

## RESEARCH REPORT

**Reversible inhibition of reproduction during regeneration of cerebral ganglia and celomocytes in the earthworm *Dendrobaena veneta*****J Okrzesik<sup>1</sup>, N Kachamakova-Trojanowska<sup>2</sup>, A Jozkowicz<sup>2</sup>, AJ Morgan<sup>3</sup>, B Plytycz<sup>1</sup>**<sup>1</sup>*Department of Evolutionary Immunology, Institute of Zoology, Jagiellonian University, Krakow, Poland*<sup>2</sup>*Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics, and Biotechnology, Jagiellonian University, Krakow, Poland*<sup>3</sup>*Cardiff School of Biosciences, Main Building, Cardiff University, Cardiff CF10 3US, Wales, UK**Accepted November 25, 2013***Abstract**

Earthworms may be subjected to mechanical/chemical stimuli and/or sub-lethal predator attacks leading to the extrusion of celomocytes and/or loss of body parts; thus, regeneration of cells, tissues and organs has adaptive value. The aim of present study on the lumbricid earthworm *Dendrobaena veneta* was to determine the interactive effects of celomocytes and the brain on the regeneration of either system after experimental depletion or extirpation, and to assess the effects of such treatments on reproductive performance. Decerebration was achieved either by amputating the first six anterior segments, or by surgery; celomocyte depletion was achieved by a standard electro-stimulation procedure. Celomocytes (amebocyte and eleocytes, respectively) were counted by hemocytometry, and riboflavin content in celomocyte lysates measured by spectrofluorimetry. The main findings were: (i) *D. veneta* regenerated anatomically intact brain, including neurosecretory cells, within 10 - 18 weeks after its removal plus celomocyte depletion (i.e. dual treatment); (ii) amoebocyte counts recovered to control levels by 10 weeks after extrusion treatment alone, but were still lower (60 %) than in controls at 18 weeks after dual treatment; eleocyte recovery after electro-stimulation alone was slow, reaching control levels only after 18 weeks, and was further retarded (31 % of controls at 18 weeks) by brain extirpation; (iii) riboflavin content was lower than controls only in the dual-treatment worms at 5 weeks; riboflavin content relative to eleocyte numbers was initially higher than controls in both treatment groups; this index was restored to control levels by 18 weeks in the electro-stimulation only treatment whilst recovery was somewhat retarded in the brain-extirpated group; (iv) celomocyte depletion treatment alone slightly impaired reproductive output, whilst brain removal had pronounced and protracted inhibitory effects. The observations engender the hypothesis that brain-derived neurosecretions and immune-competent celomocytes act in tandem to modulate neural regeneration and reproduction.

**Key Words:** *Dendrobaena veneta*; immune-neuroendocrine interactions; celomocytes; riboflavin; cocoon production; regeneration

**Introduction**

Interest in earthworms as metazoan models for studying fundamental aspects of tissue regeneration date back well over a century to the series of seminal publications by the Nobel Prize-winning geneticist Thomas Hunt Morgan (Morgan, 1897). Tissue regeneration, or the restoration of lost body parts as it has been defined (Bely and Nyberg, 2009),

whilst commonplace within members of the Annelida, is extensively variable within the phylum with some enchytraeid oligochaetes able to regenerate complete organisms from fragments comprised of a few segments (Takeo *et al.*, 2010), other taxa amongst the oligochetes and polychetes possessing different degrees of ability to regenerate anterior and posterior segments, with posterior regeneration particularly prevalent, and Hirudinea apparently incapable of any segment replacement (Bely, 2006). Earthworms are anatomically more intricate than other favoured model organisms, such as sponges, hydra and flatworms, since they are metamerically-segmented coelomates with a closed circulation and a well-developed nervous system.

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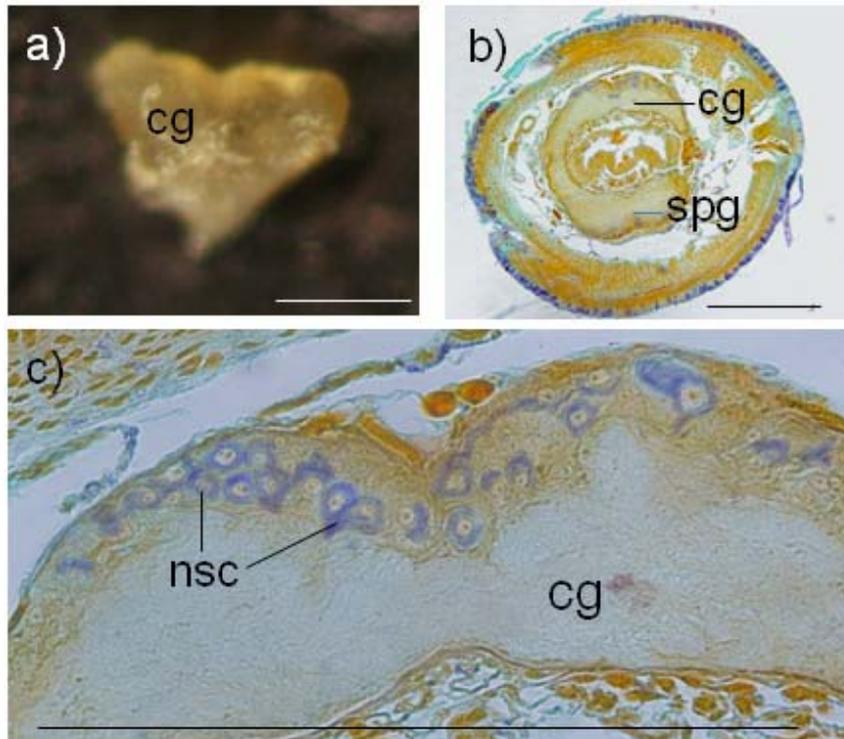
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**Fig. 1** The regeneration of surgically-extirpated cerebral ganglion in *D. veneta*. (a) Whole cerebral ganglion (cg) experimentally removed through a small dorsal incision at time 0; (b) Micrograph of a transverse section through the anterior segments of *D. veneta* fixed 18 weeks after cg extirpation; the plane of the section, stained with paraldehyde fuchsin, corresponded with the position of the fully regenerated cerebral ganglion (cg); spg - subpharyngeal ganglion. (c) A magnified view of the regenerated cerebral ganglion (cg) depicted in Fig. 1b; note the distinct cluster of neurosecretory cells (nsc). Bars = 1 mm.

