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Differential Time Course of Restoration of Experimentally Depleted Coelomocytes and Fluorophores in the Earthworm *Eisenia andrei**

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Stressed earthworms expel coelomic fluid containing several vital cellular and soluble components, thus their post-stress recovery has adaptive value. The present manuscript describes the recovery rates of coelomocytes (amoebocytes and eleocytes) and two fluorophores (riboflavin and 4-methylumbelliferyl β-D-glucuronide, MUG) after experimental extrusion by electrostimulation. Analyses were conducted at time points (from 0.5 hour to 7 weeks) by a combination of cell counts, spectrofluorimetric measurements of riboflavin and MUG, and fluorescence microscopy. Coelomic fluid retrieved 30 minutes after extrusion contained <10% of the baseline levels of amoebocytes, eleocytes and riboflavin; the depleted levels of these variables were fully restored after 3, 5, and 7 weeks post-extrusion, respectively. Restored eleocytes were richer in riboflavin than the eleocytes of worms electrostimulated at t_0. MUG was less severely depleted (to 49% of baseline) than riboflavin, and was restored to the initial level within 1 week post-extrusion. This indicates that MUG, unlike riboflavin, resides mainly within non-coelomocyte cellular location(s); moreover, this fluorophore may be a useful molecular marker for distinguishing even immunologically-compromised *E. andrei* from closely related composting species.

Key words: earthworms, coelomocytes, amoebocytes, eleocytes, riboflavin, MUG fluorophore; regeneration.

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Lumbricid worms attacked by predators or irritated by mechanical or chemical stimuli are able to expel coelomic fluid via dorsal pores in the body wall. Coelomic fluid contains several functionally vital cellular and soluble constituents, including factors integral to immune reactions (BILEJ *et al*. 2011). Consequently, the efficient recovery of coelomocytes and soluble factors after stress-related disturbance is evidently adaptive and worthy of detailed investigation.

Coelomocyte counts are restored slowly during a period of a few weeks after experimental expulsion induced by immersion in alcohol (COOPER *et al*. 1995), ultrasounds (HENDAWI *et al*. 2004) or mild electric current (ROCH *et al*. 1979), the latter procedure being applied also in the present studies. Restoration of coelomocytes was initially observed by EYAMBE *et al*. (1991) in *Lumbricus terrestris*. Subsequently, the restoration of coelomocyte numbers after experimental expulsion in the composting species *Dendrobaena.veneta* was shown to be a protracted temperature-dependent process (OLCHAWA *et al*. 2003; POLANEK *et al*. 2011; KLIMEK *et al*. 2012) connected with proliferation of free coelomocytes (HOMA *et al*. 2008). We have recently observed that the recovery of depleted coelomocytes measured 3 weeks after experimental expulsion was much more efficient in juveniles than in adults of ecologically similar composting earthworms, *D. veneta, Eisenia fetida,* and *E. andrei* (SANTOCKI *et al*. 2015). Each of these three lumbricid species possess two distinct cohorts of coelomocytes (i.e. amoebocytes and eleocytes), with the chloragocyte-derived eleocytes characteristically

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expressing autofluorescence emanating mainly from riboflavin (PLYTYCZ *et al*. 2007; PLYTYCZ & MORGAN 2011). These findings demonstrate how cell counts and riboflavin content in sampled coelomic fluid are simple and robust parameters that may be used to track the status of the earthworm immune system after experimental manipulation as well as during physiological and pathological events.

The three composting species, especially juveniles, are difficult to distinguish using classical morphological traits. Since these species are much favoured for ecotoxicological investigations, where the possibility of inter-species differences in sensitivity must be embraced, rapid and reliable high-throughput means of identifying individual worms is an important prerequisite. For this reason we have developed non-invasive markers, verified by molecular-genetic probes, to distinguish the three composting species (RORAT *et al*. 2014). Among the potentially useful markers is a fluorophore present in coelomic fluid of *E. andrei,* that is quite distinct from riboflavin. This biogenic fluorophore was identified as 4-methylumbelliferyl β-D--glucuronide (MUG) by ALBANI *et al*. (2003). In our experience, MUG was found to be present in every individual of *E. andrei* that we have investigated. However, qualitatively similar fluorescence spectra, albeit of much lower intensity, were recorded in some specimens of *E. fetida* (RORAT *et al.* 2014). Whilst the low intensity 'noise' of MUG in a minority cohort of *E. fetida* does not invalidate the use of this molecule as a biomarker for distinguishing *E. andrei* from the other species, it is important to establish how the standard technique of electrostimulation for coelomic fluid sampling affects MUG levels in *E. andrei*. Thus the core aim of present study was to measure and track the levels of MUG in *E. andrei* at time intervals after electro-extrusion of coelomic fluid and, further, to compare the MUG response pattern with the time-resolved restoration of amoebocyte, eleocyte, and riboflavin levels in the same individuals of adult *E. andrei.* To the best of our knowledge this is the first study of the post-extrusion restorative dynamics of MUG. Such baseline information yields benchmarks for future studies of the modulatory effects of various neuro-hormonal and/or environmental factors on the composting model species.

