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EXPOSURE TO EXTREMELY LOW FREQUENCY (50 HZ) ELECTROMAGNETIC FIELD CHANGES THE SURVIVAL RATE AND MORPHOMETRIC CHARACTERISTICS OF NEUROSECRETORY NEURONS OF THE EARTHWORM *EISENIA FOETIDA* (OLIGOCHAETA) UNDER ILLUMINATION STRESS

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Abstract - An *in vivo* model was set up to establish the behavioral stress response (rate of survival) and morphometric characteristics of A_1 protocerebral neurosecretory neurons (cell size) of *Eisenia foetida* (Oligochaeta) as a result of the synergetic effect of extremely low frequency electromagnetic fields (ELF-EMF – 50 Hz, 50 μT, 17 V/m and 50 Hz, 150 μT, 17 V/m, respectively) and constant illumination (420-450 lux). If combined, these two stressors significantly ($p<0.05$) increased the survival rate of *E. foetida* in the 150 μT-exposed animals, because of delayed caudal autotomy reflex, an indicator of stress response. In addition, morphometric analysis indicated that there were changes in the protocerebral neurosecretory cells after exposure to the ELF-EMF. The present data support the view that short-term ELF-EMF exposure in "windows" of intensity is likely to stimulate the immune and neuroendocrine response of *E. foetida*.

Key words: Light stress, electromagnetic field, survival rate, neuroendocrine response, *Eiseina foetida*

INTRODUCTION

Extremely low frequency electromagnetic fields (ELF-EMF) affect biological systems and induce biological changes ranging from the molecular to cell level (Goodman and Blank, 2002). Many reported results could be ascribed to a synergistic action of the ELF-EMF with a certain chemical or physical agent(s). Anomalies in the embryos of *Drosophila* sp. subjected to both electromagnetic field (EMF) and elevated temperature are more frequent than in embryos subjected to thermal stress alone (Michel and Gutzeit, 1999). It has been shown that ELF-EMF in the presence of another stressor strongly enhances the stress response to elevated temperature in the nematode *Caenorhabditis elegans* (Junkersdorf et al., 2000). Meta-analysis of data from *in vitro* studies and short-term animal studies suggest that magnetic fields do interact with other chemical and physical exposures (Juutilainen et al., 2006).

It has been well established that various stress causes changes in Oligochaeta neuroendocrine system activity (Al Yousuf, 1988; Aros and Vigh, 1959; Awasthi and Mishra, 1974; Carley, 1978; Fischer and Molnár, 1997; Kulkarni et al., 1979; Siekierska, 2003; Stevanović and Matavulj, 1988). The neuroendocrine system of Oligochaeta consists of neurosecretory neurons located in the protocerebral ganglion and ventral nerve cord. The protocerebral ganglion of Oligochaeta has many types of neurosecretory neurons. These cells are distinguishable by their size, position of their somata, stain affiliation, as well as by their ultrastructural characteristics. In *Eisenia foetida*, A_1 neurones are located dorsolaterally and close to the surface of the protocerebrum (Al You-

suf, 1988; de Morais et al., 1979; Herlant-Meewis, 1966).

Continuous light exposure is a potentially powerful stress factor, acting as a disruptor of the circadian cycle. This, in turn, may provoke neuroendocrine variations, leading to immunomodulation, behavioral changes and changes in the response of an organism to genotoxic stress (Antoch and Kondratov, 2010; Van der Meer et al., 2004). In mollusks, constant light induces behavior changes and a significant reduction in the hemocyte number, and consequently alters the immune response of these animals (Waissel et al., 1999). Constant illumination also induces the hyperfunction of neuroglandular cells in the earthworm *Lumbricus rubellus* (Stevanović and Matavulj, 1988). Al Yousuf (1988) reported that illumination leads to dramatic neurosecretory bursts of A cells in *Lumbricus terrestris* protocerebral ganglion.

Considering the findings that ELF-EMF alternates the stress response, the goal of this study was to establish the behavioral stress response (rate of survival) and morphometric characteristics (cell size) that indicate the activity level of A_1 protocerebral neurosecretory neurons of *Eisenia foetida* (Oligochaeta) after constant illumination with and without ELF-EMF exposure.

