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Cytomorphological changes in the cerebral and ventral ganglionic neurosecretory cells during copulation in epigeic earthworms

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ABSTRACT: In spite of hermaphroditism, most earthworm species reproduce by cross fertilization i.e. by the process of copulation of two mature partners. Mechanism of copulation in few earthworm species (*Lumbricus terrestris*) is known. However literature on neurosecretory control of copulation in earthworm is lacking. In the present study 30 pairs of conjugating earthworms of *Eisenia fetida* were collected from laboratory culture beds during 2016 of monsoon season. Cerebral and ventral ganglia of 10 pairs of conjugating earthworms and 20 pairs of post conjugation (2 hrs after separation, 4 hrs after separation) earthworms were fixed in Bouin's fluid for cytomorphological studies on their cerebral neurosecretory cells. Earthworms (10 numbers) debrained through anterior transection of first five segments (brain present in 3rd segment) by sterilized paragon knife were studied to observe conjugation if any. Cerebral and ventral ganglia of 5 pairs of pre-conjugating earthworms were considered as control. Cerebral and ventral ganglia of earthworms displayed chiefly two types of neurosecretory cells such as A cells and B cells. The A cells are deeply stained AF-positive cells arranged in upper cortical tier beneath the perineurium and generally exhibiting process of axonal transport. The B cells, generally larger in size than A cells, are lightly or moderately stained with or without axonal transport and lie in between cortical A cells and central fibrous neuropile. In the cerebral ganglia the A cells outnumbered the B cells, while in the ventral ganglia the opposite is true. Debrained *E. fetida* survived but did not conjugate. Secretory dynamics in the cerebral and ventral ganglionic neurosecretory cells A and B were recorded in pre-conjugating, conjugating and post-conjugating earthworms. Absence of conjugation in debrained worms and exhibition of the same in earthworms with brain and its changes in neurosecretory profile clearly indicate involvement of cerebral neurosecretion in the phenomenon of conjugation in earthworm. Our result indicates probable involvement of cerebral neurohormone in the process of conjugation in *E. fetida*.

Keywords: Copulation; Copulatory behavior; *Eisenia foetida*; *Eudrilus eugeniae*; Neurosecretory cells; Neurosecretion; Cerebral ganglia; Ventral ganglia.

1. INTRODUCTION

Earthworms in spite of hermaphroditism, practice cross fertilization (with some exception), thus receiving sperms in the same copulation [1]. The best examples of this phenomenon are *Eisenia fetida* and *Lumbricus terrestris*. Dominguez et al. [2] reported cases of self-fertilization in earthworm species such as, *Eisenia andrei*, where the individuals bend themselves, allowing their spermathecal pores to contact the ventral part of the clitellum. The spermatozoa are then transferred from the male pores to the spermathecae of the same individual. In both epigeic (*Eisenia sp.*) and anecic (*Lumbricus sp.*) earthworms conjugation generally takes place above the surface, while in endogeic species it takes place inside deeper soils [1]. Size assortative mating (selection of similar sized partners) was reported in the epigeic *E. fetida* [3], as well as, in anecic *L. terrestris* [4]. Interestingly, chemical cues have been suggested by Olive and Clark [5] in earthworms as a mechanism of finding and attracting the mate. Courtship behaviour was suggested in *L. terrestris* by Nuutinen and Butt [6] during which there were repeated short touches, executed with prostomium between the partners. Wallwork [7] described the prostomium as a sensory lobe with many chemo- receptors or sensory cells. In *L. terrestris* binding during copulation is strong and lasts for an average of 3 hours [8] and aided by 40 to 40 specialized setae to pierce the partners skin introducing a product from the setal gland [9]. Transfer of bioactive substance (i.e. allohormone) during earthworm copulation manipulating reproductive process in the partners has been advocated by Koene and Ter Maat [10]. According to Olive and Clark [5] and Edwards and Bohlen [11] reproduction in earthworm is governed and influenced by external factors such as light, temperature, moisture and food supply.

