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# Effects of Anesthetic Compounds on Responses of Earthworms to Electrostimulation

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Earthworms play an important role in biomedical research, and some surgical procedures require anesthesia. Anesthetic treatments used so far usually induce convulsive body movements connected with extrusion of coelomocyte-containing coelomic fluid that may affect experimental results. Extensive movements connected with the expulsion of coelomic fluid are exploited by immunologists as a method of harvesting immunocompetent coelomocytes from worms subjected to mild electrostimulation (4.5V). The aim of the investigations was to find anesthetic drugs without unintentional coelomocyte depletion. Experiments were performed on adult specimens of *Dendrobaena veneta*, the coelomocytes of which consist of amoebocytes and riboflavin-storing eleocytes. Earthworm mobility was filmed and extrusion of coelomocytes was quantified by detection of eleocyte-derived riboflavin in immersion fluid. Treatments included earthworms (1) immersed either in physiological saline (controls) or in a solution of one of the tested anesthetic drugs; (2) electrostimulated immediately after anesthesia, and (3) electrostimulated a second time after a 1-hour recovery period. The well-established fish and amphibian anesthetic agent MS-222 induced coelomocyte expulsion. In contrast, solutions of the mammalian local anesthetic drug, prilocaine hydrochloride (0.25-0.5%, 5-10 min) caused temporal earthworm immobilization followed by recovery, thus showing utility as an efficient earthworm anesthetic.

Key words: *Dendrobaena veneta*, immobilization, riboflavin, prilocaine, MS-222.

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Available data are consistent with the idea of pain in some invertebrates (ANDREW 2011; COOPER 2011; ELWOOD 2011). In lumbricid earthworms the central nervous system is a highly differentiated neuroendocrine structure producing neurohormones and neurotransmitters (e.g. see HARTENSTEIN 2006; HERBERT *et al*. 2009), thus the feeling of pain should be considered and avoided during all kinds of *in vivo* experiments, e.g. those on extirpation of brain or ventral nerve cord ganglia (CSOKNYA *et al*. 2002; HERBERT *et al*. 2009). Surgeries are usually performed on $CO_2$-exposed worms, or other forms of earthworm anesthesia, like warming, cooling, urethane, and chloretone treatment (OGURO *et al*. 1984). In our hands, worms anesthetized by $CO_2$-containing water, cold plate or chlorophorm vapors usually react by convulsive body movements leading to extrusion of coelomocyte-containing coelomic fluid through the dorsal pores in the body wall. The partial loss of coelomocytes, the crucial component of the earthworm immune system (BILEJ *et al*. 2011), may affect the experimental results. Expulsion of coelomocytes is fol-

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lowed by slow restoration of the initial cell numbers (e.g. EYAMBE *et al*. 1991; POLANEK *et al*. 2011; KLIMEK *et al*. 2012). The transitional depletion of coelomocytes may affect other processes within the organism. Therefore there is an urgent need to develop methods of anesthesia leading to temporary earthworm immobilization without loss of coelomocytes.

In search of efficient anesthetic drugs for earthworms, we have recently tested a local anaesthetic drug used for human anesthesia (lidocaine and prilocaine) (LIBROWSKI *et al*. 2004; STOKES *et al*. 2009) and MS-222 which is an efficient anaesthetic for aquatic animals (NEIFFER & STAMPER 2009; WEBER *et al*. 2009; VERA *et al*. 2010). The findings showed effective immobilization of earthworms with prilocaine at a concentration of 0.25-1%. Lidocaine was less effective at the applied concentrations. In sharp contrast, MS-222 had a strongly irritating effect for earthworms and induced convulsive body movements connected with discharge of coelomic fluid (PODOLAK-MACHOWSKA *et al*. 2013).

