lambda at 380 nm). The spectrofluorimetric signature of *E. andrei* is characterised by a maximum at 314 nm in the excitation spectrum, and a maximum at 370 - 380 nm in the emission spectrum. Arbitrary units (AU) of fluorescence were recorded using Microsoft Excel v. 97.

Statistical analysis

The results were expressed as means \pm standard errors. Differences between the means were determined with the Mann-Whitney U-test (STATGRAPHICS Plus 5.0), with the level of significance established at $p < 0.05$.

Results

Species identification (Fig. 2)

Worms sampled in the Sadecki Mountains and reared in the Institute of Zoology in Krakow were identified as *E. andrei* on the basis of the uniformly reddish body coloration. Celomic fluid lysates of worms from the present experiments contained fluorescence spectra similar to those characteristic for the fluorophore described by Albani *et al.* (2003) as a presumptive diagnostic molecular marker for *E. andrei* (Fig. 2, top), and riboflavin-specific fluorescence spectra (Fig. 2, bottom).

Cocoon production

Cocoon production was inhibited in unfed worms compared with their fed counterparts (17 and 37 cocoons, respectively, produced in the 7 week

treatment period, corresponding to 0.30 and 0.66 cocoons/worm/week). Cocoons were absent in worms kept for 3 weeks on filters soaked with water or antibiotic/antifungal cocktail.

Effects of antibiotic/antifungal agents on soil-derived bacteria/fungi (Fig. 3)

MTT reduction assay revealed the high amount of soil-derived bacteria multiplied in bacterial broth during overnight incubation. The amounts of bacteria in the control wells filled only with bacterial broth was high. Similarly high amounts of bacteria were detected in wells supplemented with antifungal agent, Fluconazole (F wells). In a sharp contrast, living bacteria were absent in broth supplemented with antibacterial Cefuroxime (C), and in the cocktail of these two agents (CF), as optical densities read at 570 nm were very low and almost identical to those of bacteria-free wells supplemented with PBS (controls of sterility), or with F, C, or CF (Fig. 3).

Effects of food deprivation on coelomocytes (Fig. 4)

At the end of 7-week experiments body weights were statistically significantly lower in unfed worms than in their fed ad libitum counterparts (Fig. 5). Numbers of celomocytes (CN), among them both amebocytes (AN) and eleocytes (EN), were similar in fed and unfed worms, both those counted in extruded fluid and after adjustment for reduced body mass (CN/BW, AN/BW, EN/BW). Percentages of eleocytes (E) and riboflavin content in celomocyte lysates (R) were unaffected by 7-week

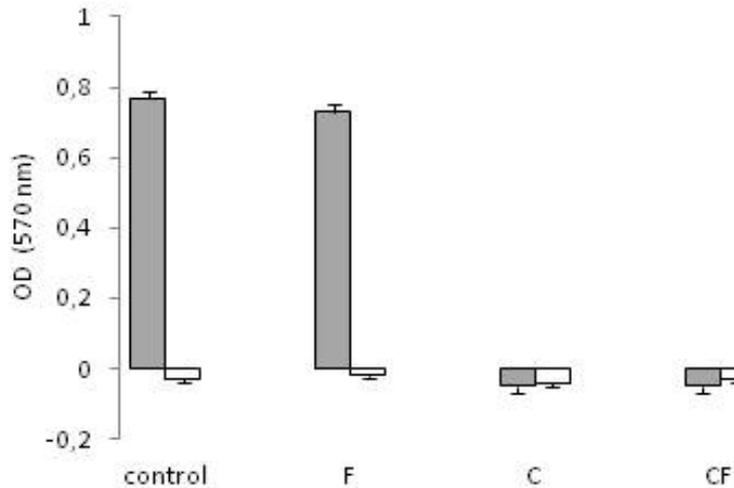


Fig. 3 Viability of soil-derived bacteria measured by MTT reduction test after overnight *in vitro* incubation in the control bacterial broth or that supplemented with antifungal Fluconazole (F), or antibacterial Cefuroxime (C) or with cocktail of these two agents (C/F). Solid and empty bars - samples with and without addition of soil-derived bacteria, respectively. OD - optical density read at 570 nm. Data are presented as means \pm SE; n = 8 wells per treatment.

food deprivation, riboflavin amount being even higher in unfed worms after adjustment for reduced body mass (RF/BW) or eleocyte number (RF/EN), but the increase was statistically insignificant (Fig. 4).