MATERIALS AND METHODS

Experimental animals

The study was performed on the earthworm *Eisenia foetida* (Oligochaeta), a test organism that has been recommended for chronic irradiation studies (Reinecke and Reinecke, 2004). Furthermore, earthworms have been established as a model for studying innate immune responses from an evolutionary perspective (Cooper et al., 2002; Field et al, 2004).

A stock culture of earthworms was maintained in a plastic container at 22±1°C, and 65-75% humidity. A commercially available brand of topsoil was used for the culture substratum. Water was added as necessary to maintain moist conditions. Laboratory rat diet pellets mixed with water were used as food; they were added weekly to the culture substratum (Gibbs et al., 1996). The earthworms used in the experiments were adults, with well-developed clitella, randomly selected from the stock culture.

Exposure protocol

For the purpose of the experiment, 54 animals divided into three groups were used. All animals were chronically exposed to continuous illumination of 420-450 lx (Luxmeter with data logger PCE-174, PCE Instruments UK Ltd, Southampton), individually, in Petri dishes (diameter 100 mm) padded with damp filter paper, for three days (72 h). Beside light exposure, the first and the second group were exposed to ELF-EMF (50 Hz, 50 μT, 17 V/m and 50 Hz, 150 μT, 17V/m, respectively), for 4 h daily during the experiment, and the third group was a sham-exposure group treated only with constant light. The surviving animals from all the groups were fixed in Bouin's fixative at the end of the experiment.

An additional 63 animals were divided into three groups. The first and second groups were individually exposed, in Petri dishes, to illumination and ELF-EMF (50 Hz, 50 μT, 17 V/m and 50 Hz, 150 μT, 17V/m, respectively), acute for 2 h or 4 h, and the third group was a sham-exposure group. From each experimental group seven animals were fixed in Bouin's fixative after 2 h and 4 h of exposure (respectively), and 24 h after initial exposure.

ELF-EMF Exposure System

Considering that coils provide a uniform magnetic field only in a limited volume at their center (Kirschvink, 1992), we made an exposure system with four square coils in order to maximize the uniformity region of the magnetic field inside a coil system. The characteristics of the ELF-EMF produced by our system were determined by Herceg et al. (2009). Measurements were performed using the EMF analyzer EFA-300 (Narda Safety Test Solutions, Hauppauge, NY, USA) with a built-in isotropic magnetic field probe.

