

Rapid Escape Response - A Behavioral Response on *Eisenia fetida*

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Abstract

A behavioral assay is described for studying locomotory behavior in annelid *Eisenia fetida*. This assay presents a method using a gustatory repellent Sodium Chloride (NaCl) to induce a locomotory response in the earthworm. This assay helps us understand the ambulation that is under control of a well co-ordinated nervous system, precisely, the Ventral Nerve Cord. To confirm this a lesion was performed on the nerve cord and functional activity under the repellent was again assayed. Results suggest that an undamaged ventral nerve cord is essential for earthworms' rapid escape responses.

Keywords: Earthworm; *Eisenia fetida*; Ventral nerve cord; NaCl; Locomotion; Assay

Introduction

How animals progressed from a simple nerve net to a complex centralized nervous system remains one of the most exciting and unsolved questions of animal evolution [1]. The nervous systems of invertebrate organisms often are designated for their spatially directed collections of neurons responsible for local control of operations, such as the thoracic or abdominal ganglia, which receive sensations and direct motoric responses for specific body segments, all under the general control of a cephalic ganglion whose role includes sensing the external environment [2-5].

Earthworms are used in an increasing number of microcosm experiments that investigate their behavior and biology or that consider earthworms an environmental factor that influences soil properties and biological interactions [6-11]. However, there exists no standardized protocol for performing comparable studies. The earthworm is a much studied species, and in recent years much work has been carried out with regard to the mechanism of its movement. Though there are several descriptions of the modes of movement, their neuronal control and the functional organization of the nervous system are less well understood. Nevertheless an understanding of the mechanisms involved is important to comparative neurophysiology since the earthworm probably stands midway in the evolution from non-myelinated to myelinated neurons and from simple nerve nets to organized reflex systems [2,4,12-15].

The main nerve trunk, i.e., the ventral nerve cord in the earthworm, lies close to the inner median surface of the body wall, and runs from segment 4 to the rear end of the earthworm. It swells slightly to form a ganglionic enlargement in each segment [14].

Among annelids, the lumbricid earthworm, *Eisenia fetida*, which is important in breaking down organic wastes, has been used commonly for research into regeneration, because it is easy to culture and handle in the laboratory. Earthworms can tolerate moderate salinity but some earthworm species like *Eisenia fetida* are reported to be highly salt tolerant with 28-day LC₅₀ of NaCl found to be 5436 mg kg⁻¹[16].

Considering these facts, we decided to use *Eisenia fetida* as our model organism. They were maintained in plastic cups containing moist tissue paper (source of cellulose) [17-21]. Test was performed in two sets, one set consisting of pre-clitellate juvenile animal and second of clitellate mature animals. Results were recorded. From this assay we determined the response of an earthworm to Sodium Chloride as well as necessity of an undamaged Ventral Nerve Cord to produce the response.

Materials and Methods

Organisms used

Earthworms of species *Eisenia fetida* bought from Yusuf Mehralli Centre (Mumbai). Using the general physiological characteristics of *E. fetida*, a confirmation of the identification of species was performed [22].

Maintenance and nutrition

Earthworms were maintained in cup cultures. Small holes were made using a thin edged screw driver in plastic cups. A thick layer of tissue paper was put inside and it was moistened with tap water. Since tissue paper is a source of cellulose, which serves as nutrient source for earthworms, they decompose the paper and survive on available cellulose. A muslin cloth was used to cover the cups. Cups were numbered serially and placed in sets of 10 each in different trays containing layers of newspapers on which water was added daily so that cups retained moisture. Clitellate and Pre-clitellate were marked differently.

Rapid escape response assay

Materials

Reagents: 200 mM NaCl solution, distilled water.

Distilled water is used as Control.

Apparatus and miscellaneous: 30 test tubes (15 ml), 10 ml pipette, test tube rack, Timer, glass bowl with tap water, paint brush, filter paper.

Method: A clean dry test tube was taken. 1 ml of the solution (distilled water or 200 mM NaCl) was added using glass pipette, taking care that it doesn't touch the walls of the test tube.

With the help of a brush, one worm from the cup culture was taken out gently and give it a brief wash in the bowl with tap water to remove any adhering castings.

The worm was picked up with the same brush, rolled and submerged in the solution. Care was taken that the worms do not touch glass wall and it is directly put into the solution.