Earthworms are relatively easy to culture and manipulate in the laboratory, and knowledge of particular aspects of their physiology and molecular genetics is substantial and burgeoning (Stürzenbaum *et al.*, 2009). Thus, they provide several advantages in terms of understanding the mechanisms underlying the regeneration of tissues and organs in a complex, albeit accessible, organism (Cho *et al.*, 2009).

Neurosecretions released by the elements of the ventral nerve cord and cerebral ganglion ('brain') are evidently involved, either directly or indirectly, in regulating not only earthworm tissue regeneration but also other vital functions such as reproduction and electrolyte balance (Alonso-Bedate and Sequeros, 1985). The remarkable ability of the earthworm to regenerate transected or ablated giant axons (Birse and Bittner, 1981), and relatively quickly (70 to 80 days) and completely the cerebral ganglion, has drawn the attention of neuroscientists seeking convenient models for studies on neuronal regeneration (Lubics *et al.*, 2002; Csoknya *et al.*, 2003). During the early phases of cephalic regeneration, when the earthworm lacks a brain and is unable to consume food, there is evidence of gross regression of developing gametes (Jamieson, 1981).

Free-floating celomocytes migrate early to the locus of wounding in earthworms, and are involved in wound plug formation and subsequent tissue remodelling by virtue of their phagocytic activities and delivery of nutrients and growth-stimulating molecules (Jamieson, 1981; Varhalmi *et al.*, 2008). A number of neuropeptides have been immunolocalised within the celomocytes of the earthworm *Eisenia fetida* (Wilhelm *et al.*, 2006). The observation (Somogyi *et al.*, 2009) that pituitary adenylate cyclase-activating polypeptide (PACAP)-like proteins not only occurs in earthworm celomocytes but it is enriched in these immune-competent cells located in the vicinity of regenerating tissues is particularly noteworthy. PACAP in vertebrates protects cells within the central nervous system against stress-induced apoptosis (Vaudry *et al.*, 2002), thus providing support for the notion (Somogyi *et al.*, 2009) of a functional link between the earthworm nervous system and celomocyte-mediated regenerative activities. Indeed, there is now a substantial body of evidence supporting the general principle of intimate interactions between the neuroendocrine and innate defense systems in invertebrates and vertebrates (Cohen and Kinney, 2007).

Samuel *et al.* (2012) recently detected autofluorescent cells in the regeneration blastema of the earthworm *Eudrilus eugeniae*, and that riboflavin (vitamin B2) was the main source of the cytoplasmic fluorescence. Moreover, these authors also observed that injected riboflavin promoted regeneration in a dose-dependent mode. Whilst it is plausible that earthworms may contain more than one type of riboflavin-containing autofluorescent cells, it has been conclusively demonstrated that one cohort of celomocytes, the eleocytes (wandering chloragocytes), are particularly riboflavin-rich in some lumbricid species such as compost-inhabiting *E. fetida* and *Dendrobaena veneta* (Kozioł *et al.*, 2006; Cygal *et al.*, 2007; Plytycz and Morgan, 2011; Sulik *et al.*, 2012; Rorat *et al.*, 2013). It is significant that earthworms possess the temperature-dependent capacity to regenerate their grossly depleted celomocyte community after experimental extrusion, with amoebocyte numbers recovering within a few weeks whilst eleocyte numbers were fully recovered much later, depending on the ambient temperature (Homa *et al.*, 2008; Klimek *et al.*, 2012). These reported differences in restoration kinetics between the two morphologically and functionally distinct celomocyte types is entirely consistent with the differences in proliferative behaviour of amoebocytes and eleocytes after wounding and tissue grafting (Parry, 1976). Both the loss of celomocytes and body segments may occur under natural conditions due to mechanical stimuli, autotomy of toxin- or waste product-laden tissues, or sub-lethal predation (Bely and Nyberg, 2009), thus the regeneration of cells and tissues in earthworms has potential adaptive value.

Just as there has been a long-term interest in tissue regeneration in earthworms, there has also been considerable research activity centered on the impact of environmental toxicants on earthworm celomocytes (Plytycz *et al.*, 2007; Plytycz and Morgan, 2011) whose momentum continues apace into the nanotechnology age (Hayashi and Engelmann, 2013). Despite these efforts much remains to be known and understood about the interactions within the neural-immune axis in the context of cell proliferation and regeneration. Therefore, the primary aim of the present study was to determine the effect of brain extirpation on the temporal recovery of amoebocyte and eleocyte populations in *D. veneta* after electro-stimulated expulsion. These changes, including the riboflavin content of eleocytes, were tracked in relation to the morphology of the regenerating brain. A subsidiary aim was to monitor the effects of brain removal and regeneration on reproductive output in terms of cocoon and hatchling counts, given that neurosecretions probably regulate restorative growth and reproduction at some level in earthworms (Alonso-Bedate and Sequeros, 1985).

## Materials and Methods

### Earthworms

Adult specimens of *D. veneta* from a commercial supplier (EKARGO, Krepa Slupska) were reared at the Institute of Zoology, Jagiellonian

University, Krakow under controlled laboratory conditions (16 - 19 °C; 12:12 LD, 25 % humidity). They were kept in plastic boxes with perforated lids in the soil from a commercial supplier (PPUH BIOVITA, Tenczynek, Poland). The worms were fed ad libitum on boiled nettle (*Urtica dioica*) leaves and the commercial soil of proper humidity was exchanged at 4 - 6 week intervals to avoid its intoxication by metabolic products.