Effects of antibiotics on worm celomocytes (Fig 5)

One-week dermal exposure to soft filter papers soaked with water or Cefuroxime/Fuconazole (CF) aqueous solution had no adverse effects on worm viability, while worms exposed for 3 weeks to antibiotic/antifungal agents showed impaired mobility thus experiments were terminated. Autopsy revealed presence of ingested pieces of filters in worm intestines indicating that animals were penetrated by antibacterial/antifungal cocktail both via the derma and through the digestive tract.

At the end of 1-week experiments body weights were similar in worms maintained on a substrate of filter paper as the only nutritional source, and moistened either with water (C - control group) or antibiotic/fungicide Cefuroxime/Fuconazole (CF) aqueous solution (A group) (Fig. 5A). Body weights (BW) were unaffected during 1-week CF exposure, while the percentages of eleocytes (E %) tended to be increased. In the worms exposed to antibiotics/fungicides (A groups), also numbers of celomocytes, among them amebocytes and eleocytes tended to be increased. Amount of riboflavin was unaffected, both that measured in celomocyte lysates and after adjustment for body mass (RF and RF/BW, respectively), but that concerning riboflavin adjusted to eleocytes number (RF/EN) was lower in cells from worms exposed to antibacterial/antifungal cocktail than that in water exposed worms, but the difference was statistically insignificant (Fig. 5A).

At the end of 3-week experiments (Fig. 5B) body weights (BW) and percentages of eleocytes (E %) among coelomocytes were similar in worms maintained on the water- and C/F-soaked filter papers as the only nutritional source (Fig. 5B). In the worms exposed to antibiotics/fungicides, numbers of amebocytes tended to be increased (CN), eleocytes (EN) were unaffected, while amount of riboflavin tended to be reduced, both total and adjusted for body mass (RF and RF/BW, respectively), with differences being statistically insignificant, but that concerning riboflavin adjusted to eleocytes number (RF/EN) was higher in the control worms than that from the A (CF-treated) group, the difference being close to significance ($p = 0.06$) (Fig. 5B).

Discussion

It is well established that riboflavin is important for the proper functioning of the innate immunity of both animals (e.g., Powers 2003; Verdrengh and Tarkowski, 2005) and plants (Dong and Beer, 2000; Asai *et al.*, 2010; Yoshioka *et al.*, 2011), as well as being a signalling molecule in bacterial quorum sensing (Rajamani *et al.*, 2008; Atkinson and Williams, 2009). In earthworms riboflavin acts as a chemoattractant for celomocytes what may facilitate the targeted recruitment of immune-competent celomocytes to the site of pathogen invasion (Mazur *et al.*, 2011). Therefore, accumulation of riboflavin in chloragosomes of chloragocytes and chloragocyte-derived eleocytes is probably of adaptive value for earthworm species, and eleocyte riboflavin stores are mobilized mainly in a case of pathogen invasion to support immune functions (Plytycz and Morgan, 2011).