Convulsive body movements are common in lumbricid worms under natural conditions, e.g. when irritated by predators or investigators, thermal shock, sudden light exposure, or contact with some chemicals. This ability is used by immunologists for non-invasive quantitative coelomocyte retrieval under strictly controlled experimental conditions, i.e. by irritating worms with a mild electric current (ROCH 1979), ultrasounds (HENDAWI *et al*. 2004), or 5% ethanol (COOPER *et al*. 1995). In the present experiments we applied this peculiar earthworm response to electrostimulation for testing the effects of prilocaine and MS-222 on coelomocyte extrusion.

The coelomocytes of lumbricid species consist of amoebocytes (the classical immunocompetent cells) accompanied in some – but not all – species by autofluorescent eleocytes (CHOLEWA *et al*. 2006). Autofluorescence of the latter cells is dependent on accumulation of fluorophores including riboflavin (vitamin B2) (KOZIOL *et al*. 2006; PLYTYCZ *et al*. 2006; PLYTYCZ & MORGAN 2011). The fluorescent self-marking of eleocytes makes them suitable for rapid quantification, applied in the present paper concerning coelomocyte expulsion by electrostimulated lumbricid earthworms, *Dendrobaena veneta.*

The aim of the experiments was to examine the effects of prilocaine and MS-222 on earthworms measured by temporary inhibition of body movements and/or coelomocyte-derived fluorophore extrusion during electrostimulation applied immediately after drug treatment and after a 1-hour recovery period.

## Material and Methods

### Earthworms

Adult earthworms *Dendrobaena veneta* (Oligochaeta; Lumbricidae), purchased from a commercial supplier (EKARGO, Słupsk) were reared in commercial soil (PPUH BIOVITA, Tenczynek) under controlled laboratory conditions (17°C; 12:12 LD). The worms were kept in plastic boxes with perforated lids and the moisture level was checked weekly. 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.

### Anaesthesia and recovery

Adult (clitellate) earthworms were individually immersed in small Petri dishes (5 cm diameter) filled with 3 ml PBS (controls) or in PBS-solution of appropriate concentrations of prilocaine hydrochloride (Sigma Aldrich) (2%-0.125%), or MS-222 (Tricaine methanesulfonate; Ethyl 3-aminobenzoate methanosulfonate salt; Sigma-Aldrich; Fluca) (1%-0.0125%) for 5-30 minutes and worm behavior was recorded. Then the earthworms were transferred individually to wells with PBS of the 6-well plate and their mobility was assessed and filmed (Fig. 1). This was followed by worm recovery on wet filter papers. The procedure was repeated one hour later. The coelomocyte-containing fluid was harvested and analysed in respect of riboflavin content.

### Measurement of earthworm mobility

Earthworm mobility was recorded in a 6-well plate (Nunc) adapted to the application of 4.5V electric current and covered with a lid with grids enabling earthworm localization. Adult earthworms were immersed individually in wells filled with 3 ml of physiological saline (PBS) and their mobility was filmed for 15 seconds before application of an electric current (spontaneous mobility) and during 60-second irritation with a 4.5V electric current (induced mobility). Changes of earthworm location within particular areas of the grid were counted in one-second intervals. After testing, the worms were transferred to plates with PBS-soaked filter papers for 1-hour recovery and the procedure was repeated. The coelomocyte-containing PBS was harvested from wells and used for analysis of riboflavin content in coelomocyte lysates.

### Riboflavin content

Riboflavin contents were spectrofluorimetrically quantified in: 1) samples of fluid used for worm anesthesia at various concentrations of anesthetic drugs with 0% concentration as a control; 2) samples of fluid used for electrostimulation immediately after anesthesia; 3) samples of fluid used for electrostimulation after a 1-hour recovery period.

Riboflavin was detected and quantified by spectrofluorometric measurements performed on 2 ml coelomocyte-suspension lysates (lysed with 2% Triton; Sigma-Aldrich) using an LS50B Perkin-Elmer Spectrofluorimeter as described previously (CYGAL *et al*. 2007)

## Results

### Earthworm mobility

After immersion in PBS worms exhibited low spontaneous mobility which increased rapidly after applying the electric current, with most evident convulsive body movements during the initial 15 seconds of electrostimulation. After preincubation of the earthworm in the appropriate concentration of the anesthetic drug both spontaneous and induced body movements were completely abolished (Fig. 1).