### Experimental scheme

Earthworms of similar body weights (0.7 - 0.9 g) were selected from the stock culture and divided into control and experimental groups. Within each experimental series the number of worms and soil content in the control and 'treatment' boxes were the same, *i.e.*, 10 worms per box with 400 g of soil in preliminary experiments, and 15 worms per box with 500 g of soil in three experimental series of the main experiment. Control groups consisted of untreated worms (group 'C'). Worms whose celomocytes were extruded by mild electro-stimulation (4.5 V, 30 sec), as described below, were designated the 'V' treatment group.

### Pilot experiments

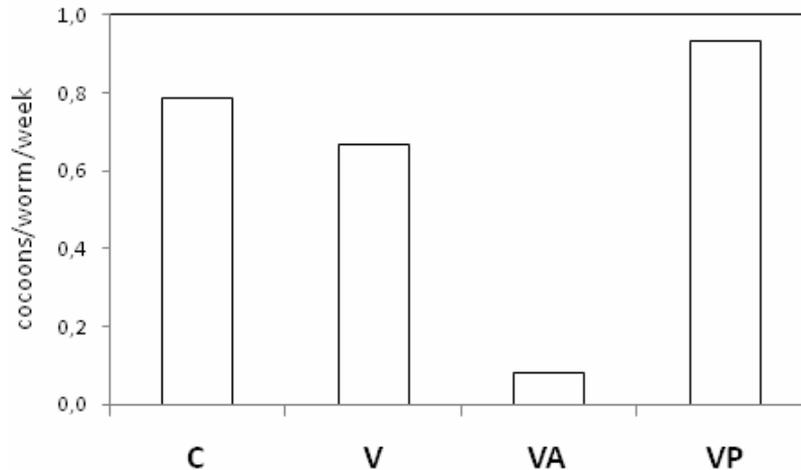
In a pilot experiment, groups of electro-stimulated worms had either 6 anterior or 6 posterior body segments amputated by transection; these treatment groups were designated 'VA' and 'VP', respectively. Therefore the exploratory experiment comprised of treatment groups C, V, VA and VP, each maintained in a separate box, with 10 individual worms per box. This experiment was informative, but in the case of the critical VA treatment it was not possible to distinguish the effects of neurosecretory disturbance from those of interrupted ability to consume food. For this reason, a second more refined experiment was conducted where the cerebral ganglion as a discrete entity was removed.

### Main experiments

In the main experiment, the 'new' treatment was the surgical extirpation of the cerebral ganglion in worms that had previously been electro-stimulated, and designated group 'VE'. Therefore the main experiment comprised of C, V, and VE treatment groups of worms kept in separate boxes, 15 worms per box. These experiments were repeated three times. At the start of main experiments (*i.e.*, on day 0) 15 earthworms from each of the treatment groups were placed on moist filter paper on the surface of fresh soil to recover from any experimental manipulation they had experienced, and after recovery they burrowed into the soil. At weeks 5, 10 and 18 post treatment, 5 worms from each of the C, V, and VE treatment boxes were weighed and subjected to the extrusion of celomocytes by electric shock followed by removal and fixation of the first 6 segments for the histological evaluation of cerebral ganglion regeneration (see below).

### Body segments amputation and cerebral ganglia extirpation

Our attempts to anaesthetise earthworms either by immersion in CO<sub>2</sub>-containing cold water, or cooling



**Fig. 2** Cocoon production rates (expressed as number of cocoons per individual worm per week) by *D. veneta* during the 6-week maintenance period after the initiation of treatment. Group C = untreated control worms; Group V = worms whose coelomocytes were extruded at  $t_0$  by electro-stimulation alone; Group VA = worms treated by electro-stimulation followed by amputation of first 6 anterior (cerebral) segments at  $t_0$ ; Group VP = worms treated by electro-stimulation followed by amputation of the 6 terminal (caudal) segments at  $t_0$ . In each case the treatment groups comprised of 10 worms.

them on a cold plate with or without exposure to chloroform, resulted in uncontrolled partial celomocyte extrusion which would obviously confound the celomocyte restoration observations. Therefore for present purposes controlled celomocyte extrusion was induced by the electro-stimulation. Electro-stimulation conveniently caused a temporary state of immobilisation that facilitated effective brain extirpation by transection or surgery without any form of anaesthesia. In the VA and VP treatment groups, celomocyte extrusion was immediately followed by transection of either the 6 anterior (VA) or 6 posterior (VP) body segments. In the VE treatment, celomocyte extrusion was followed by cerebral ganglion extirpation by cutting the circumpharyngeal connectives through a small dorsal incision in the 3<sup>rd</sup> segment; surgery was performed under a dissecting microscope. The removed cerebral ganglia were fixed in 4 % formalin. At the end of each experiment, cerebral ganglia of some representative worms from each treatment group were removed and fixed in 4 % formalin for a gross anatomical observation; in some representative cases the first 6 anterior segments were removed and fixed in Bouin's solution, dehydrated in a graded series of ethyl alcohol, rinsed in benzene, and embedded in paraffin wax according to the Sikierska (2002) protocol. Serial wax sections (6  $\mu$ m thick) were mounted on glass slides and stained with paraldehyde fuchsin for neurosecretion (Cameron and Steel, 1959).

#### Cocoon and juvenile counts

Cocoons were manually collected and counted every time the soils in the treatment boxes were replaced with fresh soil. The cocoons were cultured further at 16 - 19 °C either in the soil or individually

on moist filter paper in the wells of 96-well plate until hatching to determine their viabilities as expressed by hatchling counts.