Materials and Methods

Earthworms

Eisenia andrei (Lumbricidae, Annelida) derived from a laboratory culture at the Lille University (France), their taxonomic identity having been confirmed by barcoding and several molecular markers (RORAT *et al*. 2014). They have been reared for several generations in commercial soil (PPUH BIOVITA, Tenczynek) under controlled conditions (17°C; 12:12 L/D) in the Department of Evolutionary Immunology of the Institute of Zoology, Kraków, Poland. The worms were kept in plastic boxes with perforated lids and the moisture level was checked weekly. Worms were fed *ad libitum* with a mixed diet comprised of dried/boiled nettle (*Urtica dioica*) and dandelion (*Taraxacum officinale*) leaves, boiled/dried tea leaves, and powdered commercial mouse pellets.

Experimental scheme

Adult specimens of similar body weights (in total 60 worms of 0.6-0.7 g body weight) were used for 3 series of experiments. At the start of each series expulsion of coelomocyte-containing coelomic fluid was performed by electrostimulation at the start of experiments (time zero), and then the groups of 5 worm each were electrostimulated for coelomic fluid retrieval at selected time intervals representing different levels of temporal resolution: (i) intervals within the first 24 hours post-extrusion, (ii) intervals within the first week post-extrusion, and (iii) intervals up to 7 weeks post-extrusion. Coelomocytes were extruded at time 0, and their recovery was analysed in groups of 5 worms at each time point marked on X-axes of Figures 1, 3, and 5.

Coelomic fluid extrusion

Coelomic fluid with suspended coelomocytes was expelled through the dorsal pores of electrostimulated earthworms, as described previously (RORAT *et al*. 2014). Coelomic fluid extrusion was always performed on depurated earthworms kept overnight on wet filter papers, and then gently washed/dried. Then 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 tetra-acetic acid (EDTA; Sigma-Aldrich) to avoid cell clumping and electrostimulated for 30 sec with an electric current (4.5V) as described previously (RORAT *et al*. 2014). 2ml samples of the extruded coelomocyte suspensions were used for spectrofluorimetric analysis, while 1 ml was used for microscopic analyses and cell counts in haemocytometer.

Spectrofluorimetry and analysis

Spectrofluorometric measurements were performed on 2 ml coelomocyte-suspension lysates (lysed with 2% Triton; Sigma-Aldrich) using an LS50B Perkin-Elmer Spectrofluorimeter. Emis-