Figure 1 illustrates the locations of 6 specimens of *D. veneta* at four successive 1-second time intervals (A, B, C, D) during 4.5V electrostimulation. The earthworms in wells No. 1, 2, and 3 did not change their locations versus grids on the lid during the 4-second observation period as they were immobilized by pretreatment by immersion in prilocaine chloride (5 min, 0.5% solution). In contrast, the control earthworms were pretreated by 5 min immersion in physiological saline PBS only, thus they were very mobile and changed their locations within wells No. 4, 5, and 6.

An example of quantification of *D. veneta* mobility is visualized for the earthworm located in the encircled well No. 6 in Figure 1. Orange dots within the grids mark areas which differ in the presence/absence of the earthworm body between Figure 1C and 1D, i.e. in a one-second interval of time. After the first 60-sec electrostimulation, eartworm mobility in response to 4.5V irritation is regained already 15 minutes later (data not shown).

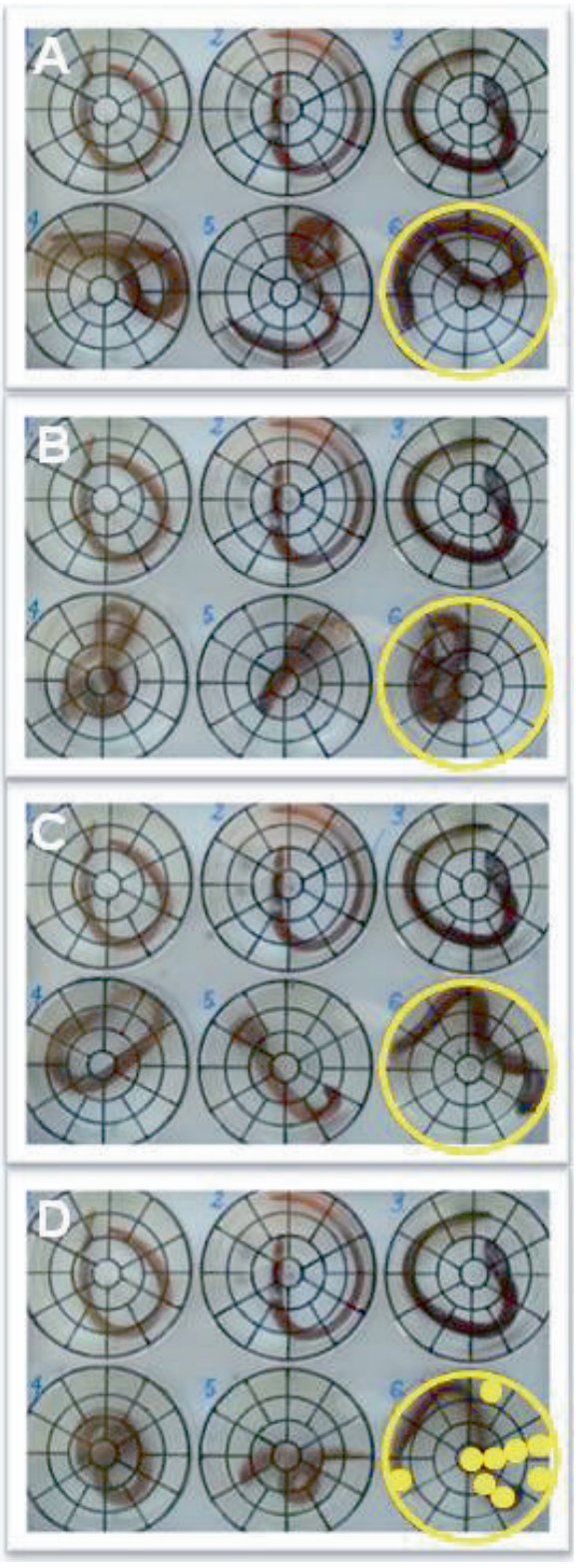


Fig. 1. *Dendrobaena veneta* immobilization/mobility at representative four successive 1-second time intervals (A, B, C, D) during electrostimulation (4.5V) and the principle of quantification of earthworm mobility by comparisons of earthworm locations within grids of a