#### Celomocyte extrusion

Earthworms were stimulated for 30 sec with a 4.5 V electric current to expel coelomic fluid with suspended celomocytes through the dorsal pores as described previously (Plytycz *et al.*, 2006). In summary, the earthworms were placed individually in Petri dishes containing 3 ml of extrusion fluid (phosphate-buffered saline, PBS, containing 2.5 g/l ethylenediamine tetra-acetic acid, EDTA (Sigma-Aldrich), to prevent cell clumping). Freshly prepared 2 ml suspensions were used for spectrofluorimetry, and the remaining sample from each worm was fixed in 2 % formalin for flow cytometry and for celomocyte counting in a haemocytometer.

#### Spectrofluorimetry

The spectrofluorimetric measurements were performed on celomocyte suspension-lysates (2 % Triton; Sigma-Aldrich) using a Perkin-Elmer Spectrofluorimeter LS50B. Excitation spectra were recorded between 300 - 520 nm ( $\lambda = 525$  nm), while emission spectra were recorded between 380 - 700 nm ( $\lambda = 370$  nm). The spectrofluorimetric signatures of unbound riboflavin are two excitation maxima (370 nm and 450 nm) and one emission maximum (525 nm). Arbitrary units (AU) of fluorescence at 525 nm emission wavelength were recorded using Microsoft Excel v. 2007.

#### Statistics

Recorded parameter values for earthworms and their celomocytes were expressed both as direct means and as fold-differences in relation to the

values for the appropriate control groups. Data were expressed as  $X \pm SE$  and analysed by ANOVA with post-hoc Tukey's test;  $p < 0.05$  was established as the level of significance.

## Results

### *Cerebral ganglion regeneration*

Figure 1 shows the gross morphology of a freshly removed cerebral ganglion (Fig. 1a). A micrograph shows a transverse histological section through the 3<sup>rd</sup> anterior segment of an earthworm that had been surgically depleted of suprapharyngeal (cerebral) ganglion 18 weeks earlier (Fig. 1b), and a micrograph depicting the paraldehyde fuchsin-stained neurosecretory cells of a regenerated cerebral ganglion at 18 weeks post-surgery (Fig. 1c). It is evident that *D. veneta* can regenerate the morphology and (apparently) the function of its cerebral ganglion within 18 weeks of extirpation.

### *Cocoon and hatchling (juvenile) counts*

The pilot experiment revealed that the cumulative numbers of cocoons laid during the first 6 post-operative weeks by the C, V, VA, and VP treatment groups of worms, 10 animal per group, were 47, 40, 5, and 57, respectively, *i.e.*, 0.8, 0.7, 0.1, and 0.9 cocoons per worm per week, respectively (Fig. 2). Celomocyte loss by electro-stimulation only slightly impaired reproductive output (group V). In contrast, the removal of anterior segments containing the suprapharyngeal and subpharyngeal ganglia, as well as the mouth and pharynx, almost completely inhibited cocoon production (group VA); whilst removal of posterior segments (group VP) appeared to stimulate cocoon production during the early regeneration phase.

Cocoon production by worms in each treatment group of the representative series of the main experiment in relation to intact controls is presented in Figure 3. Compared with controls (C), cocoon production rate in the celomocyte-deprived worms (V group) was lowered to 72 % during the first 5 post-operative weeks, then increased to 83 % of the control level between weeks 6 and 7, and then attained values (ranging from 90 % to 110 %) similar to controls at four time intervals up to 18 weeks. In contrast, in the brain-extirpated worms (VE group) cocoon production was almost completely abolished during the first 5 post-operative weeks; between weeks 6 and 7 it reached a level equivalent to 19 % of the control, and then oscillated to some extent between 31 % and 52 % of the control level between weeks 8 and 18 (Fig. 3).

Figure 4 shows the numbers of cocoons and hatchlings produced by worm per week in the C, V, and VE groups from the representative series of the main experiment. In C and VE groups the numbers of hatchlings were consistently equal to or slightly higher than cocoon numbers, indicating that most cocoons contained one embryo. In contrast, in the VE group, only 3 cocoons and no hatchling were produced up to the 5<sup>th</sup> week after surgery; the numbers of juveniles were much lower than the numbers of cocoons produced during subsequent postoperative weeks (Fig. 4).

The reproductive performance by worms from three experimental series of the main experiment in relation to intact controls is presented in Fig. 5. Compared with controls (C), cocoon production rate in the celomocyte-deprived worms (V group) were lowered to 75 % during the first 5 post-operative weeks, and then attained values similar to controls at 10 and 18 weeks (103 % and 95 %, respectively). In contrast, in the brain-extirpated worms (VE group) cocoon production was almost completely abolished during the first 5 post-operative weeks (till 5 % of the control), and then reached a level equivalent to 23 % and 30 % of the control at 10 and 18 postoperative weeks, being still significantly lower than those in the C and V groups (Fig. 5a).

Numbers of juveniles attained similar values in C and V groups at 5, 10 and 18 post-operative weeks. In contrast, in the VE group, none juveniles hatched from cocoons produced up to the 5<sup>th</sup> week after surgery. The numbers of juveniles were consistently very low throughout experimental period, as they reached only 5 % and 15 % of the control of by the 10<sup>th</sup> and 18<sup>th</sup> postoperative weeks (Fig. 5b). Thus, at 18 weeks after celomocyte retrieval and brain extirpation the reproductive performance of VE worms was still only ~15 % of the observed outputs in C and V worms (Fig. 5b).

### *Earthworm body weights*

The mean body weights of worms in the V and VE treatment groups within the main experiment did not differ significantly from control values at post-operative weeks 5, 10, and 18 (data not shown).