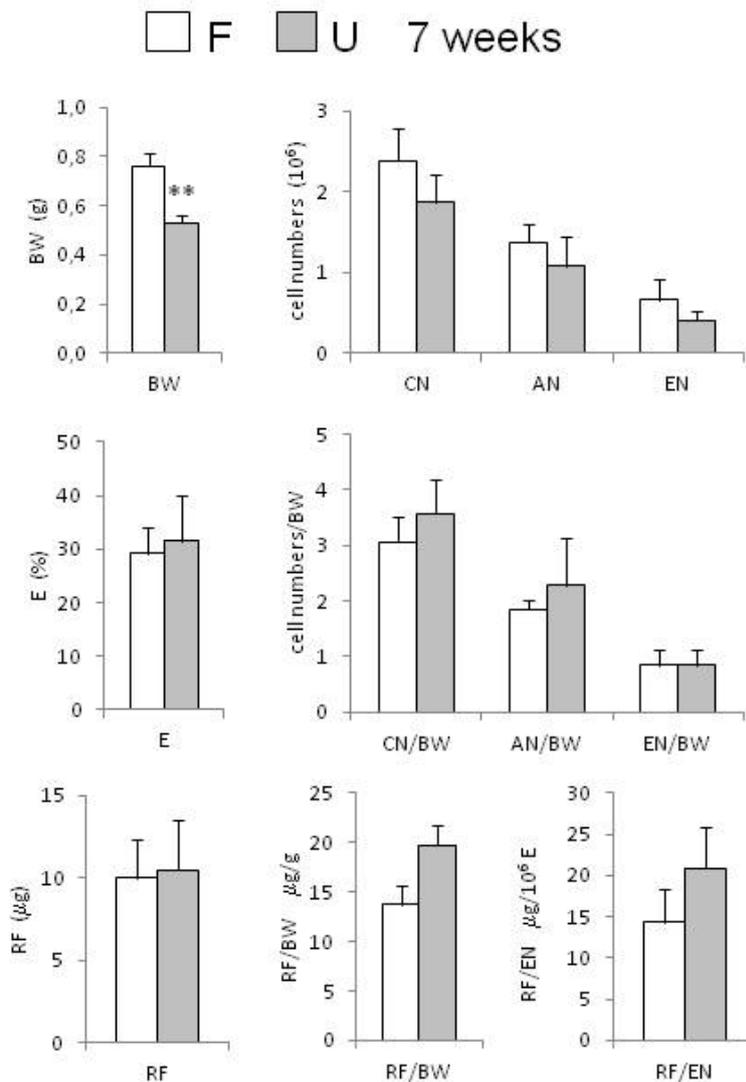


Fig. 4 Effects of 7-week food deprivation on adult *E. andrei*. Worm groups fed ad libitum on nettle and dandelion leaves were designated 'F' (open bars), and unfed groups were designated 'U' (solid bars). Body weights (BW), percentages of eleocytes (E, %), celomocyte numbers (CN), among them amoebocyte numbers (AN) and eleocyte numbers (EN), and body weight-adjusted (CN/BW; AN/BW; EN/BW); total riboflavin content (RF) in extruded celomocytes, body weight-adjusted riboflavin content (RF/BW), and riboflavin content adjusted to extruded eleocyte numbers (RF/EN). Data are presented as means \pm SE; $n = 8$ worms per group. Lack of statistically significant differences according to Mann-Whitney U-test between F and U treatments ($p > 0.05$).

Our working hypothesis was that riboflavin stores in eleocytes are exhausted in food-deprived worms and especially in those fed only on filter papers and exposed dermally/orally to solution of antibacterial and antifungal agents. Contrary to our expectations, these experimental manipulations had no or little effects on riboflavin status in eleocytes of *E. andrei*.

In the first part of the present study we revealed that the riboflavin contents in eleocyte-rich celomocyte lysates were very similar in *E. andrei* fed ad libitum on nettle and dandelion leaves and in worms deprived of plant-derived food. Dietary restriction over a period of a few weeks has previously (Piotrowska *et al.*, 2010; Polanek *et al.*,

2011) been observed to have a little or no effect on the riboflavin status of circulating immune-competent cells in other earthworm species. Consequently, we excluded such a possibility that the main sources of the vitamin for earthworms is a plant-based detritivorous diet.