Role of neurosecretion in the process of reproduction in earthworms was first highlighted by Tombes [12] who opined that the cerebral A type neurosecretory cells (NSCs) appeared to be active during the process. He further reiterated that copulation and oviposition coincided with vacuolation of the neurosecretory perikarya, resulting in reduction in staining in the cell body, as well as, in axons [12]. Later Golding and Whittle [13] reported that in earthworms, the cerebral A type NSCs with ultra-structural characteristics of typical peptide secreting cells located in the cortical region of the ganglia enlarged and had signs of secretory activity during sexual maturation and meiotic activity within the gonads. These cells probably secretes cerebral gonadotrophic hormone [5, 14]. Earlier Hagadorn [15] and Malecha [16] reported gametotrophic role of α cells (homologous to A cells of oligochaetes) present in the cerebral ganglia of leech, *Theromyzon rude* and *Hirudo medicinalis* respectively. Later Ranganathan and Parthasarathi [17] correlated histological changes in the ovaries of earthworms with the cytological changes in the cerebral A and B type NSCs.

In fact hermaphroditism with cross fertilization is a remarkable feature in earthworms. Although literatures on neurohormonal influence of cerebral ganglia in gonad maturation in earthworms are available [13], there are no reports on the role of neurosecretory cells in the central nervous system during the process of conjugation in earthworms. So the aim of our present investigation was to study the behavioural and physiological changes during conjugation in *Eisenia fetida*, *Eudrilus eugeniae* and *Perionyx excavatus*. Neurosecretory changes in the central nervous system during conjugation in earthworms are based on *Eisenia fetida*.

2. MATERIALS AND METHODS

Perionyx excavatus, *Eudrilus eugeniae* and *Eisenia fetida* were cultured separately in wooden boxes (45 cm × 30 cm × 30 cm) containing 10 days old partly decomposed cow dung. As earthworms are photonegative, the culture box was covered with black cloth over an iron mesh supported by wooden frame,

so that worms were maintained in dim light. As epigeic earthworms viz *Eisenia*, *Eudrilus* and *Perionyx* copulate at surface, the dim light in no way hampered their biological processes. Three to five pairs of *Perionyx excavatus*, *Eudrilus eugeniae*, and *Eisenia fetida* each in conjugating conditions, were taken out from the stock cultures and placed on petri dishes in different dates and time to study their behaviour and physiological changes if any under the laboratory conditions (T 26-28°C, RH 70-85%) during June to August 2016.

Five pairs of *Eisenia fetida* were 'debrained' amputating the first five segments by sterilized paragon knife. 'Debrained' worms were kept in earthen pots (2L) covered with a black cloth and containing partly decomposed cow dung, the natural food of epigeic earthworms. Substrate moisture was maintained at about 60%-70 % by periodic sprinkling of water with a hand sprayer. As the experiment was carried out in a well ventilated room, the outside and inside temperature of the room did not differ to a great extent. On daily basis the worms were checked for conjugation events if any for about two months. Amongst the three species, only *Eisenia fetida* was selected for studying neurosecretory activity during conjugation, because this worm undergoes conjugation at the surface of wastes in the day time under laboratory conditions and they are also less sensitive to touch during conjugation. From the clitellate earthworms, *Eisenia fetida* of similar age group (60-70 days old), thirty pairs of conjugating earthworms were collected at different times during monsoon. Cerebral and ventral cord ganglia of 10 pairs (20 individuals) of conjugating earthworms, 20 pairs of post conjugation (10 pairs of worms, 2 hours after separation, and 10 pairs of worms 4 hours after separation) earthworms and 10 pairs of control earthworms before conjugation were fixed in aqueous Bouins fluid for cytomorphological studies.

Bouins fixed cerebral, subesophageal and ventral ganglia (at clitellar region) were dehydrated and embedded in paraffin (58-60°C). Serial sections (7 µm thick) were stained with both simplified Aldehyde Fuchsin [18] and Gomori's Chrome Alum- Haematoxylin Phloxin [19] staining techniques following acid permanganate oxidation. Photographs were taken by Leica DM-1000 bright field microscope.

Nucleo-cytoplasmic (NP) indices of the neurosecretory cells (NSCs) (20 cells for each cell type) were determined by measuring the maximum diameter of the cell body (perikaryon), as well as, the nuclei. Palcovit's formula ($V = \pi/6 \times LD^2$) was applied to determine the volume of the nucleus, as well as, the cell. The nucleo-cytoplasmic (NP) index based on the average value of the ratio $V_n/(V_c-V_n)$ (V_n = volume of nucleus, V_c = cell volume) has been considered to determine neurosecretory activity. NP indices were then statistically analysed by one way ANOVA followed by Tukey test to check significant differences if any among the control and experimental sets. Indices of neurosecretory activity were correlated with the amount of neurosecretory material (NSM) present within cell body.