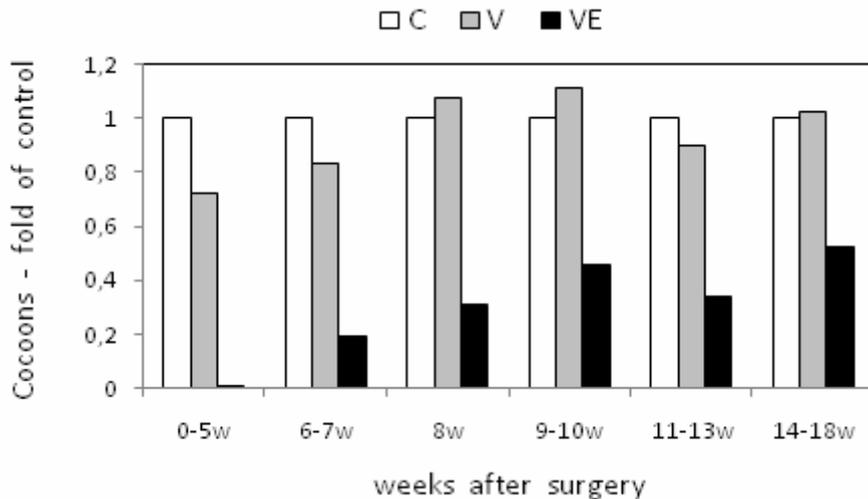
### *Amebocyte and eleocyte counts*

The number of amebocytes (AN) in the celomocyte extrusion-only treatment group (V) was reduced to 49 % at 5 weeks after electro-stimulation, but recovered to the control level by 10 weeks. Amebocyte numbers in the worms treated with both electro-stimulation and brain extirpation (VE group) were significantly lower than in the non-manipulated control worms even 18 weeks after surgery; the values at post-operative weeks 5, 10, and 18 were 21 %, 45 %, and 62 %, respectively (Fig. 6a).

Compared with amebocytes, the post-treatment recovery of eleocytes was slower. Eleocyte numbers (EN) in the V group reached those in the control worms only after 18 weeks, with levels of 40 % and 70 % relative to controls at post-treatment weeks 5 and 10. Recovery of eleocytes was even slower in the brain-extirpated group (VE), with numbers equivalent to 18 %, 30 %, and 31 % of controls 5, 10, and 18 weeks, respectively, after electro-stimulation and surgery (Fig. 6b).

### *Riboflavin content*

Riboflavin content, measured by spectrofluorimetry in celomocyte lysates, was considered to be proportional to the peak of emission at 525 nm (RF), and expressed as values relative to the corresponding control values at given post-treatment intervals (Fig. 6c). Riboflavin content was also adjusted to account for eleocyte counts (RF/EN), and again expressed in relation to the control value (Fig. 6d).



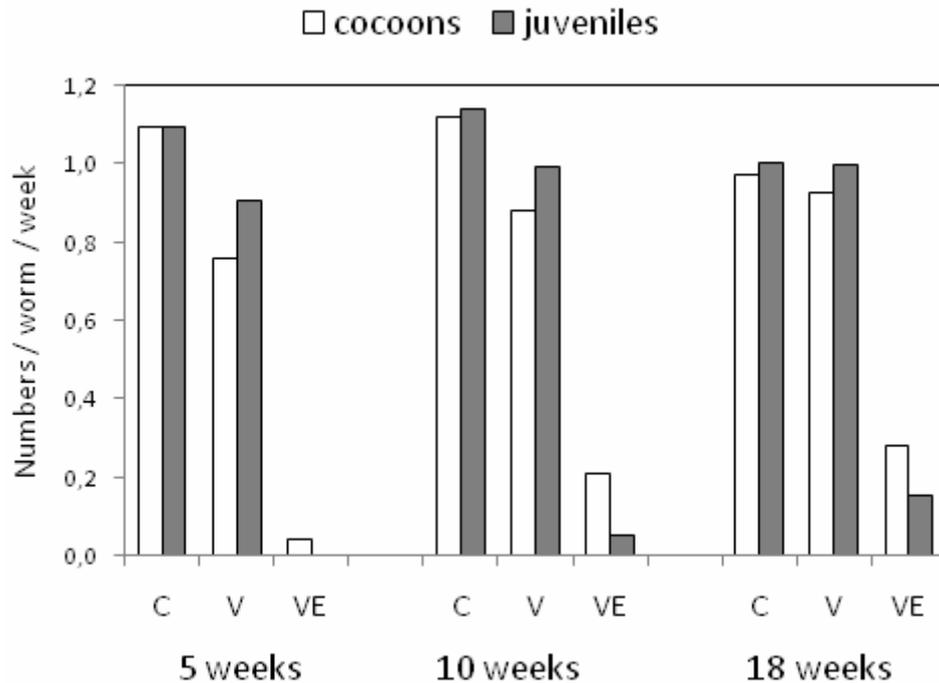
**Fig. 3** Cocoon production by treated groups of *D. veneta* relative to untreated controls. Cocoons were counted at different time intervals up to 18 weeks after the initiation of the treatments. The data are presented as a ratio of the measured number of cocoons per individual worm per week in a given treatment group to the same parameter in control worms during the same time interval. Group C = untreated control worms; Group V = worms whose coelomocytes were extruded at  $t_0$  by electro-stimulation alone; Group VE = worms treated by electro-stimulation followed by surgical extirpation/removal of the cerebral ganglion (brain) at  $t_0$ . Data from the representative experimental series.