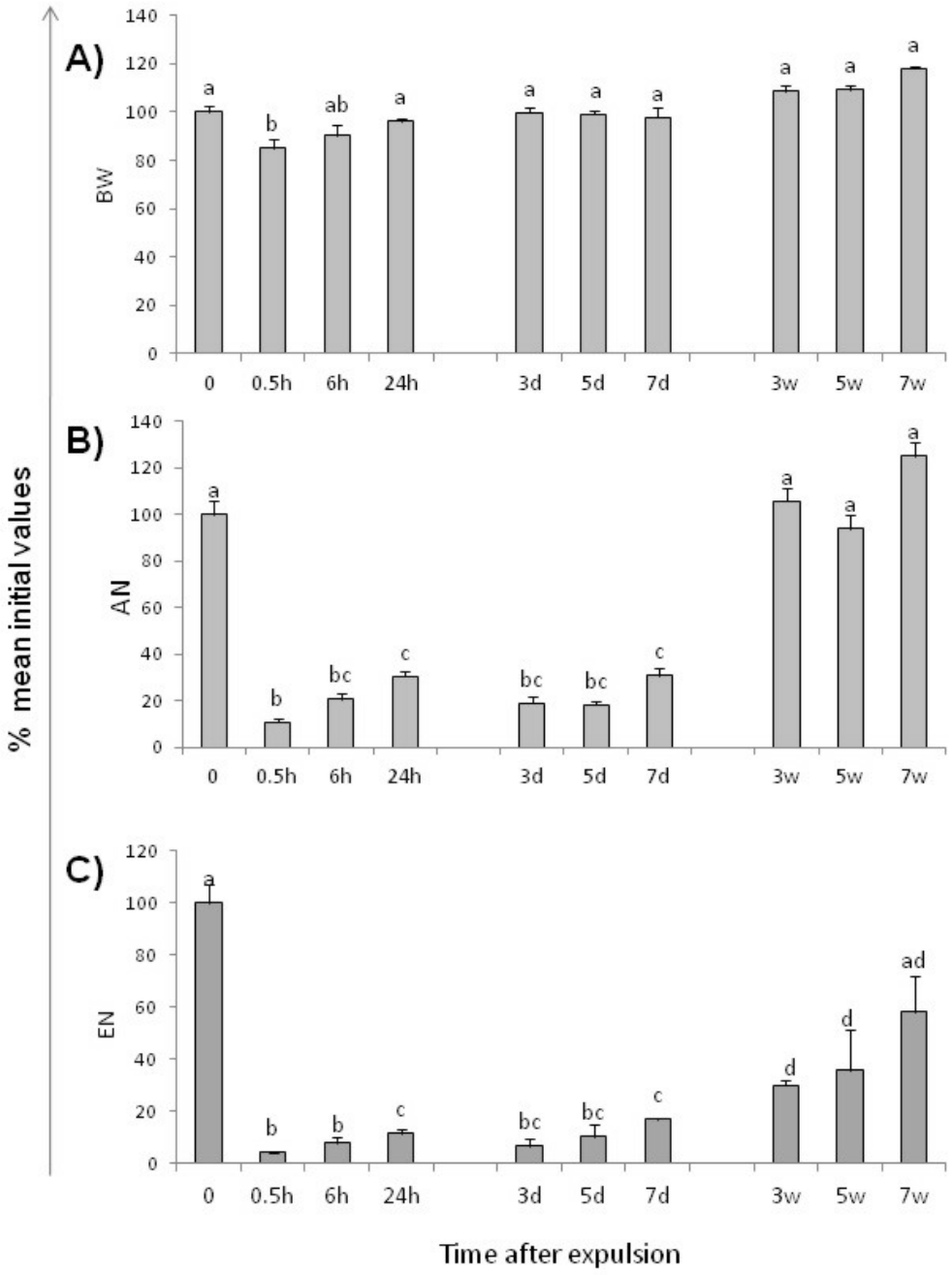

Fig. 1. Time courses of restoration of A) body weights (BW), B) numbers of amoebocytes (AN), and C) numbers of eleocytes (EN) of adult specimens of *E. andrei* depleted by expulsion of coelomic fluid induced by electrostimulation performed at the start of experiments and measured at time zero (0), and then after 0.5, 6, and 24 hours (h); 3, 5, and 7 days (d); 3, 5, and 7 weeks (w). Mean values calculated as percentages of those measured at time 0 ± SE; numbers of samples at each time point n = 5.

sion 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 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. Arbitrary units (AU) of fluorescence were recorded using Microsoft Excel v. 97. Amount of riboflavin in the sample is proportional to the maximum at 525 nm on the emission spectrum and 450 on the excitation spectrum.

MUG fluorophore (4-methylumbelliferyl β-D--glucuronide; MUGlcU; MUG) was detected by ALBANI *et al*. (2003) as an *E. andrei* specific fluorophore with emission spectra between 340

and 480 nm (lambda at 320 nm) and excitation spectra between 300 and 510 nm (lambda at 380 nm). The amount of MUG in a sample is proportional to the maximum at 370 nm on the emission spectrum and 314 nm on the excitation spectrum, as described previously (RORAT *et al*. 2014).

Eleocytes fluorescence intensity

For measurements of fluorescence intensity of eleocytes, samples of 3 specimens of *E. andrei* coelomocytes retrieved at time zero were compared with samples retrieved 3 days later from the same worms. Photographs of coelomocytes extruded for the first time and on day 3 of restoration process (captured with Carl Zeiss Axio Imager fluorescence microscope) were exported to ImageJ (Rasband, W. S., ImageJ, U. S. National Institute of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016) and converted into gray scale. In the program, 30 fluorescent eleocytes from each group (visible as white and gray on black background) were selected with freeform selection tool. Then, from the analyze menu "set measurements" option was chosen and "mean gray value" was selected from the checkboxes. Next, "measure" option was selected from the analyze menu and the results were presented in a popup box as a stack of values, which were calculated using Microsoft Excel 2003. Data were expressed as means with standard errors. Differences between means were determined with Statistica 10 (StatSoft Inc.) by non-parametrical Wilcoxon matched pairs test, with the level of significance established at $P<0.001$.

Statistical analysis

Coelomocyte- and fluorophore-connected parameters were calculated using Microsoft Excel version 97. Results concerning restoration of coelomocytes/fluorophores were expressed as means ± standard deviations, and within each series recalculated as percentages of the respective mean values at the start of experiments. Then data from particular experiments were combined and analyzed jointly. Differences between groups were determined by ANOVA with post-hoc Tukey's test or Mann- Whitney's U test (Statgraphics Plus 5.0), with the level of significance established at $P<0.05$.

Results

Loss and restoration of coelomocytes

Electrostimulation treatments caused efficient extrusion of coelomocyte-containing coelomic fluid and resulted in a transient loss of 14% of the initial body weight, but the initial body weight was fully restored 6 hours after electrostimulation (Fig. 1A).