These observations may be interpreted in a number of ways. First, it is feasible that if riboflavin is not consumed to counteract a pathogen challenge during a given period, then the vitamin budget could be maintained by recycling directly or indirectly from senescent eleocytes to newly formed eleocytes. Second, earthworms may possess enough riboflavin stored internally elsewhere, for example in the chloragogenous tissue, to maintain optimal levels in

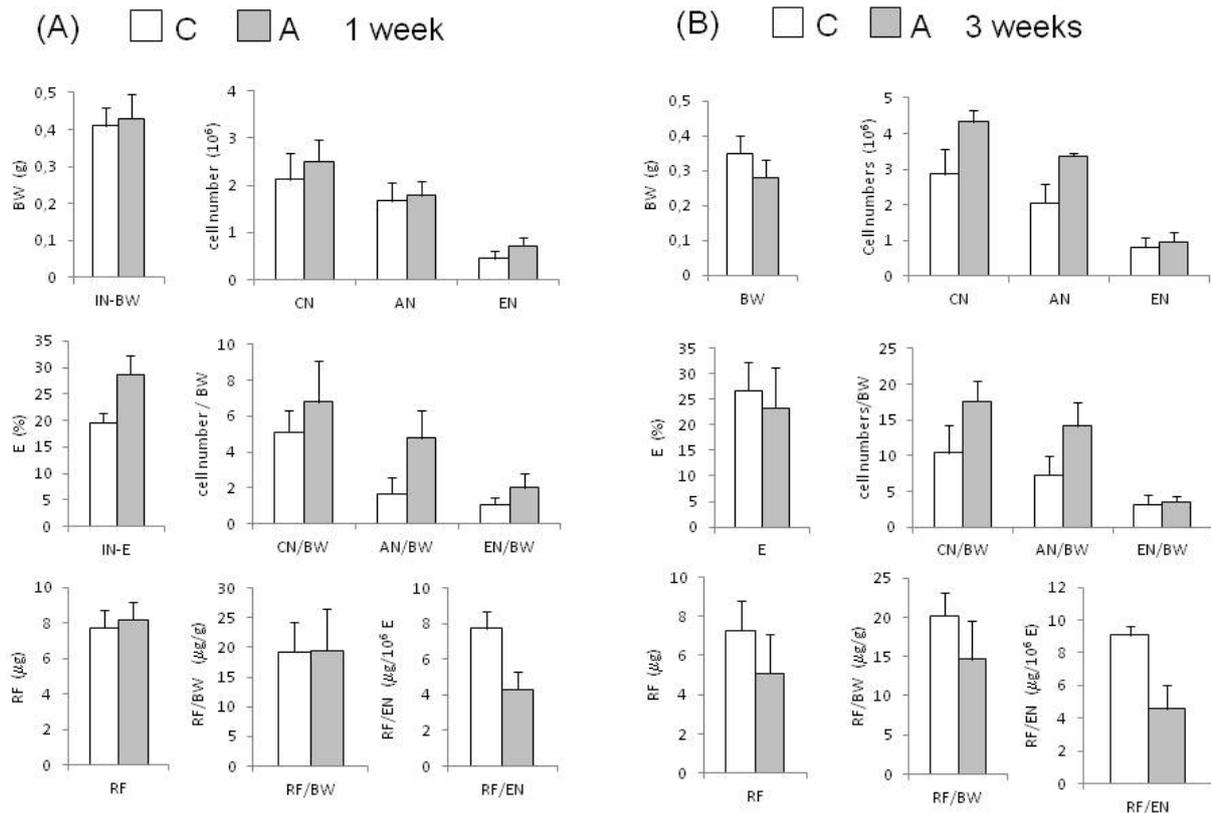


Fig. 5 Effects of 1 week (Fig. 5A) or 3-weeks (Fig. 5B) antibacterial/antifungal treatments on adult *E. andrei*. Worms were kept on filter paper soaked either with water (control, C, open bars) or with a cocktail of antibiotic/antifungal agents (A, solid bars). Body weights (BW), percentages of eleocytes (E, %), celomocyte numbers (NC), among them amoebocyte numbers (AN) and eleocyte numbers (EN), and body weight-adjusted (CN/BW; AN/BW; EN/BW); total riboflavin content (RF) in extruded celomocytes, body weight-adjusted riboflavin content (RF/BW), and riboflavin content adjusted to extruded eleocyte numbers (RF/EN). Data are presented as means \pm SE; $n = 5$ worms per group. Lack of statistically significant differences according to Mann-Whitney U-test between C and A treatments ($p > 0.05$).

the eleocytes stores over the time period. Third, during a period of dietary restriction riboflavin may be replenished from endosymbiont source or sources, possibly driven by upregulation through metabolic feedback. It is not possible to infer from present knowledge which of these possibilities is the most likely. Measuring systemic riboflavin-binding protein, and the immuno-localisation of its membrane-bound counterpart (Foraker *et al.*, 2003), would be helpful in this regard.

In order to check if the main source of earthworm riboflavin is intestinal microflora the second part of experiments was performed. In an attempt to reduce if not eliminate the gut flora (Thakuria *et al.*, 2008) earthworms were maintained for a period of several weeks on a filter paper substrate spiked with water (control) or a cocktail of antibiotic and antifungal agents. Post mortem revealed that worm intestines were filled with papers, what strongly supports that Cefuroxime/Fluconazole cocktail, which successfully killed soil bacteria, might eliminate the worm gut flora. Contrary to our expectations, the riboflavin accumulation within the eleocytes was only slightly