network in encircled well No. 6. Immobilized worms in the upper row (wells No. 1, 2, and 3) were pretreated by 5-min immersion in 0.5% prilocaine hydrochloride, while mobile control worms in the lower row (wells No. 4, 5, and 6) were pretreated by 5-min immersion in PBS. Yellow dots on Figure 1D indicate the number of changes of earthworm locations within well No. 6 between the frame on Figures 1C and 1D.

### Effects of prilocaine on earthworm mobility

Figure 2 shows concentration-dependent and time-dependent anesthetic effects of prilocaine on adult specimens of *D. veneta*. Worms were com-

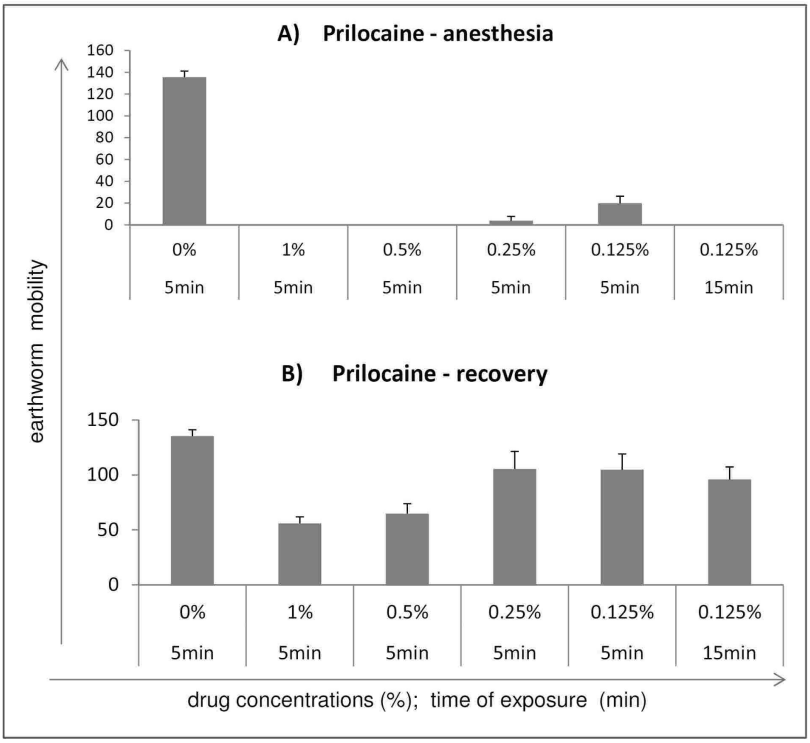


Fig. 2. Effects of prilocaine exposure on electric shock-induced (4.5V) mobility of earthworms *Dendrobaena veneta* electrostimulated immediately after anesthesia (A) and for a second time after a 1-hour recovery period (B). Mobility was measured as the sum of changes of locations within grids during the initial 15 seconds of electrostimulation (compare with Figs 1A-D). Means + SE, n = 6 earthworms.

pletely immobilized after 5 minute immersion in 1%, 0.5% and 0.25% solution of prilocaine, while 0.125% solution was fully efficient only after 15 min immersion (Fig. 2A). The effects were reversible as evidenced by partly regained mobility measured after a 1 hour recovery period (Fig. 2B).