3. RESULTS AND DISCUSSION

3.1. Courtship behaviour

Interesting courtship behaviour like repeated short touches by the prostomium was recorded prior to conjugation in *Eisenia fetida*. Similar mating behaviour was reported by Nuutinen and Butt [6] in the *Lumbricus terrestris*. Presence of water borne molecules attarctin and temptin as mate attraction pheromones were reported to be involved in mate selection in earthworms [20].

During conjugation in *E. fetida*, two individuals were attracted to each other with secretion of profuse mucus from the mucus secreting cells present in the epidermis. There were increase in the activity of mucus secreting cells in the epidermis of conjugating earthworms than those in the non-conjugating control and post

conjugated experimental earthworms (Fig. 2 a-c). During sexual congress, the two worms lie side by side with their “head” region in opposite direction (Fig. 1 a, b). The worms align in such a manner that the clitellum of one earthworm lies opposite to the segments that bear the openings of the spermathecae (sperm containing sacs) of other worms. The underside of their bodies is held firmly together with setae (tiny bristle) and the sticky mucus that coats both the worms and binds them together for about an hour or so [21].



Figure 1. Showing conjugation in a. *Eudrilus eugeniae*, b. *Eisenia fetida*.

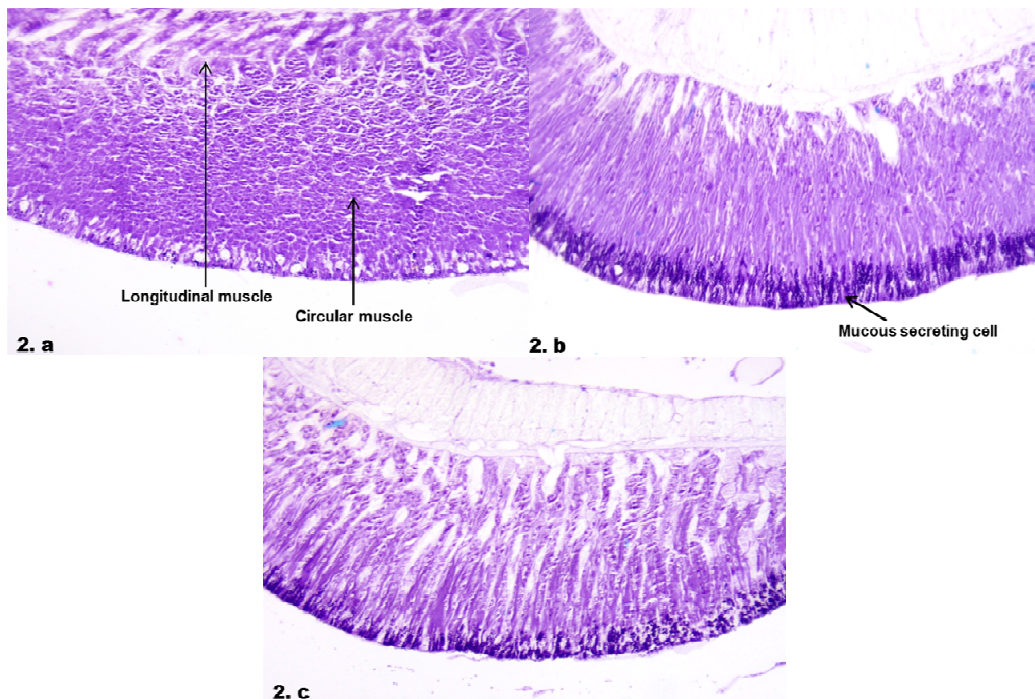


Figure 2. Transverse section of body wall of *E. fetida* showing distribution of longitudinal muscle, circular muscle & mucus secreting cells from inside outwards. a. Before conjugation, b. During conjugation and c. After conjugation (AF stain, 10X).

Native earthworm, *Perionyx excavatus* was highly sensitive to touch during conjugation, so that a slight touch to a conjugating pair led to their quick separation. Exotic species such as *Eisenia fetida* (European earthworm) and *Eudrilus eugeniae* (South African earthworm) were less sensitive to touch during conjugation. So in spite of slight touch or disturbances, *E. fetida* and *E. eugeniae* did not separate, instead their copulation continued for about 50 minutes and 70 minutes (mean time period for 3 pairs each) respectively. Tight binding during conjugation was probably due to involvement of specialised setae in the region of spermatheca and genital areas [22].