Despite the significantly lower eleocyte numbers relative to controls at 5 and 10 weeks in earthworms subjected to experimental celomocyte extrusion (V), and at 5, 10, and 18 weeks in earthworms subjected to celomocyte extrusion and brain extirpation (VE) (Fig. 6b), the riboflavin content in celomocyte lysates was significantly lower only in the VE group at 5 weeks post-treatment (Fig. 6c). However, the amount of riboflavin as a function of eleocyte counts (RF/EN) was considerably higher (up to  $\sim 3$ ) than control values in the VE earthworm group at 5, 10, and 18 weeks post treatment, but with an apparently steady downward trend toward values observed in untreated animals (Fig. 6d). The mean RF/EN value in celomocyte extrusion-only worms (V treatment group) was significantly higher ( $\sim 3$ ) than in control worms at 5 weeks post-treatment, but by week 10 was not significantly different from the control mean value, an observation substantiated by the V versus C comparison at 18 weeks (Fig. 6d). At 18 weeks post-treatment, the significantly higher mean RF/EN value observed in the brain-extirpated group (VE) compared with celomocyte extrusion-only treatment group (V) implied that surgery retarded recovery (Fig. 6d).

## Discussion

The annelid central nervous system (CNS) is a highly differentiated neuroendocrine structure which produces neurohormones and neurotransmitters (e.g., Takahama *et al.*, 1998; Hartenstein 2006; Wilhelm *et al.*, 2006; Herbert *et al.*, 2009). In earthworms the CNS is comprised of the ventral nerve cord (VNC) consisting of segmentally

repeated ganglia joined across the midline by commissures and longitudinally by connectives. All VNC ganglia are uniform in anatomical organization except for the first VNC ganglia; these are fused to form the suboesophageal ganglion which is connected by paired circumpharyngeal connectives to a dorsal cerebral ganglion, loosely referred to as the 'brain'. The time-course of regeneration of extirpated cerebral ganglia has been described in three lumbricid earthworm species: the brain was observed to have regained its original morphological fidelity within 6 weeks in *Lumbricus terrestris* (Aros and Vigh, 1962), within 5 - 6 weeks in *Allolobophora chlorotica* (Koritsanszky and Hartwig, 1974), and within 10 weeks in *E. fetida* (Lubics *et al.*, 2002). In the present study, *D. veneta* reformed a histologically complete brain, including previously described neurosecretory cells (Siekierska, 2003), within 18 weeks after surgical extirpation combined with the additional restorative burden of replacing celomocytes extruded by electro-stimulation. This apparent delay in brain regeneration observed in the dual treatment leads us to postulate a bi-directional relationship between the neural and immune system of earthworms. It has long been recognized that neurosecretions promote the regeneration of, for example, amputated caudal segments, and that the activities of immunocompetent celomocytes (Bilej *et al.*, 2011) at the locus of regeneration are crucial and maybe functionally orchestrated by neurosecretions (Somogyi *et al.*, 2009). On the other hand, the regeneration of the brain itself, whilst largely dependent by the suboesophageal ganglion via the connectives (Lubics *et al.*, 2002) and possibly aided by neurotrophin-like molecules (Davoli *et al.*, 2002), appears also to be nurtured by



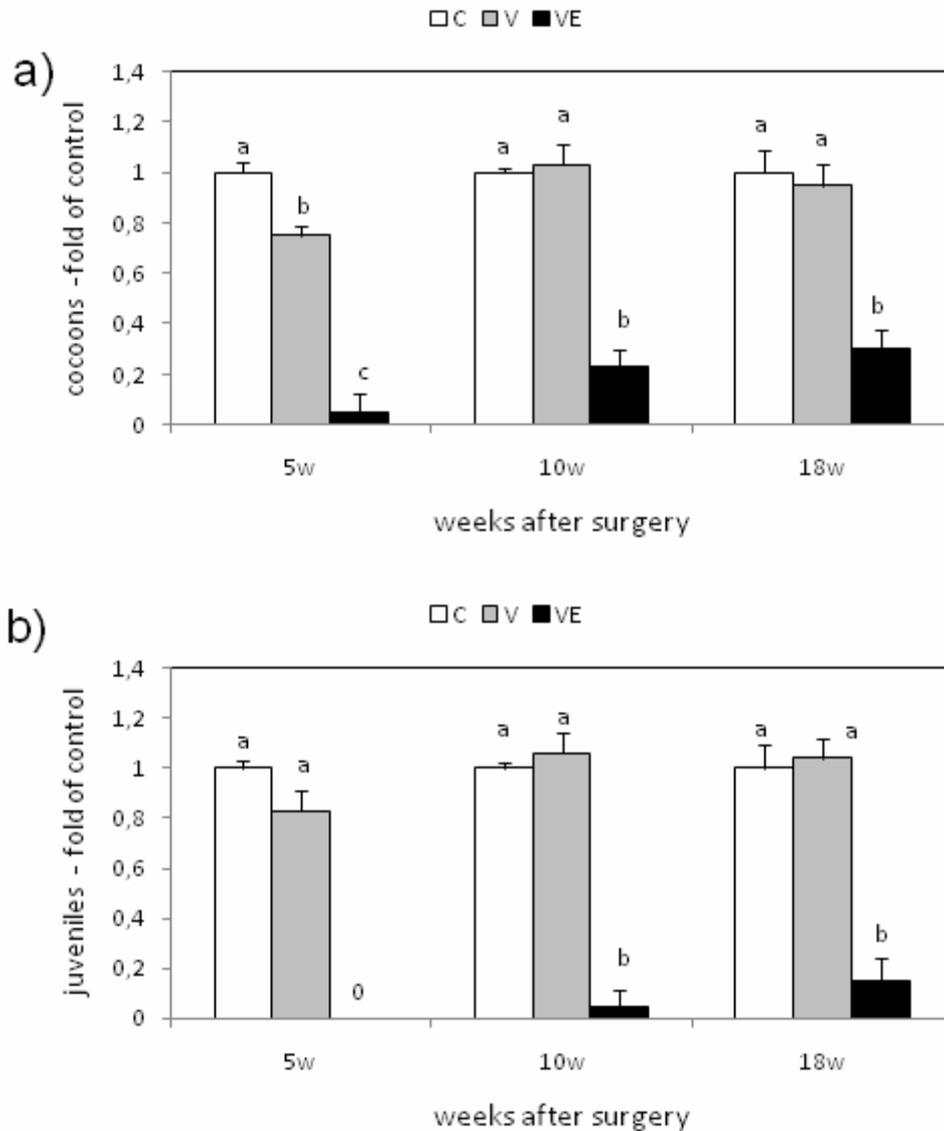
**Fig. 4** Reproductive outputs of control and treated earthworm groups measured at 5, 10 and 18 weeks after the initiation of treatment (cf. Fig. 3). Data expressed as number of cocoons or juvenile worms per adult worm in a given treatment group per week. C = untreated control worms; Group V = worms whose coelomocytes were extruded at  $t_0$  by electro-stimulation alone; Group VE = worms treated by electro-stimulation followed by surgical extirpation/removal of the cerebral ganglion (brain) at  $t_0$ . Data from the representative experimental series.