## Effects of anesthetics on coelomocyte-derived fluorophore extrusion

Earthworm electrostimulation was accompanied by expulsion of a yellowish fluid visible to the naked eye, as it contained coelomocytes, among them riboflavin-storing eleocytes (PLYTYCZ & MORGAN 2011). Spectrofluorimetry of Triton-lysed samples of immersion fluid revealed the presence of riboflavin spectra. The emission spectrum of a riboflavin-containing sample has a peak at 525 nm proportional to riboflavin content, preceded by the very distinct peak X at 410-420 nm characteristic for Triton lysates of *D. veneta* coelomocytes (Fig. 3A).

For the example illustrated in Figure 3A, the amount of riboflavin extruded during the first electrostimulation was high (app. 45 arbitrary units, Fig. 3A, thick solid black line) and dropped to app. 18 AU during the second electrostimulation performed 1 hour later (Fig. 3A, dotted black line).

Figure 3 illustrates that each earthworm was tested for extrusion of coelomocytes into 3 subsequent extrusion fluids: (1) control PBS or anesthetic drug solution (grey lines); (2) PBS used for the first electrostimulation (thick solid black lines); (3) PBS used for the second electrostimulation after 1-hour recovery period (dotted black lines).

Riboflavin and accompanying X-fluorophore are completely or almost completely lacking in samples of PBS or 0.25% prolicaine solution after 5-minute immersion (horizontal grey lines on Figures 3A and 3B). By sharp contrast, very distinct spectra of fluorophores are visible in samples of MS-222 solutions in both 1% and 0.1% concentrations (grey lines on Figures 3C, D).

Electrostimulation after PBS immersion induced pronounced riboflavin and X fluorophore extrusion (black line in Figure 3A), while signatures of fluorophores were negligible after prilocaine anesthesia (Fig. 3B) or low after MS-222 exposure (Figs 3C, D). After 1-hour recovery, riboflavin and X fluorophore were lower than those after the first stimulation of the control PBS-treated worm (Fig. 3A), while they were very pronounced in the worm after prilocaine anesthesia (Fig. 3B). Low amounts of fluorophores were still present in recovering worms after MS-222 exposure (Figs 3C, D).