The differences in the rates and patterns of post-extrusion recovery by amoebocytes and eleocytes are illustrated in Figs 1B, 1C. Half an hour after initial electrostimulation the number of amoebocytes was 11% of the t_0 value and then increased to 21% and 30% during the first 6 and 24 hours, respectively. During consecutive 1 week time intervals amoebocyte percentage fluctuated between 18 and 31%, and was restored to approximately the t_0 level after 3 weeks (Fig. 1B).

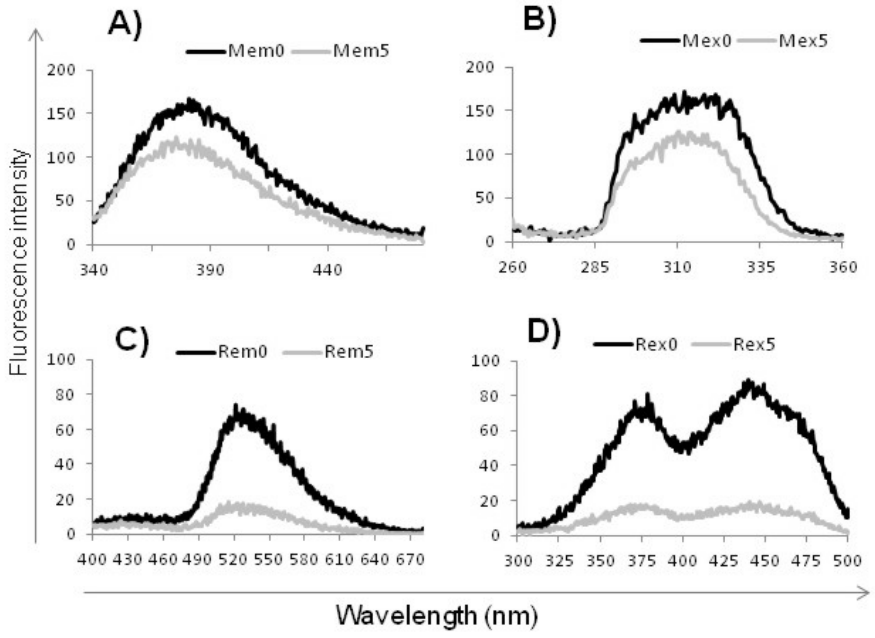

Fig. 2. Representative fluorescence spectra of emission (left) and excitation (right) of MUG fluorophore (A, B) and riboflavin (C, D) in celomocyte lysates retrieved at time 0 (dark lines) and after 5-day restoration (grey lines) in *E. andrei*.

Loss of eleocytes was much more severe: half an hour after extrusion their numbers was 4% of the initial t_0 value, then fluctuated between 7% and 17% during the first post-extrusion week, and then increases to 20%, 36%, and 58% at weeks 3, 5 and 7, respectively, with only the week 7 value being statistically similar to the initial t_0 value (Fig. 1C).

Loss and restoration of fluorophores

Fig. 2 shows examples of MUG and RF fluorescence spectra recorded at t_0 and after a 5-day recovery period. It was noteworthy that MUG levels were almost completely restored within this short time-frame, whilst riboflavin recovery at this point was negligible.