The stop watch was started immediately; the time taken for the worm to climb completely out of the solution is recorded for a maximum of 2 min for preclitellate worms and 4 mins for clitellate worms. The first four steps were repeated for another readings; a new tube was used for every reading. The assay was repeated on all sets of worms. A total of 140 worms were used – 60 clitellate and 60 preclitellate. (plus an additional 10 clitellate and 10 pre-clitellate)

The protocol was performed once in 24 hours on earthworms with an undamaged ventral nerve cord.

Protocol to introduce lesion on ventral nerve cord [14]

- The earthworm was subjected to 1% chloroform for 1-2 mins.
- It was taken on a clean blotting paper.
- It was turned to its ventral buff side and near the 23-24 segments a oblique cut was made ensuring it just causes damage to ventral nerve cord and does not cause cut to entire body of worm.
- Cut was made using a sharp blade.
- Earthworm was washed with tap water to remove traces of chloroform.
- A sham lesion on the dorsal side was performed on 20 worms separately (10 clitellate, 10 Pre-clitellate).

The assay was again performed in the same manner as mentioned above on the 0th hour of the lesion for each worm.120 earthworms were lesioned on the VNC. Results were recorded.

Results

Behavioral assay on juvenile *Eisenia fetida* with an undamaged ventral nerve cord

The assay was performed on 60 pre-clitellate animals. Each worm was subjected to the control Distilled water for 2 mins and then to 200 mM NaCl for two minutes. Time was decided based on reviewed data as well as first taking out an average time required for earthworms to escape 200 mM NaCl. The time of escape was recorded for each earthworm. It was seen that all the earthworms do not show any movement in distilled water within the period of 2 minutes. In case of test tube containing 200 mM NaCl the earthworms use peristaltic movement and climb the wall of the glass test tube within the 2 minutes in order to escape the gustatory repellent. Results of two sets were as follows: (Tables 1 and 2)

Earthworm No.	Control (D/W)	200 mM NaCl
1	X	00:04
2	X	00:03
3	X	00:02

4	X	00:07
5	X	00:05
6	X	00:06
7	X	00:06
8	X	00:12
9	X	00:07
10	X	00:04

Table 1: Set 1.

Earthworm No.	Control (D/W)	200 mM NaCl
11	X	00:05
12	X	00:02
13	X	00:05
14	X	00:07
15	X	00:04
16	X	00:04
17	X	00:05
18	X	00:05
19	X	0.07
20	X	0.04

Table 2: Set 2.

Table shows response of individual earthworms on subjecting them with D/W and NaCl. Here timings are indicated minutes:seconds. X indicates failure to escape in 2 minutes.

Similar results were observed for the rest 40 Pre-clitellate animals.

Behavioral assay on mature *Eisenia fetida* with an undamaged ventral nerve cord

Assay was performed in a similar manner. However it was seen that response time required in clitellate animals was greater than juvenile ones. We first assayed the time required for the clitellate animal to escape 200 mM of NaCl and then checked if they could stay in distilled water for a period slightly greater than average time required by all mature animals. Thus, we set a protocol for 4 minutes which was greater than the time required for most clitellate animals to escape 200 mM NaCl. Results were recorded in a similar manner. (Figures 1 and 2; Table 3)

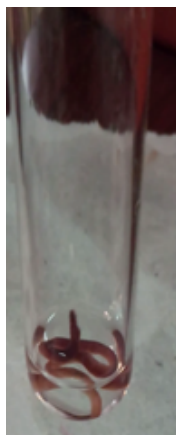


Figure 1: Indicates earthworm in D/W.

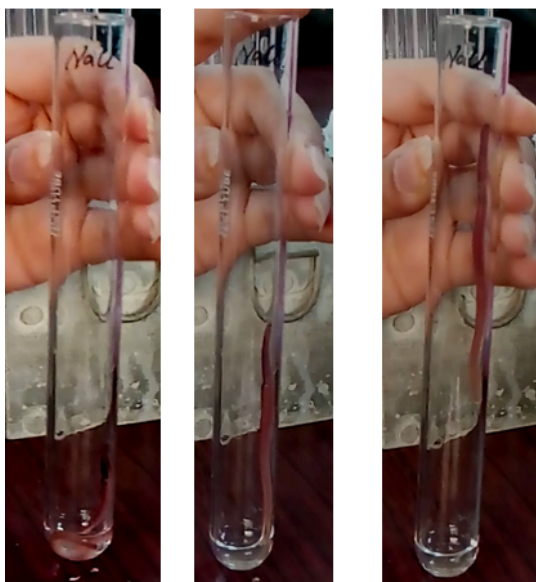


Figure 2: Indicates the recordings after earthworm was subjected to 200 mM NaCl.