celomocytes. It is plausible that members of the celomocyte community deliver nutrients, signaling molecules, growth factors, and vitamins, as well as providing various protective roles in the vicinity of neural damage and repair (Jamieson, 1981; Varhalmi *et al.*, 2008). Whilst it is imprudent to attempt a consolidation of this notion because of the relatively meager state of knowledge about the composition and function of celomocytes, it is pertinent to note that the functional intimacy of the neural and immune systems has been more thoroughly expounded in another taxon of clitellate annelids, the leeches, where the term “neuroimmune system” is accepted in the lexicon (Salzet and Macagno, 2009).

Expulsion of celomocyte-containing coelomic fluid may be induced by various chemical and physical stressors, such as thermal, chemical, and mechanical stimuli, including sub-lethal predator attacks. Stress-inducing stimuli can ultimately reconfigure the neuroendocrine-immune network at molecular, cellular, and physiological levels (Adamo, 2012). The indiscriminate loss of a significant proportion of the free-floating celomocytes of *D. veneta* in the present study evidently modified the process of brain regeneration. It is presently unclear what roles the main celomocyte types, amebocytes and eleocytes, play in space and time during regeneration. Klimek *et al.* (2012) reported that the amebocyte count in *D. veneta* returned to control levels within 4 weeks after experimental extrusion in

otherwise intact worms. This recovery rate is rather faster than observed in the present study, but both studies agree that the recovery of eleocyte counts lags significantly behind that of amebocytes. This is not surprising given the different life cycles of the two cell types. Parry (1975) found that ‘free’ amebocytes are mitotically active; eleocytes, on the other hand, are detached mature chloragocytes (Klimek *et al.*, 2012) with consequential higher replenishment inertia. The compositional distinctiveness of amebocytes (Somogyi *et al.*, 2009) is also reflected in their functional capacities as displayed, for example, by the selective uptake of metallo-nanoparticles (Hayashi and Engelman, 2013). Thus, it is tempting to conclude that amebocytes play more prominent roles than eleocytes in brain regeneration, particularly during the early phases, because eleocyte counts are still very low at 18 weeks post-treatment at a time when the brain has fully recovered its morphological integrity. However, our observations indicate that cell counts alone may be misleading.

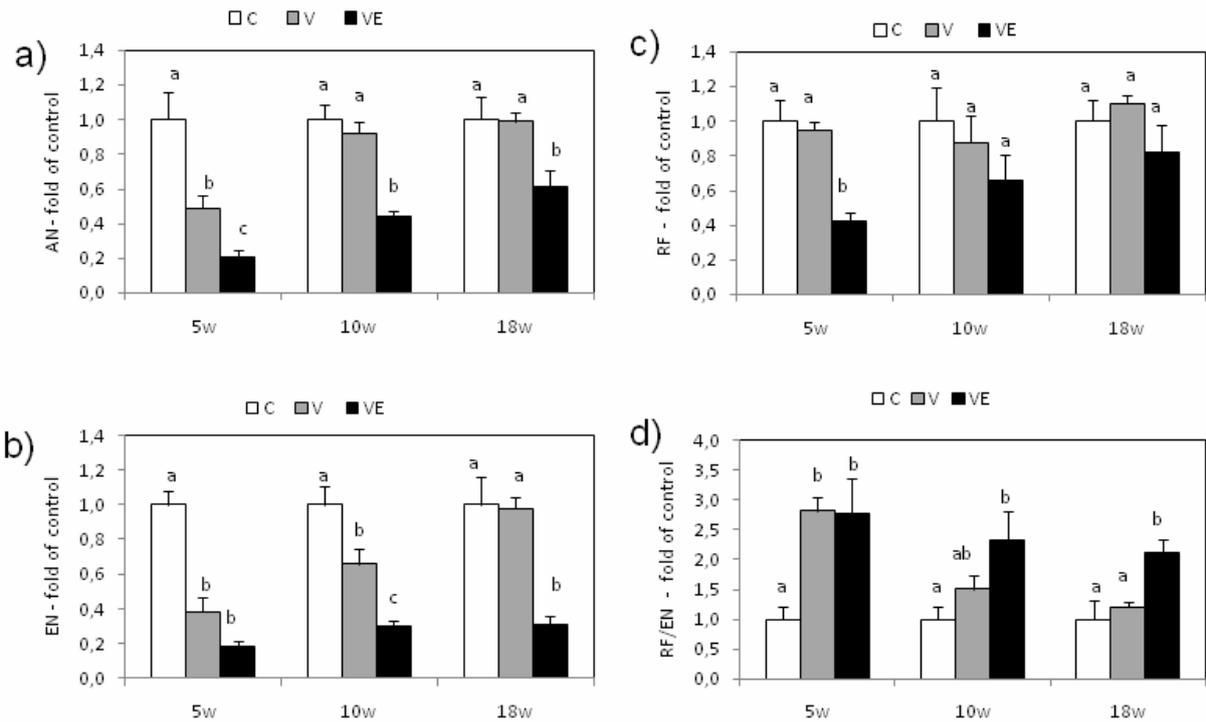
Eleocytes, but not amebocytes, exhibit autofluorescence, a signal confined to their intracellular ‘chloragosome’ granules (Plytycz *et al.*, 2007) and derived primarily from riboflavin (vitamin B2) (Kozioł *et al.*, 2006; Cygal *et al.*, 2007; Sulik *et al.*, 2012). Riboflavin (vitamin B2) plays an important role in immunity of animals (Verdrengh and Tarkowski, 2005) and plants (Zhang *et al.*, 2009). The recent findings by Samuel *et al.* (2012) that