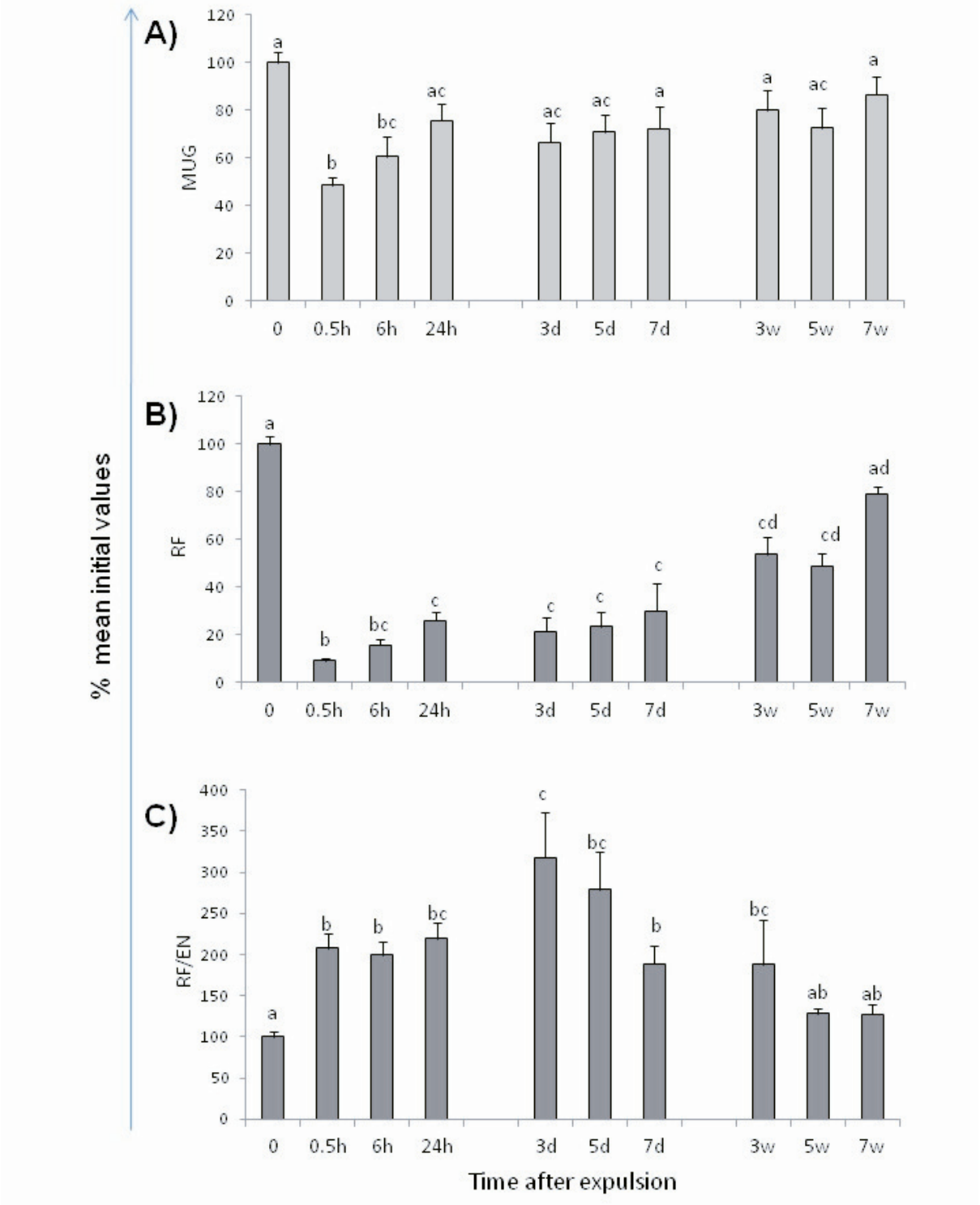

Fig. 3. Time courses of restoration of A) MUG fluorophore, B) riboflavin, and C) fluctuations of RF/EN ratio in coelomic fluid of adult specimens of *E. andrei* subjected to electrostimulation at the start of experiments and measured at time zero (0), and then after 0.5, 6, and 24 hours (h); 3, 5, and 7 days (d); 3, 5, and 7 weeks (w). Mean values calculated as percentages of those measured at time 0 ± SE; numbers of samples at each time point n = 5.

MUG fluorophore as a reliable molecular biomarker of *E. andrei*

The content of MUG fluorophore was significantly reduced (to 49% of t_0 value) within 30 minutes after extrusion of coelomic fluid, but recovered rapidly, reaching 61% of the time zero value by 6 hours post-extrusion and 85% (i.e. statistically indistinguishable from the t_0 value) by 24 hours post-extrusion. With some minor discontinuities, measured MUG values from 1 day to 7 weeks were generally maintained at levels comparable to the initial value (Fig. 3A).