After exchange of sperms, the worms separate and move away, each carrying the sperms of the other in its spermathecae. The clitellum of each worm secretes albumin like substance that hardens when comes in contact with outside air and forms a band around the body. The worms wriggle out of this band which slides forward over the female reproductive openings, collect eggs shed by the worm and then over the openings of spermathecae to collect sperms received during mating [21]. The band closes at both ends forming a cocoon with sperms and eggs inside facilitating fertilisation inside the cocoon. As the cocoon passes over the prostomium, its terminal ends seal up to produce tight envelope. Fertilization is thus external and successive cocoons are produced until the store off eggs and sperms has been exhausted. The wall of this envelope subsequently hardens and changes colour as it dries up [7, 23].

3.2. Effect of 'brain removal' on conjugation and cocoon production

Our present study indicated that the 'debrained' earthworms (*E. fetida*) did not conjugate up to 50 to 60 days following which they conjugated and produced cocoons. According to Nanda and Chaudhuri [24] cephalic nerve ring in the top soil endogeic megascolicid earthworm, *Metaphire peguana* was regenerated completely within six weeks after cephalic transection. In epigeic earthworm *Eisenia fetida*, brain was regenerated within 30 days after amputation of cephalic segments containing brain [25]. In *Eudrilus eugeniae* cephalic nerve ring consisting of cerebral ganglia and subesophageal ganglia were regenerated with typical arrangement of AF +ve neurosecretory A type and B type cells around the central fibrous neuropile within 40-50 days [26]. Recent studies of Banik and Chaudhuri [27] revealed that regeneration takes place at a much faster rate in epigeic earthworms compared to endogeic and anecic earthworms. Delay to start cocoon laying in *E. fetida* following brain regeneration was due to time for reconstruction of blood vascularisation in regenerated brain [12] and also delay in the appearance of AF +ve A type cells in the regenerated cerebral ganglia (after 40 days) in earthworm [26]. Earlier Golding and Whittle [13] advocated that cerebral peptidergic A type cells are involved in the process of reproduction in earthworm. This indicates that presence of brain is essential for earthworms to conjugate and to produce cocoons. Furthermore, external factors such as temperature (26-28°C), food supply in the form of cow dung rich in bacteria and the presence of dim light were favourable for regeneration of cephalic segments along with cerebral ganglia (in the 3rd segment) followed by conjugation and subsequent cocoon laying.

3.3. Neurosecretory changes during conjugation and post conjugation periods

Coupled with behavioural and physiological changes, cytological alterations in the cerebral and ventral ganglionic (clitellar region) NSCs during conjugation and post conjugation periods in *E. fetida* were recorded.

3.3.1. Pre-conjugation control

Cerebral, subesophageal and ventral ganglia (clitellar region) exhibited 2 types of NSCs- deep stained A cells and moderately stained B cells, which were in different phases of secretory activity (Fig. 3 a, b). Both A cells and B cells accumulated neurosecretion before conjugation. A cells with homogeneously stained colloidal secretion were located peripherally and displayed axonal transport (Fig. 3a) to the "zone of accumulation" at the margin of fibrous neuropile which was distributed with AF positive neurosecretory materials (NSM). Moderately stained B cells were located between the fibrous neuropile and A cells. The B cells had discrete secretory granules and distinct axon oriented nuclei. In contrast to the ventral ganglia, A cells outnumber B cells in the cerebral ganglia.