reduced in the antibiotic-exposed worms fed only with antibiotic-soaked filter paper. It is worth to notice that such dermal exposure to filter papers soaked with metal chlorides was very efficient in studies on effects of heavy metals on coelomocytes of several earthworm species, putatively by disruption of balance between coelom-invading pathogens and worm immune system (e.g., Wiczonek-Olchawa *et al.*, 2003; Olchawa *et al.*, 2006; Plytycz *et al.*, 2011).

It is not known whether the antibiotic is trafficked through the earthworm body and has a negative impact on endosymbionts such as *Verminephrobacter* (e.g., Lund *et al.*, 2010a, b). However, investigations on insects provide concrete evidence that bacterial endosymbionts do synthesise and supply riboflavin to their hosts (Nakabachi and Ishikawa, 1999). More recently, studies have shown that the depleted genomes of the bacterial endosymbionts of certain insect species have retained ancestral genes encoding essential amino acids and components of the riboflavin-synthesis pathway (Moran *et al.*, 2003). In an exquisite example of co-evolution, it has been

observed that different endosymbiont species within a given insect host species retain complimentary components of common and essential metabolic pathways (McCutcheon *et al.*, 2009). It is not implausible that endosymbionts play similar nutritive roles in earthworms, but there is no direct evidence for the phenomenon. The fact that *Verminephrobacter eiseniae* is vertically inherited via the cocoons of *Eisenia fetida* (Davidson *et al.*, 2008) does, however, raise the intriguing possibility that the riboflavin requirements of the developing embryo are supplied directly by this or other endosymbiont species within the egg capsule. Another possible mechanism for keeping a relatively constant riboflavin content in earthworm coelomocytes would be the 'insertion' of riboflavin biosynthesis genes into the earthworm genome by horizontal DNA transfer from microbes. This is not as implausible as it may appear because such lateral transfer of fungal carotenoid genes into arthropods is well documented phenomenon (Moran and Jarvik, 2010; Altincicek *et al.*, 2012). Given the ubiquity of riboflavin in earthworms, and the immunological benefits provided by the vitamin, it is important to seek the presence of genes regulating riboflavin biosynthesis in lumbricid earthworms.

Acknowledgements

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