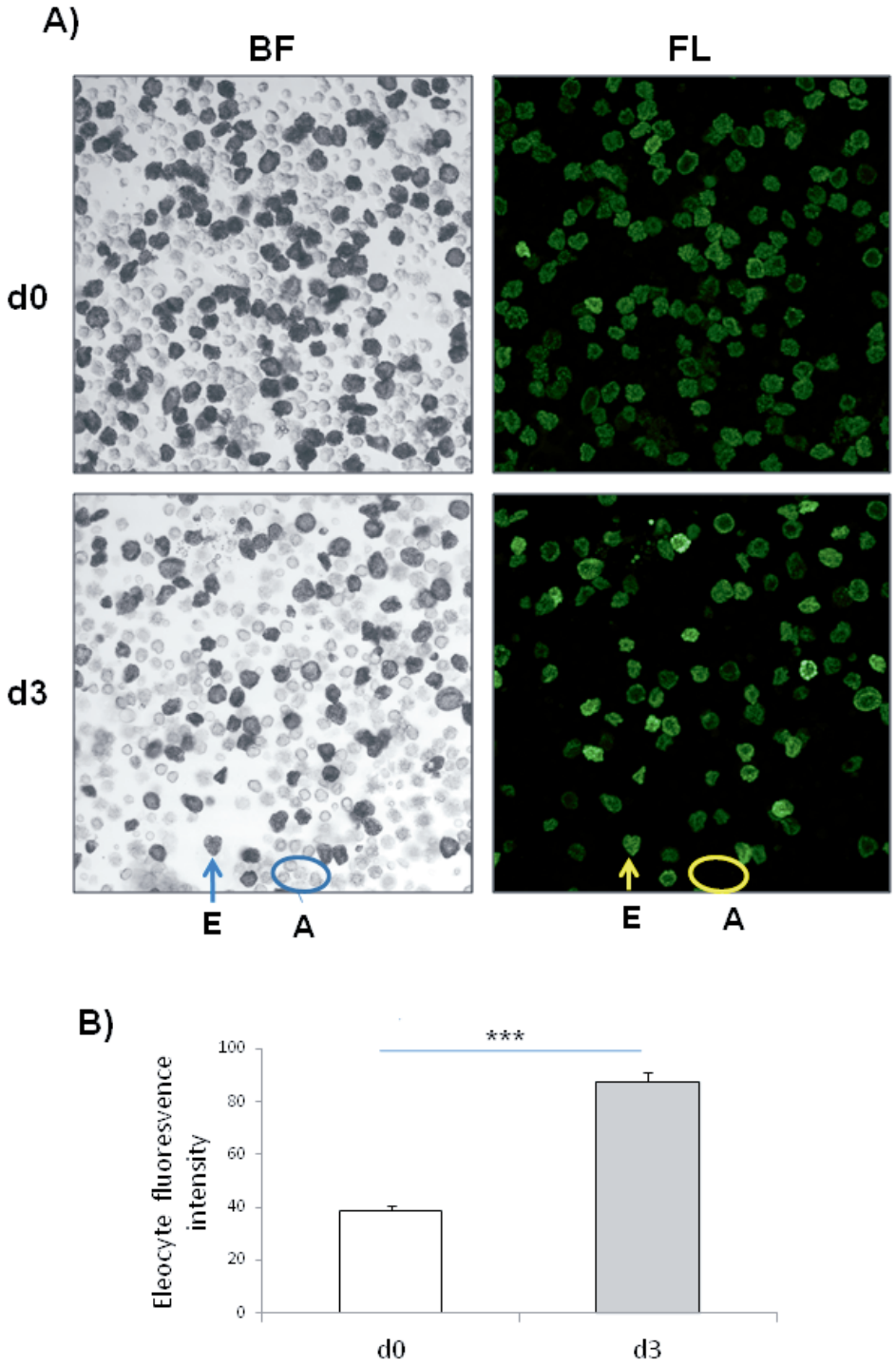

Fig. 4. Changes in fluorescence intensity of eleocytes of adult specimens of *E. andrei* retrieved by electrostimulation at the start of experiments (d0) and from the same worms three days later (d3). A – Amoebocytes (exemplified by A) and autofluorescent eleocytes (exemplified by E) visible in the bright field (BF) and in blue light (FL) under fluorescent microscope. B – Results of analysis of intensity of eleocyte fluorescence. Means ± SE from 3 worms, 30 eleocytes per worm. The difference between d0 and d3 is statistically highly significant.

Riboflavin

Half an hour after initial electrostimulation, spectrofluorimetric analysis of riboflavin (RF) content in coelomocyte lysates was only 9% of its initial t_0 baseline value. Subsequently, the riboflavin content fluctuated between 15% and 30% during the first week, and later more-or-less steadily increased to 54% (3rd week), 49% (5th week), and 77% (7th week); the latter value statistically similar to the baseline value (Fig 3B).

Riboflavin content per eleocyte (RF/EN) was 2 or 3 times higher from at 30 minutes and up to 3 weeks after electrostimulation, and only later was restored to baseline level (Fig. 3C).

Eleocyte fluorescence intensity

Fig. 4A shows fluorescence microscopy images of coelomocytes samples retrieved at the start of experiments (t_0) and three days later. In the bright field there are clearly visible both amoebocytes (pale) and strongly granulated eleocytes (dark). Fluorescence images of the same samples showed only green eleocytes but no amoebocytes. Green fluorescence was much more intense in coelomocytes retrieved 3-days post-extrusion than in coelomocytes at t_0. These morphological observations were statistically confirmed by quantitative analysis as fluorescence intensity of eleocytes on day 3 post-extrusion was twice as high as in eleocytes at t_0 (Fig. 4B).

Summary of main results

A transient loss and differential "actual" time courses of restoration of experimentally depleted coelomocytes and fluorophores in *E. andrei* are summarized in Fig. 5; numbers of amoebocytes were restored faster than numbers of eleocytes (Fig. 5A), and amount of MUG fluorophore was restored much faster than that of riboflavin (Fig. 5B). The contrast between the post-extrusion temporal changes in MUG- and riboflavin-mediated fluorescence signals was noteworthy: apart from an immediate decrease MUG fluorescence was relatively resilient, whereas riboflavin (RF) recovery was slow and protracted after a sharp early slump.