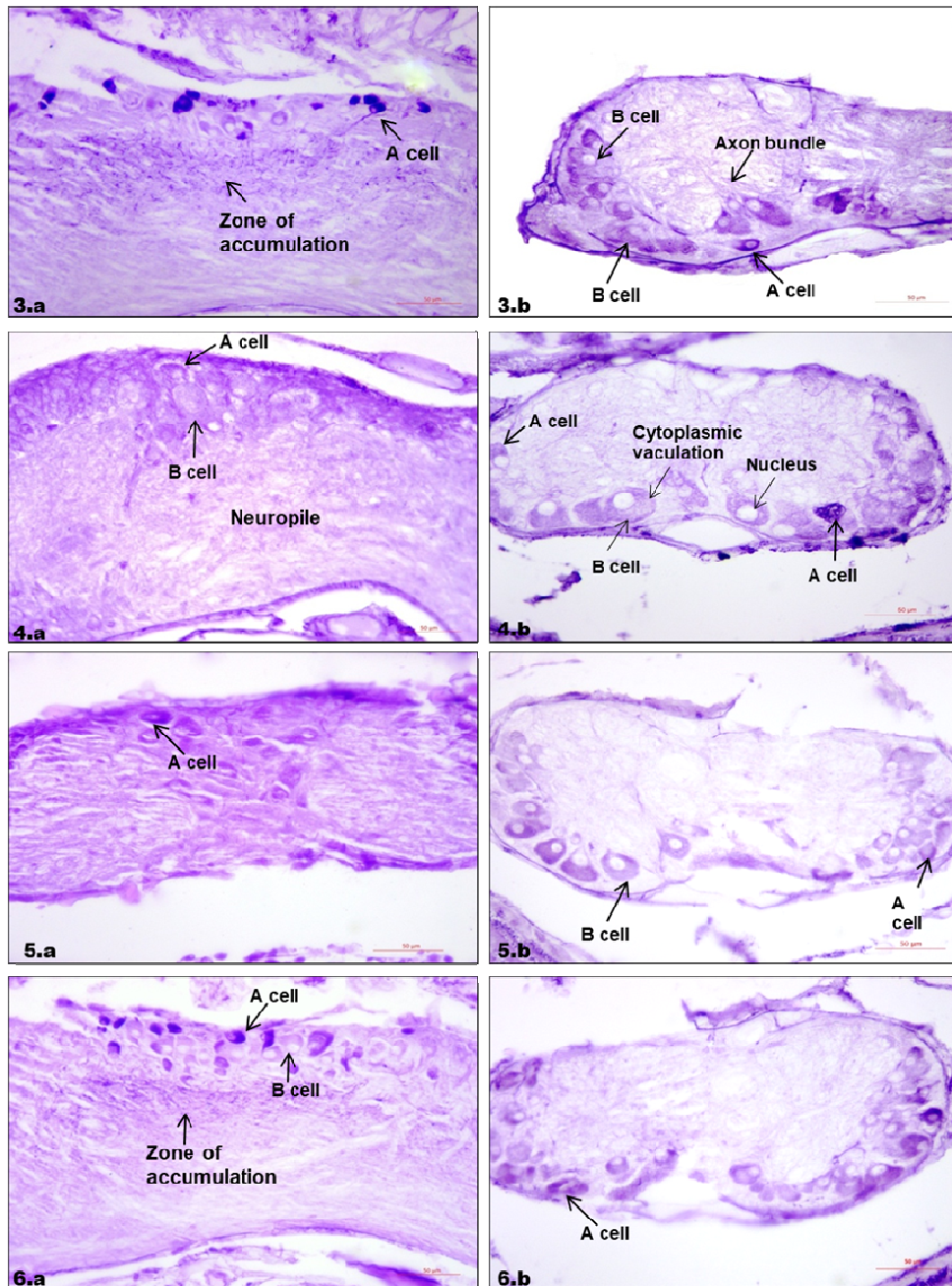


Figure 3-6. T.S. of *E. fetida* showing distribution of neurosecretory cells and central neuropile in the cerebral ganglia (CG) [3a-6a] and ventral nerve cord ganglia (VG) [3b-6b], AF, 40X. 3a, 3b - CG and VG before conjugation (control group); 4a, 4b - CG and VG during conjugation; 5a,5b - CG and VG 2 hours after conjugation; 6a, 6b - CG and VG 4 hours after conjugation.