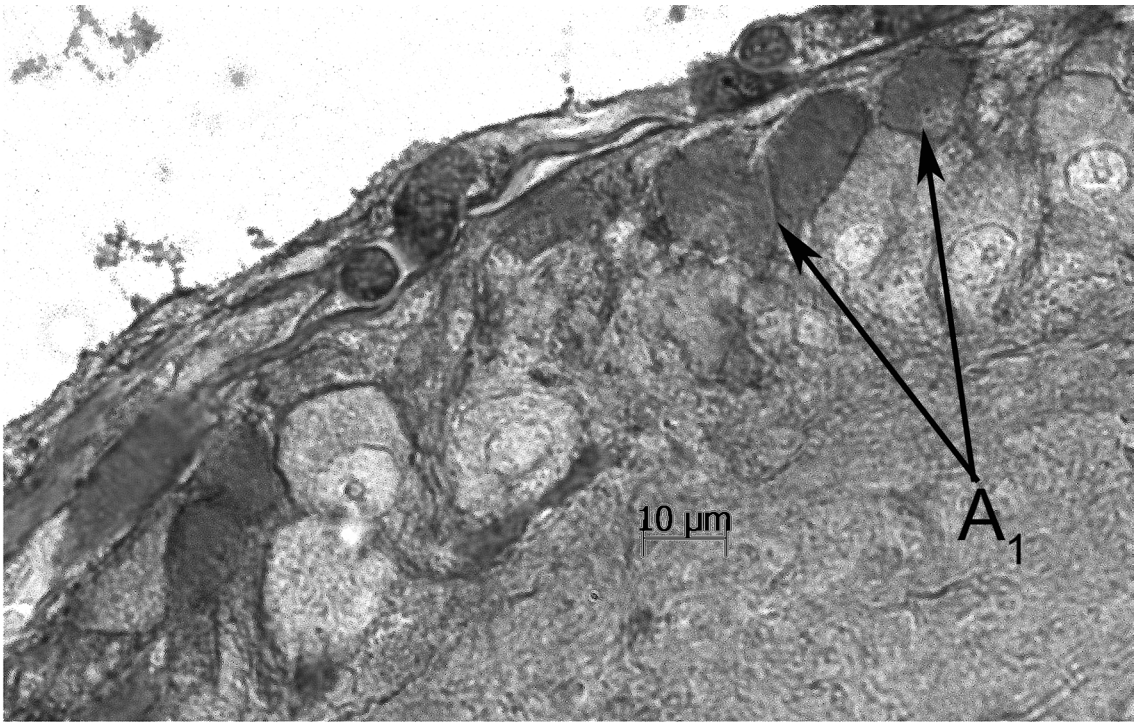

Fig. 1.- Protocerebral A_1 neurosecretory cells in transverse cross-section of *Eisenia foetida* protocerebral ganglion

The exposure system was energized at 50 Hz, 220 V *via* a transformer with the required output. The system was linear with regard to the current intensity, and produced the electric field of 17 V/m and a magnetic field with flux density (B) of 50 µT and 150 µT. The EMF produced by the coils was in a vertical direction with regard to the geomagnetic field of 40 µT.

In the central region of the coils, the magnetic field was uniform and the coils provided a region of nearly uniform magnetic field sufficiently large for the exposure of a maximum nine Petri dishes.

Histological procedure

The *Eisenia foetida* samples fixed in Bouin's fixative was separated to the front of the body behind the clitellum, rinsed, dehydrated in serial ethanol concentrations, impregnated in xylol and paraffin wax. Serial 5 µm-thick whole body cross-sections were stained using the Alcian blue-PAS-orange G technique. In serial cross sections of protocerebrum, the A_1 neurosecretory cells (n=20) were easily selected based on their morphological characteristics (Fig. 1). Namely, the neurosecretory material in these cells acquired *different shades* of homogeneous blue. The nucleus of the neurosecretory cells was often obscured by an abundance of neurosecretory material (Stevanović, 1976).

Morphometry

The sizes of the neurosecretory neurons (in μm^2) were calculated as the mean value of product multiplication of their longer (a) and shorter (b) axes (a x b in μm^2). The cross sections of the protocerebral ganglion were digitized by an image processing and analysis system, AxioVision Rel. 4.8.1. (Carl Zeiss MicroImaging GmbH, Germany), linked to an Axio Imager 1 light microscope (Carl Zeiss, Germany) with an AxioCam MRc 5 digital camera. Digitized sections were further analyzed and measurements were performed using the image analysis software Digimizer 4.0.0.0. (MedCalc Software, Belgium).

Table 1. Survival rate of *Eisenia foetida* during 72 h of continuous illumination, with and without ELF-EMF exposure.

	Sham-exposure		50μT		150 μT	
	Survived	Lethal	Survived	Lethal	Survived	Lethal
No	8	10	10	8	14	4
rate	44.44%	55.56%	55.56%	44.44%	77.78%*	22.22%

* - statistically significant ($p<0.05$, Fisher's exact test)

Table 2. The size (μm²) of *Eisenia foetida* protocerebral A_1 neurosecretory cells exposed to 50 Hz ELF-EMF and constant illumination in different time intervals. The control group taken from stock culture (C), sham-exposure, i.e. group exposed only to constant illumination (S), 50μT and constant illumination exposed group (50 E) and 150 μT and constant illumination exposed group (150 E)

	A_1 neuron size (μm²)	
	C	115,60
2 hours	S^a	$130,21^{b,c,f,g,h,i,j,k}$
	50 E^b	$113,74^{a,c,d,e,i,l}$
	150 E^c	$106,11^{a,b,d,e,f,g,h,i,j,k,l}$
4 hours	S^d	$129,55^{b,c,f,g,h,j,k}$
	50 E^e	$129,03^{b,c,f,g,h,j,k}$
	150 E^f	$116,83^{a,c,d,e,l}$
24 hours	S^g	$117,89^{a,c,d,e,l}$
	50 E^h	$116,16^{a,c,d,e,i,l}$
	150 E^i	$123,49^{b,c,h,j,k,l}$
72 hours	S^j	$116,00^{a,c,d,e,i,l}$
	50 E^k	$116,62^{a,c,d,e,i,l}$
	150 E^l	$132,34^{b,c,f,g,h,i,j,k}$

index – values indicated by same letters denote significant difference among experimental groups ($p<0.05$, Mann-Whitney test).