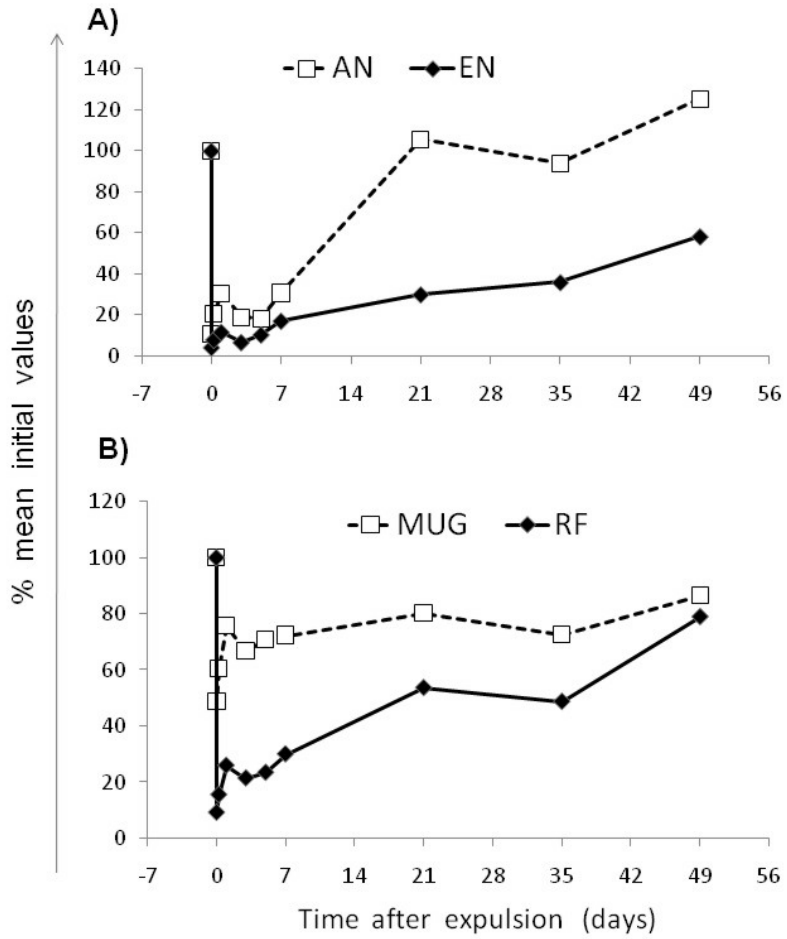

Fig. 5. 'Actual' time courses of restoration of (A) numbers of coelomocytes (amoebocytes, AN, and eleocytes, EN), and B) fluorophores (MUG and riboflavin, RF) present in coelomic fluid of adult specimens of *E. andrei* depleted by electrostimulation at the start of experiments and measured at time zero (0), and then at time points up to 49 days after depletion, expressed as percentages of the respective values at time 0 considered as 100%.

Discussion

Coelomocytes

Electrostimulation of earthworms induces sudden expulsion of coelomic fluid containing freely floating morphotic elements, the coelomocytes (COOPER *et al.* 2002). Coelomocytes comprised in *E. andrei* of two major cell types: non-autofluorescent amoebocytes and eleocytes with autofluorescent granules, the so-called 'chloragosomes' (PLYTYCZ *et al*. 2007; RORAT *et al*. 2014). Faster restoration of amoebocytes than eleocytes after coelomocyte extrusion has previously been observed in other composting earthworm species: *D. veneta* (HOMA *et al*. 2008; POLANEK *et al*. 2011; KLIMEK *et al*. 2012; SANTOCKI *et al*. 2015), and *E. fetida* (SANTOCKI *et al*. 2015). Fast recovery of amoebocyte numbers may reflect the importance of this cell type in the immune-defence of the organisms (BILEJ *et al*. 2011; OTTAVIANI 2011), and is probably achieved by the proliferation of amoebocyte stem cells located in intersegmental septa and somatopleural peritoneum (PARRY 1976). Proliferation of free-floating amoebocytes in the coelomic cavity (PARRY 1976; HOMA *et al*. 2008) may also be contributory.

Eleocytes almost certainly have a more protracted life-cycle compared with amoebocytes. They are considered to be the detached, free-floating, derivatives of attached chloragocytes precursors whose multi-functional roles include carbohydrate storage and metabolism, the sequestration of essential and non-essential metals, and probably haem synthesis (MORGAN *et al*. 2002), and cytolytic molecules (KAUSCHKE *et al*. 2007; PROCHAZKOVA *et al*. 2006) including proteins of lysenin family (OPPER *et al*. 2013; MACSIK *et al*. 2015; in preparation).

It is plausible that both amoebocytes and eleocytes are morphologically and functionally distinct lineages of the same stem cells, and that the differences in the complexity and duration of their respective life cycles may explain the differences in their restorative patterns and kinetics after non-discriminatory experimental coelomocyte depletion.