9	X	03:51
10	X	02:15
11	X	02:21
12	X	03:00
13	X	01:00
14	X	01:04
15	X	00:30
16	X	02:00
17	X	01:02
18	X	03:23
19	X	03:10
20	X	02:13

Table 3: Response of individual earthworms on subjecting them with D/W and NaCl.

Table shows response of individual earthworms on subjecting them with D/W and NaCl. Here timings are indicated minutes:seconds. X indicates failure to escape in 4 minutes.

Behavioral assay on juvenile *Eisenia fetida* with a lesioned ventral nerve cord

Each pre-clitellate earthworm was subjected to control as well as 200 mM NaCl just after causing a lesion on the ventral nerve cord. The 0th hour readings were recorded in the same manner. No movement was seen in distilled water in 2 minutes. Then the worm was removed from D/W and introduced in 200 mM NaCl. No attempts of any escape were made by the worm of any kind in the two minutes.

Behavioral assay on mature *Eisenia fetida* with a lesioned ventral nerve cord

0th hour readings on clitellate animal was taken in the similar manner. No movement was seen in both control as well as 200 mM NaCl in the given 4 minutes assay.

Behavioral assay on juvenile as well as mature *Eisenia fetida* with a sham lesion on the dorsal body

In both the cases no response was seen in distilled water in 2 minutes for juvenile and 4 minutes for mature worms. A locomotory response was observed in 200 mM NaCl in both cases just like the previously recorded response. Thus a sham on the dorsal side did not disrupt the well co-ordinated locomotory response, as nerve cord was undamaged (Figures 3-7).

Earthworm No.	Control (D/W)	200 mM NaCl
1	X	02:21
2	X	03:26
3	X	01:23
4	X	01:25
5	X	00:32
6	X	02:15
7	X	01:37
8	X	03:38



Figure 3: Shows preclitellate worm post lesion on ventral nerve cord.



Figure 4: Shows clitellate worm post lesion on ventral nerve cord.

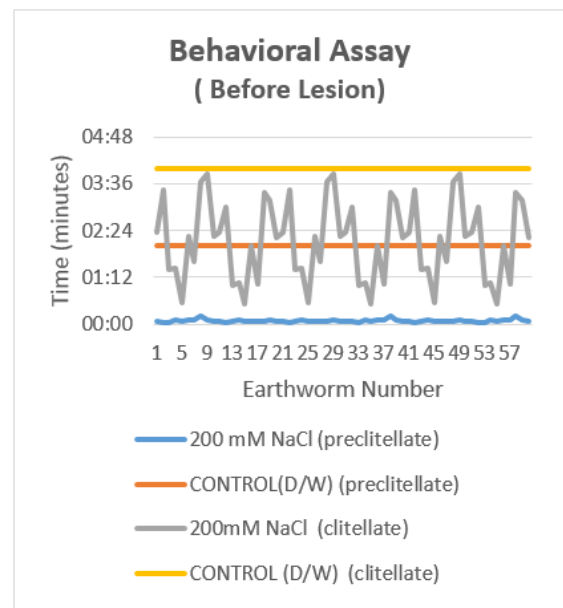


Figure 5: Behavioral assay before lesion.

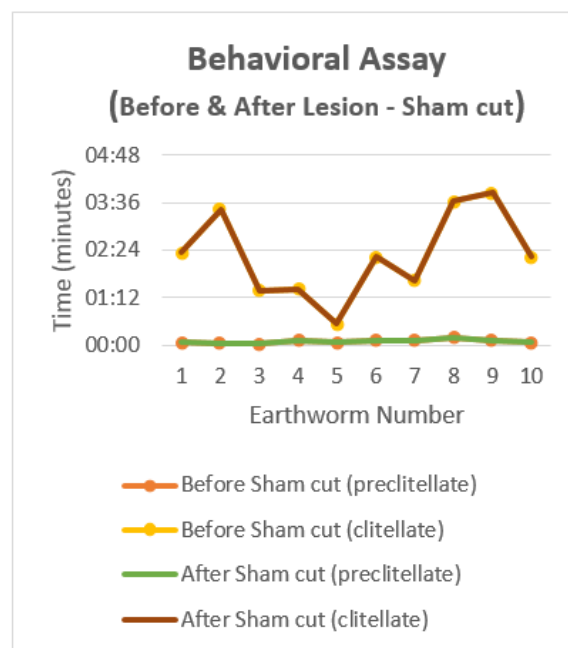


Figure 6: Behavioral assay before and after lesion-sham cut.