Data were statistically analyzed by Fisher's exact test for testing the differences between proportions for small samples, and by the Mann-Whitney test.

RESULTS

Continuous illumination for 72 h in combination with ELF-EMF significantly ($p<0.05$) increased the survival rate of the 150 μT ELF-EMF-exposed group of animals compared to the sham-exposed group (Tab. 1).

Caudal autotomy, as a survival strategy behavior of earthworms, was observed in both the sham-exposed and ELF-EMF-exposed groups (Tab.1). As it was found in all three groups, we assumed that autotomy was a result of continuous illumination-induced stress.

The first sign of autotomy, i.e. yellow coelomic fluid excretion, was noted after less than 24 h in the sham-exposed group (Fig. 2a), but only in 5% of the animals. Autotomy took place after 48 h. In this group, only 11% of the animals did not respond to light stress with autotomy.

In the group exposed to the EMF of 50 μT, coelomic fluid excretion was noted after less than 24 h in 22% of animals, while autotomy started after 48 h.

Before the third exposure to the field of 150 μT, coelomic fluid excretion was noted after 48 h, but in

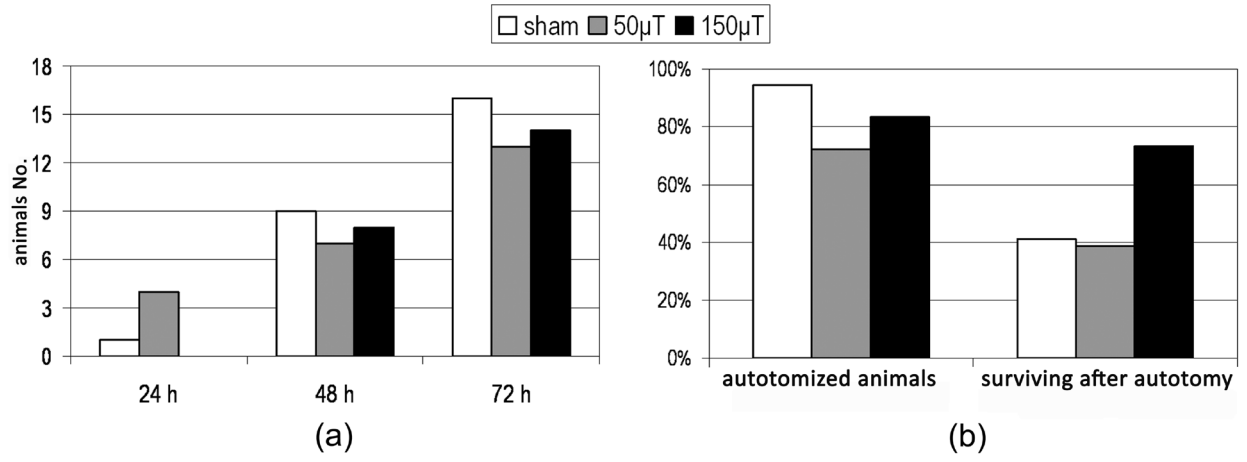

Fig. 2. (a) Number of autotomized animals, including animals with a first signs of autotomy and (b) percentage of autotomized animals and percentage of animals that survived after autotomy, following three days of constant illumination and 4 h/day ELF-EMF exposure/ sham-exposure of *Eisenia foetida*

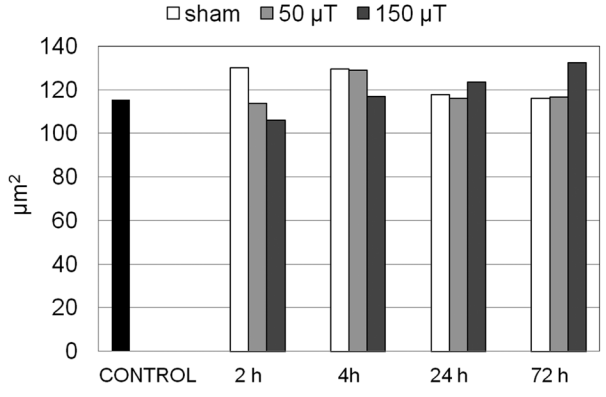

Fig. 3. Effect of constant illumination and the ELF-EMF exposure on size of *Eisenia foetida* A_1 neurosecretory cells during acute and chronic exposure.

only 10% of the animals. After 72 h, a high percentage (77.78%) of the animals that survived underwent caudal autotomy.