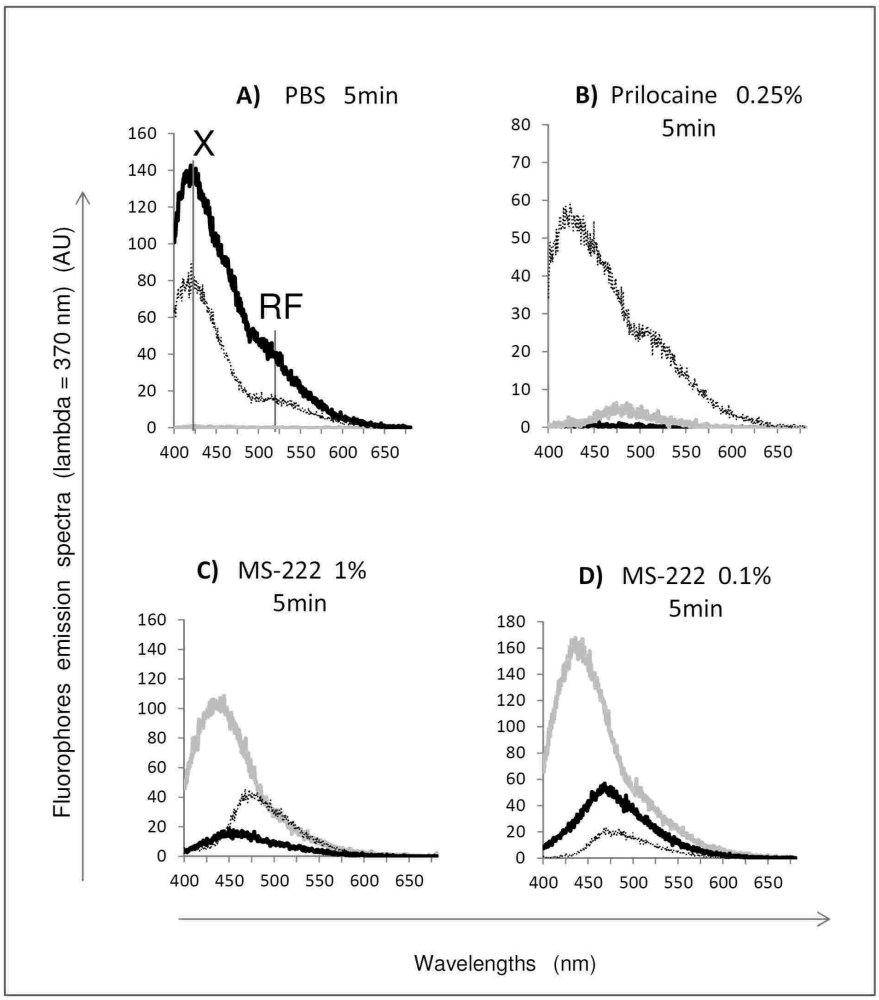


Fig. 3. Representative examples of the effects of anesthetics on extrusion of fluorophore-containing coelomocytes by *D. veneta* during: (1) immersion in PBS (control) or anesthetic solutions (grey lines); (2) 1-minute electrostimulation (4.5V, 5 min) immediately after drug exposure (thick black lines); (3) 1-min electrostimulation applied after a 1-hour recovery period (dotted black lines). A – Control worms immersed in PBS only; B – prilocaine immersion; C-D – MS-222 immersion. RF – peak of riboflavin; X – peak of X-fluorophore.

### Comparison of effects of prilocaine and MS-222 immersion on riboflavin extrusion

Figure 4 shows the contribution of fluorophore to the emission spectrum at 525 nm (mainly riboflavin) present in (1) PBS or drug solution used for anesthesia (grey parts of bars), (2) PBS used for the first electrostimulation (black parts of bars), and (3) PBS used for the second electrostimulation after 1-hour recovery (open parts of bars) in the whole riboflavin extruded from the representative worms considered as 100%. The control worm extruded the majority of riboflavin (above 70%) during the first electrostimulation.

Earthworms immersed in prilocaine at concentrations equal or higher than 0.5% exhibited short convulsive body movements connected with riboflavin extrusion followed by immobilization and lack of response to electrostimulation. Immersion in 0.25% prilocaine solution for 5 or 10 minutes in practice did not induce riboflavin extrusion. The amount of riboflavin extruded by prilocaine-anesthetized worms was also very low (8% and 1%) during the first electrostimulation, while 92% and 98% of the extruded riboflavin was present in the fluid used during the second electrostimulation after a 1-hour recovery period. In conclusion, prilocaine may serve as an efficient anesthetic drug for earthworms.

Immersion of worms in 1% MS-222 solution did not inhibit worm mobility and induced a massive riboflavin expulsion into the immersion fluid (42-63% after 5-min immersion in 1%-0.06% drug, and 40-minutes immersion in 0.025% MS-222), while longer exposure (50 and 60 min) to lower concentrations (0.012% and 0.006%) was less noxious for earthworms (10% and 2% riboflavin expulsion, respectively). After MS-222 immer-