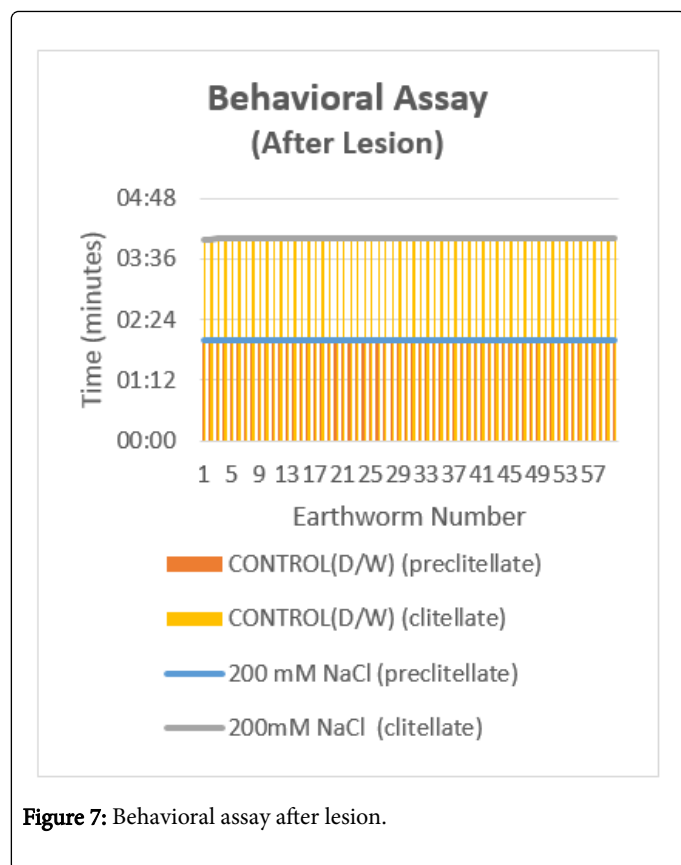


Figure 7: Behavioral assay after lesion.

Discussion

This work signifies the role of the ventral nerve cord in the locomotory behavior of the model organism. *Eisenia fetida*, is used for various ecotoxicological studies and more recently has been commonly used to study regenerative mechanisms. This neuronal control on movement has been stated by Gardener and Drewes [12]. To confirm the significance of the ventral nerve cord in the control of locomotion, an assay using a gustatory repellent was set up. Sodium chloride has been tested with various organisms such as *Drosophila* and *C. elegans* to record a chemosensory response [23-25]. Salinity tests to check adaptability as well as survival rates to Sodium Chloride have been performed in *Eisenia fetida* [8,16,26,27]. Tactile impulses to NaCl on nerve cord of earthworm have been recorded and an observation that a concentration from 200 mM to 1000 mM shows greater electrical stimulation to touch responses has been made [4,12,20].

In order to set up a chemical assay, for confirmatory tests, standard protocols used for *C. elegans* and *Drosophila* during chemosensory behavioral tests were modified for our organism [23,25,28].

Distilled water was used as a control as Roots has reported the behavior of *L. terrestris* submerged in water placed in glass tubes and has observed no significant movement or escape behavior [29,30].

As 200 mM was lowest concentration at which significant electrical stimulation was recorded, it was taken as test concentration [20]. Pre-clitellate earthworms showed a faster escape behavior than mature clitellate animals. Reason for this phenomenon was compared to other organisms like *C. elegans*, which have shown an age related decline in locomotory responses [31,32].

On damage on the ventral nerve cord both juvenile as well as mature worms showed loss in activity [33-35]. In order to confirm that this loss in movement was solely due to lesion on ventral nerve cord, a set of 10 animals of both pre-clitellate and clitellate type were subjected to a sham muscular lesion on the dorsal side of the body wall [36-40]. These animals showed no change in locomotory behavior as compared to their previous activity as observed before sham cut [41,42].

Thus it was evident to show the importance of the ventral nerve cord in the escape response shown by earthworm *Eisenia fetida*.

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