The percentage of autotomized animals was high in all three groups, ranging from 72.32% in the 50 μT-exposed group to 94.45% in sham-group (Fig. 2b), but there was no statistically significant difference among the groups regarding the autotomy rate. On the other hand, the survival rate of autotomized animals was noticeably higher in the 150 μT-exposed group (73.34%) than in both the 50 μT and sham-exposed groups (38.46% and 41.18%, respectively). The established differences in survival rates are on the borderline of statistical significance. Nevertheless, they indicate a trend in increasing survival success of the autotomized animals in the 150 μT-exposed group.

The morphometric analysis of the protocerebral ganglion indicated that there were changes in the size of A_1 neurosecretory cells both in acute and chronic exposure to ELF-EMF compared to the sham-exposed group (Tab. 2).

Acute treatment with constant illumination significantly increased the size of A_1 neurosecretory cells, but after 24 h of exposure, the size of these neurosecretory cells returned to control level and maintained this level during the next 72 h of exposure.

The size of the A_1 neurosecretory cells decreased during the first 2 h of exposure in both the 50 μT- and 150 μT-exposed groups. However, the size of A_1 neurosecretory cells in 50 μT-exposed group was significantly higher than in 150 μT-exposed group (Fig. 3).

After 4 h of exposure, the size of the A_1 neurosecretory cells in the 50 μT-exposed group was signifi-

cantly increased than after 2 h. The A_1 neurosecretory cell size in this group matched the size of the cells in the sham-exposed group that was the same as after 2 h of exposure. The size of A_1 neurosecretory cells in the 150 μT-exposed group was also significantly increased in comparison to 2 h, but this increase was significantly lower when compared with the 50 μT-exposed group (Fig. 3).

A significant decline in the size of A_1 neurosecretory cells was recorded 24 h after initial exposure in the 50 μT-exposed group and in the sham-exposed group. In these groups, the size of the cells showed no statistically significant difference compared with the control. However, the size of the A_1 neurosecretory cells in the 150 μT-exposed group was significantly increased in comparison to the other groups, as well as to the size of the cells in the 2 and 4 h 150 μT-exposed group. In addition, in this group, the size of the cells was significantly higher than in the control (Fig. 3).

Chronic exposure caused a further, although not significant, decrease in A_1 neurosecretory cell size in both the 50 μT- and sham-exposed groups. The neurosecretory cell sizes in these groups was not significantly different compared to the control. On the other hand, the size of the A_1 neurosecretory cells showed an increasing trend in the group treated with an ELF-EMF of 150 μT, and the size of these cells was significantly higher compared to all other groups (Fig. 3).

DISCUSSION

It is well known that organisms can be protected against a potentially lethal stress by pre-exposing them to a low dose of the same or different stress. The stimuli that induce this protective response, among others, include EMF (Bjorasen et al., 2004; Cuppen, 2010; Di Carlo et al., 2001, 1998; Elmusharaf et al., 2007)

In the present study we noticed that exposure to ELF-EMF (50 Hz, 17 V/m, 50 μT / 150 μT) during constant illumination stress increased the survival rate of *Eisenia foetida* depending on the electromagnetic field intensity. The animals in all the experimental groups manifested caudal autotomy as a stress response to constant illumination. This, however, occurred later in the animals exposed to 150 μT than in those exposed to 50 μT and the sham-exposed animals. Subsequently, the survival rate during the 3-day experiment was significantly enhanced by 150 μT treatment.

In Oligochaeta, autotomy occurred as a response of the innate immune system, following the encapsulation of foreign material and pathogens (Field et al., 2004) and/or as a response to noxious stimulation (Lesiuk and Drewes, 1999; Vidal and Horne, 2003). Caudal autotomy represents a precursory sign of lethality and together with self-constriction and rigidity indicates neurotoxicity in toxicity tests (Gong et al., 2008), as well as in irradiation with β rays (Hertel-Aas et al., 2007).