Riboflavin

It is highly likely that riboflavin is the main fluorophore responsible for fluorescence of eleocytes of both *E. andrei* and *E. fetida* (PLYTYCZ *et al*. 2006; RORAT *et al*. 2014). It is therefore plausible to hypothesise that expelled eleocytes and/or their granules were responsible for the mucus fluorescence observed by HEREDIA *et al*. (2008) in *E. foetida* (*sic*).

Riboflavin (vitamin B2) plays an important role in immunity of animals (e.g. VERDRENGH & TARKOWSKI 2005) and plants (e.g. ASAI *et al*. 2010), is responsible for quorum sensing in bacteria (e.g. ATKINSON & WILLIAMS 2009) and supports regenerative processes (JOHNSON *et al*. 2012). The amount of riboflavin stored in eleocytes of lumbricid worms is species-specific (PLYTYCZ *et al*. 2006) and changes in a species-specific manner in response to various edaphic factors (PLYTYCZ *et al*. 2011; PLYTYCZ & MORGAN 2011), but also due to expulsion of coelomocyte-containing coelomic fluid. In the latter case its initial content is restored very slowly (KLIMEK *et al*. 2012; SANTOCKI *et al.* 2015).

Freshly detached free-floating eleocytes probably have a higher riboflavin content that their older counterparts, indicating that during the 'free' phase of their cellular life cycles the eleocytes progressively release riboflavin for nutritional, immune-potentiating, and regenerative purposes (JOHNSON *et al*. 2012). A significant increase of the ratio of riboflavin content to eleocyte numbers (RF/EN) during the early weeks after extrusion of coelomic fluid was recorded in our present study and in previous studies (POLANEK *et al*. 2011; KLIMEK *et al*. 2012; SANTOCKI *et al*. 2015). However, the possibility that riboflavin is also released directly to coelomic fluid from attached chloragocytes cannot be dismissed nor, as suggested by SULIK *et al*. (2012), can alternative or additional riboflavin sources including symbiotic bacteria be excluded. Further studies are necessary to resolve this issue.

MUG fluorophore

MUG fluorophore was described as a molecular fingerprint of *E. andrei* (ALBANI *et al*. 2003) but its biological role is not known yet. Our present knowledge indicates that among compostiong earthworm species MUG is always present in *E. andrei*, is not expressed in *D. veneta*, whilst some individual specimens of *E. fetida* appear to possess a MUG-like fluorophore of much lower intensity than that consistently observed in *E. andrei* (RORAT *et al*. 2014; SANTOCKI *et al*. 2015). Studies on the putative origin of this fluorophore in the minority cohort of *E. fetida* are in progress. Despite the fact that molecular-genetic phenotyping techniques have demonstrated that *E. fetida* and *E. andrei* are true species (PEREZ-LOSADA *et al*. 2005; OTOMO *et al*. 2009), we cannot overlook the possibility that some individuals within our laboratory cultures are genetic hybrids. Our use of COI mitochondrial gene barcoding (RORAT *et al*. 2014; SANTOCKI *et al*. 2015) on randomly sampled individuals does not negate the possibility that hybrids could have been included inadvertently in our ex-

periments. Although DOMINGUEZ *et al*. (2005) and REINECKE & VILJOEN (1991) concluded that *E. andrei* and *E. fetida* were reproductively isolated, ANDRE (1963) and SHEPPARD (1988) observed the production of *E.andrei-E.fetida* hybrids.

The advantage of establishing a non-genetic marker like the MUG fluorophore that can be used for contemporaneous taxonomic screening of individual earthworms alongside cytological assays of immune-cell status is self-evident. Furthermore, the MUG has the potential to be a sensitive, robust and specific distinguishing marker of *E. andrei* even when its immune system is disturbed. Relatively low intensity MUG signal may also be a reliable biochemical trait enabling individual *andrei*/*fetida* hybrids to be discerned.

The present observations indicate that the MUG fluorophore derives not only from coelomocytes but is released to coelomic fluid from a number of different cell types lining the coelomic cavity and/or from other (bacterial?) sources (in progress).

Conclusions

Stressed earthworms expel coelomic fluid containing several vital cellular and soluble components, thus their post-stress recovery has adaptive value. Numbers of amoebocytes are restored much faster than numbers of riboflavin-containing riboflavin. Freshly restored eleocytes are richer in riboflavin than mature eleocytes from undisturbed worms, but the initial level of riboflavin is restored slowly. In contrast, MUG fluorophore specific for *E. andrei* is less severely depleted and relatively quickly reaches the initial level. Such information is important for investigators of *Eisenia* sp. physiology, immunology, and/or ecotoxicology as it shows that 1) MUG fluorophore is a robust marker of *E. fetida*; 2) intraspecific variations/fluctuations in cellular and soluble components of coelomic fluid may be dependent not only on worm age/body size and modified by environmental factors, but also may be caused by stress-induced depletion of coelomocyte-containing coelomic fluid followed by gradual restoration of particular components in a differential rate.

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