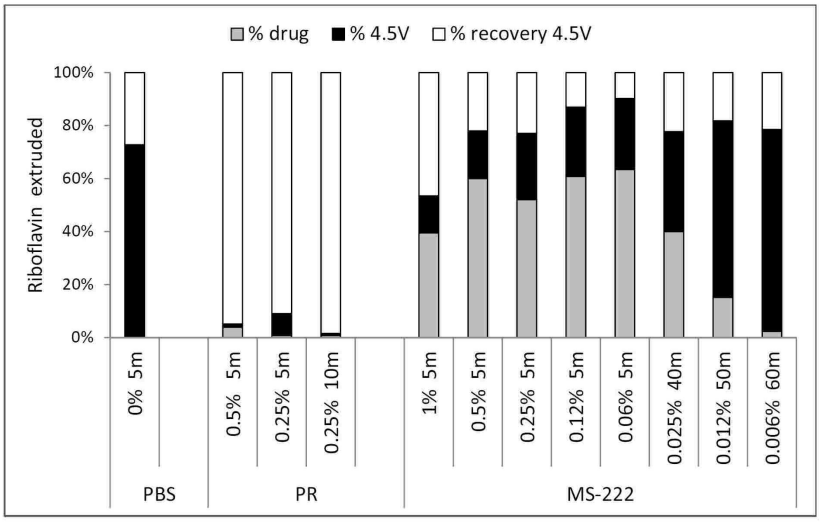


Fig. 4. Percentages of riboflavin content extruded by specimens of *D. veneta* into fluids used for: immersion in PBS, prilocaine (PR), or MS-222 (grey parts of bars); electrostimulation performed immediately after PBS/drug immersion (black part of bars); electrostimulation performed after 1-hour recovery period. Representative examples.

sions, worms still reacted to electrostimulation, expelling increasing amounts of riboflavin with decreasing drug concentrations; the remaining riboflavin was still present in PBS used for the second electrostimulation. In conclusion, MS-222 cannot be used for worm anesthesia as it is noxious for them, induces expulsion of riboflavin, and does not prevent its further expulsion by electrostimulation.

## Discussion

Various chemical and physical agents are used for immobilization or anesthesia in invertebrates (OGURO *et al*. 1984; COOPER 2011). In the present paper we applied partial immersion in PBS-solutions of hydrochloride of prilocaine, routinely used for local nerve blocks (TADDIO *et al*. 1998) and for spinal anesthesia in mammals (YAMADA *et al*. 2008) or Tricaine methane-sulfonate (TMS; MS-222) routinely used for anesthesia of fish (NEIFFER & STAMPER 2011) or amphibians (e.g. JOZKOWICZ & PLYTYCZ 1998).

MS-222 was noxious for earthworms and induced extrusion of coelomocytes including riboflavin-storing eleocytes, thus this drug seems to be inappropriate for earthworm anesthesia. Prilocaine at high concentrations (1-2%) was also noxious for worms and induced muscle reflexes, but at appropriate concentrations caused temporary worm immobilization followed by recovery.

Temporary immobilization of earthworms may be useful in various laboratory practices, both for proper species identification, or amputation of a few segments for DNA analysis, or for advancing surgical procedures, e.g. for studies on regeneration.

The identification of earthworm species is difficult. Traditional methods rely on morphological criteria based on observation of formalin- or alcohol-fixed adult specimens exhibiting a clitellum, with special attention to the details visible under a dissecting microscope (e.g. KASPRZAK 1986; SIMS & GERARD 1985). Drug-induced temporary immobilization should be sufficient for supravital observations of the important clues allowing identification of earthworm species. However, KING *et al.* (2008) showed there is unprecedented diversity of morphologically identical cryptic species within lumbricid earthworms which can be distinguished by analysis of some conserved DNA sequences, mostly mitochondrial cytochrome oxidase I (COI) gene. For the latter purpose, DNA is extracted either from ethanol-fixed whole earthworms (e.g. KING *et al*. 2008) or from their amputated tail tips (e.g. PLYTYCZ *et al*. 2009; KLARICA *et al*. 2012). Amputation of a few tail segments may be done without anesthesia of earthworms; however the effect of this procedure on the well-being of the animals should be investigated. In the case of mammals, ARRAS *et al.* (2007) studied the impact of tail biopsy on the physiological and behavioral parameters of mice. They stated that both metoxyflurane (MOF) and diethylether (ether) anesthesia induced remarkable alterations in the analyzed parameters and did not improve mouse well-being following tail biopsy routinely taken for DNA analysis.