Autotomy is a phenomenon strictly connected to stress and immunity. Stress can increase or decrease the immunological ability depending on the intensity, duration and species (Ottaviani and Franceschi, 1996). An immune response is not only caused by the presence of non-self cells. It also requires the presence of promoters called "danger signals", such as heat shock proteins as well as interferon, interleukins and other cytokines. Other factors, such as stress or shock from salt, cold or heat, beside the damage caused by pathogens, have been identified to stimulate the production of "danger signals" (Engelsma et al., 2003; Huising et al., 2003). Summarizing the results of previous studies, Cuppen et al. (2007) concluded that short-term ELF-EMF exposure, possibly repeated, can produce "danger signals" that can trigger the immune system activation feedback mechanism in the presence of pathogens.

Our results indicate that 4 h of exposure to 150 μT during the first 24 h increased the survival rate of the animals under light-induced stress compared to sham-exposed animals. In addition, the autotomy reflex, as an indicator of stress response,

was delayed in the 150 μT-exposed group. On the other hand, exposure to an EMF of 50 μT had no such effect. Prolonged exposure, over a 48-h period, resulted in the induction of the autotomy reflex in all three groups, indicating a high level of stress response, presumably due to overwhelming light-induced stress. After 72 h of exposure, mortality in the sham-exposed and 50 μT-exposed groups was 56% and 45%, respectively. However, in the 150 μT-exposed group mortality was 22%, even though only 21% of the animals survived this period without induced autotomy. The decrease in mortality is most likely a result of delaying of the autotomy reflex, i.e. the EMF exposure induced protection. The observed increase in the survival rate after autotomy in the group exposed to 150 μT and to continuous illumination, along with the evidence stated above, also indicates an enhancement in the immune response of these animals.

The results of this research have also shown that constant illumination changes the morphometric parameters of the A_1 neurosecretory cells of the protocerebrum. The changes indicate the increase in the synthesis and release of neurohormones from neurosecretory neurons in acutely constant illumination-exposed i.e. sham-exposed group. Chronic constant illumination, however, does not change the size of cells compared to the control.

Although acute 2 h, 50 μT ELF-EMF exposure prevented an increase in the A_1 neurosecretory cell size, and allowed their size to remain unchanged compared to the control, after four hours and during further exposure, this effect was lost. In the groups exposed to 50 μT for 4 h, 24 h and 72 h, the size of A_1 neurosecretory cells varied in the same way as in the sham-exposed group, indicating that the 50 μT ELF-EMF has no synergetic effect with constant illumination other than the initial.

In the group that was exposed for 2 h to 150 μT ELF-EMF, we recorded a significant decrease in the A_1 neurosecretory cell size. However, after 4 h of exposure to the designated field, there was a significant increase in cell size, and this trend continued until the end of the experiment. After 72 h of exposure, the size of the A_1 neurosecretory cells in the 150 μT-exposed group was significantly higher than in all the other groups, including the control. This change in the size of the neurosecretory cells indicated a trend of increasing activity of neurosecretion and the induction of the synthesis and release rate of neurohormones from neurosecretory neurons. This phenomenon was, we assume, due to the synergistic effect of ELF-EMF and constant illumination

The neurohormones of the A_1 neurosecretory cells of the protocerebrum in *Eisenia foetida* are somatotropic, thus regulating cell reproduction and regeneration (Al Yousuf, 1988; Al Yousuf, 1992; Al Yousuf et al., 1992a). ELF-EMF, depending on the strength, influences the level of secretion of neurohormones that influence A_1 neurosecretory cell size. Although acute exposure to 150 μT in combination with illumination stress initially leads to a significant decrease in the A_1 neurosecretory cell size, prolonged exposure to 150 μT ELF-EMF resulting in an increase trend in cell size. However, this effect was not observed after chronic exposure to 50 μT ELF-EMF. As a result of 72 h 150 μT exposure, the size of the A_1 neurosecretory cells was significantly higher compared to the size of cells in the 50 μT group and the sham-exposed group. We suppose that the increase in the A_1 neurosecretory cell size in the protocerebral ganglion of animals exposed to 150 μT and to continuous illumination indicates its higher synthetic activity, which may be in connection with the observed increase in their survival rate after autotomy, and in such a way indicates the connection between the immune and endocrine systems in *E. foetida*.

Although further investigations, especially of the mechanism of action, are needed, the present data support the view that short-term ELF-EMF, in "windows" of intensity, is likely to stimulate the immune response, thus extending the survival rate of *Eisenia foetida* under illumination stress. Our results indicate that the presumed immune stimulation may be associated with the changing levels of neurosecretory activity in the protocerebral ganglion induced by ELF-EMF.

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