**Fig. 5** Reproductive outputs of control and treated earthworm groups measured at 5, 10 and 18 weeks after the initiation of treatment (cf. Fig. 3). Data expressed as number of cocoons or juvenile worms per adult worm in a given treatment group per week. C = untreated control worms; Group V = worms whose coelomocytes were extruded at  $t_0$  by electro-stimulation alone; Group VE = worms treated by electro-stimulation followed by surgical extirpation/removal of the cerebral ganglion (brain) at  $t_0$ . All values relative to those measured in respective control samples considered to be 1. Data were combined from three different replicated experiments. Data presented as  $X \pm SE$  ( $n = 5 - 15$ ). At a given time interval post initiation of treatment, columns with the same lower-case letter were not statistically (Mann-Whitney) different at  $p < 0.05$ .

riboflavin augments regeneration of amputated earthworm body segments are very instructive; riboflavin not only promoted blastema growth but, when co-injected with antibiotics, it blocked the inhibitory effects of the antibiotics on blastema growth. It is a curious observation in our study that, in the presence of a brain, the riboflavin content of celomocyte lysate reached the control level by 5 weeks post-extrusion even though the eleocyte count was still significantly below control at this point. Even in brain-extirpated worms the riboflavin content of the lysate was similar to controls by 10

weeks after treatment, although the neural disruption significantly retarded eleocyte count recovery. Interpreting the disparity between the rapid restoration of riboflavin status and the lag in riboflavin-storing cell (eleocyte) counts is difficult. It is evident that the cohort of chloragocyte-derived eleocytes detached in response to electro-stimulated extrusion possesses higher riboflavin content, but what is the source of the 'extra' vitamin? One possibility is that stress-mediated neuro-immune reconfiguration (Adamo, 2012) is involved. This could entail the trafficking of riboflavin



**Fig. 6** Regeneration of coelomocyte system after 5 (5w), 10 (10w), and 18 weeks (18w) after experimental treatments: (a) amebocyte counts (AN); (b) eleocyte counts (EN); (c) amount of riboflavin (RF) in celomocyte lysates measured by spectrofluorimetry; (d) the relative amount of riboflavin in celomocyte lysates expressed as a function of eleocyte counts (RF/EN); all values relative to those measured in respective control samples considered to be 1. C = untreated control worms; Group V = worms whose coelomocytes were extruded at  $t_0$  by electro-stimulation alone; Group VE = worms treated by electro-stimulation followed by surgical extirpation/removal of the cerebral ganglion (brain) at  $t_0$ . Data were combined from three different replicated experiments. Data presented as  $X \pm SE$ ,  $n = 5$ . At a given time interval post initiation of treatment, columns with the same lower-case letter were not statistically (Mann-Whitney) different at  $p < 0.05$ .

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 endo-symbionts, the main source of riboflavin for earthworms according to Sulik *et al.* (2012), are somehow regulated by factors involved in the neuro-immune system linked to regeneration pathways. These hypotheses warrant robust investigations. However, whatever the source of the riboflavin, it is evident that riboflavin content is restored sufficiently rapidly after electro-stimulated celomocyte depletion in otherwise intact worms, and in worms subjected to celomocyte depletion after brain extirpation, to potentially play a role in brain regeneration analogous to that described by Samuel *et al.* (2012) in the regeneration of amputated segments.

Cocoon production is inhibited both in starved earthworms and in earthworms restoring experimentally extruded celomocytes (Polanek *et al.*, 2006). These observations were putatively confirmed in the present study, where caudal amputation did not impair cocoon production, whilst cerebral amputation of segments containing mouth, pharynx, and cerebral and subpharyngeal ganglia completely inhibited reproductive output for several

weeks after transection. However, the transection of anterior segments precludes distinguishing the effects of interrupted feeding ability from the effects of removing a key source of neurosecretions as well as the nutritional cost of regenerating vital organs. In our main experiment, reproduction was completely inhibited in worms with surgically extirpated brains and subsequently recovered slowly during the 18 week post-operative period, but had not fully restored to control levels even when the brain was apparently fully regenerated. There is some evidence that both restorative growth and reproduction in earthworms are controlled by neurosecretions (Alonso-Bedate and Sequeros, 1985). Moreover, an oxytocin-related neuropeptide, anetocin, eliciting stereotypical egg-laying behaviour patterns (Oumi *et al.*, 1996), is secreted by the cerebral ganglia (Takama *et al.*, 1998) with receptors located within urine-forming nephridial tubules in the clitellum region (Kawada *et al.*, 2004). Sound microscopic evidence has been published indicating that brain removal causes immediate and profound structural disruption within the gonads of the earthworm *D. veneta*, but that the damage is largely repaired at 20 days after decerebration (Siekierska, 2002, 2007). These observations

combined with our findings suggest that the attenuated suppression of reproductive output in worms with surgically excised brains and depleted celomocytes is at least partly, directly or otherwise, a consequence of the experimental manipulation of the immune-competent circulating cells.

In conclusion, the present study provides some evidence that the neurosecretory brain of earthworms and the celomocytes act in concert to regulate fundamentally important functions such as tissue regeneration and reproduction. Over a century after the pioneering tissue regeneration work of Morgan and others, the detailed investigations of the molecular-genetic and physiological basis of these interactions within the "neuroimmune system" of terrestrial oligochaetes is long overdue.

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