Earthworms are used for sophisticated types of surgeries, e.g. those connected with regeneration of anterior or posterior body segments (for a review see BELY 2006; ZORAN *et al*. 2010), or in

particular their nervous system (CSOKNYA *et al*. 2002; HERBERT *et al*. 2009). The remarkable ability of the earthworm cerebral ganglion to regenerate has long been known and has drawn the attention of neuroscientists as a convenient model for regeneration experiments (e.g. LUBICS *et al*. 2002). In most mentioned studies, segment amputations or brain extirpations were performed in worms subjected to $CO_2$ anesthesia (e.g. LUBICS *et al*. 2002). In our hands, worms stressed by various forms of anesthesia applied so far, like $CO_2$-containing water, cold plate, chlorophorm vapors, and 5% ethanol, usually extrude coelomocyte-containing coelomic fluid through the dorsal pores of the body wall. An extrusion of coelomocytes, being the crucial component of earthworm immunity (BILEJ *et al*. 2011) is always followed by the gradual but slow restoration of these cells (COOPER *et al*. 1995; POLANEK *et al*. 2011; KLIMEK *et al*. 2012). Putatively, temporary impairment of earthworm immunity affects closely interacting arms of the homeostatic systems of the body, namely the neurohormonal system and the immune system which share receptors and mediators (OTTAVIANI & FRANCESCHI 1997; LUBICS *et al*. 2003; OTTAVIANI 2011) thus the depletion of one system may affect the other.

In the present study, the efficiency of anesthesia by partial immersion in prilocaine solution was assessed by earthworm immobilization resulting in the lack of response to electrostimulation and the lack of riboflavin in lysates of immersion fluid. The latter criterion cannot be applied to all lumbricid species as only some of them (*Eisenia* sp., *Dendrobaena veneta*, *Allolobophora chlorotica*, *Dendrodrilus rubidus*, *Octolasion* sp.) possess freely floating riboflavin-storing eleocytes in coelomic fluid, while others (*Lumbricus* sp., *Aporrectodea* sp.) possess negligible percentages of eleocytes among coelomocytes (reviewed by PLYTYCZ & MORGAN 2011). In the latter group of species, the efficiency of anesthetics may be assessed only by immobilization and microscopic examination of immersion fluid in respect of the presence/counts of extruded coelomocytes.

It is known that mammalian species and/or strains differ in response to anesthetics (e.g. AVSAROGLU *et al*. 2007) thus further studies on lumbricid worms should be focused on establishing species-specific and size/age-specific concentrations of anesthetic drugs. We also expect that anesthetic effects on ectothermic earthworms will be dependent on the ambient temperature and on time of adaptation to a new thermal regime (e.g. PLYTYCZ & JOZKOWICZ 1994; STANKIEWICZ & PLYTYCZ 1998; KUREK *et al*. 2001) and may also vary in the annual cycle (e.g. KUREK & PLYTYCZ 2003).

## Conclusions

We have developed a simple method for recording earthworm spontaneous and induced mobility which may be used for testing efficiency of anesthetic drugs. For mobility assessment it is convenient to compare a few seconds of the spontaneous mobility in immersion fluid with the first 15 seconds of mobility induced by electrostimulation (4.5V electric current). Mobility of *D. veneta* may be completely inhibited by appropriate concentrations (0.25-0.5%, 5-10 min) of prilocaine without signs of extrusion of coelomocyte-containing coelomic fluid. A fish and amphibian anesthetic, MS-222, is inappropriate for lumbricid earthworms, at least for *D. veneta*, because it is noxious for worms and induces extrusion of coelomocytes. We assume that earthworms may serve as uncontroversial models for preliminary testing of new anesthetic drugs which may replace some procedures performed so far on mammalian species. The method described in the present paper should be further refined by studies on the effects of applied drugs on earthworm cell and tissues with special attention to coelomocytes, and on the effects of regeneration processes.

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