3.3.2. Conjugation

During conjugation there was sudden drop in number of AF positive A cells in its posterior dorsal part of cerebral ganglia of earthworms. The A cells which still exist had voluminous nuclei, cytoplasmic vacuoles and intense depletion (Fig. 4a) indicating efferent release of neurohormone from cerebral neurosecretory A cells due to afferent stimulus produced through sexual contact between the partners and passed through the ventral nerve cord. The B cells also had scanty secretory granules and abundance of cytoplasmic vacuoles. "Zone of accumulation" was devoid of any NSM indicating its utilisation during conjugation. The B cells in

the mid ventral part of the ventral nerve cord ganglia of the genital region appeared to have moderately stained AF positive secretory granules and axon oriented voluminous nuclei (Fig. 4 a, b). The A cells at the lateral part of ventral ganglia displayed shedding of staining intensities and appearance of cytoplasmic vacuoles. The subesophageal ganglionic neurosecretory cell in general, showed no remarkable changes during copulation. According to Tombes [12], copulation and oviposition in earthworms, coincide with cytoplasmic vacuolations of cell body, resulting in reduction in the amount of neurosecretion in the cell body, as well as, axons. Following oviposition, when the egg chamber was exhausted, very little neurosecretion appeared in the A cells. In the present study, both A type and B type NSCs in the cerebral and ventral ganglia had significant increase ($P < 0.05$) in cell size (hypertrophy), as well as, nucleoplasmic indices (Table 2 a, b) indicate hyper activity of these cells to promote copulation by producing copious mucous and albumen secretion from the glandular cells of the epidermis (Fig. 2b). While mucus secretion contributes to the process of conjugation and subsequent cocoon formation, albumen secretion from the glandular cells of epidermis provides nourishment for growing embryos inside the cocoon.

3.3.3. Two hours after conjugation

In the cerebral and ventral ganglia of 2 hours conjugation, AF staining intensities of A and B neurosecretory cells gradually increased with accumulation of neurosecretion in the cell body, decline in cell and nuclear volume, as well as, drop in their nucleocytoplasmic ratios (Tables 1, 2a and 2b, Fig. 5 a, b).

3.3.4. Four hours after conjugation

In the 4 hours post conjugating earthworms, secretory profile of A and B cells in the cerebral and ventral ganglia and “zone of accumulation” enriched with AF positive neurosecretion (Fig. 6 a, b) simulated the conditions of pre-conjugating control earthworms.

Table 1. A and B cell and nuclear diameter (μ) in the central nervous system of *E. fetida* during pre-conjugation (control), conjugation and post conjugation periods (n=20).

CNS	Cell types	Control		Conjugating		2 hrs PC		4 hrs PC	
		CD \pm SD	ND \pm SD	CD \pm SD	ND \pm SD	CD \pm SD	ND \pm SD	CD \pm SD	ND \pm SD
Cerebral ganglia	A cell	8.2 \pm 0.69	3.34 \pm 0.15	10.34 \pm 1.18	4.9 \pm 0.25	9.5 \pm 0.35	4.3 \pm 0.19	7.9 \pm 0.73	3.37 \pm 0.19
	B cell	14.67 \pm 0.26	6 \pm 0.332	15.76 \pm 0.62	8.07 \pm 0.42	15 \pm 0.325	7.77 \pm 0.346	14.79 \pm 0.357	6.732 \pm 0.3
Ventral nerve cord	A cell	11.37 \pm 0.68	5 \pm 0.182	13.85 \pm 0.9	6.84 \pm 0.313	13.616 \pm 0.646	5.9 \pm 0.2	11.9 \pm 1.07	5.23 \pm 0.327
	B cell	15.72 \pm 0.67	7.7 \pm 0.49	19.85 \pm 0.851	10.8 \pm 0.47	18.32 \pm 1.03	9.163 \pm 0.33	17.69 \pm 0.9	8.33 \pm 0.35

SD: Standard deviation; CD - cell diameter; ND - nuclear diameter; n = numbers of cells observed.

Table 2a. Nucleo-cytoplasmic Index of A- and B cells in cerebral ganglia of *E. fetida* before, during, and after conjugation (n=20).

NPI*	Control	Conjugating	2 hrs post conjugation	4 hrs post conjugation
A cells	0.044 \pm 0.003 ^a 0.029 – 0.06	0.083 \pm 0.006 ^b 0.053 – 0.119	0.065 \pm 0.003 ^c 0.05 – 0.086	0.049 \pm 0.004 ^a 0.027- 0.065
B cells	0.059 \pm 0.002 ^a 0.046 – 0.072	0.112 \pm 0.005 ^b 0.093 – 0.137	0.117 \pm 0.003 ^b 0.104 – 0.135	0.079 \pm 0.002 ^c 0.064 – 0.091

Values represent Range; Mean \pm (SE); Dissimilar letters indicating significant difference at 5% level of significance;

* One way ANOVA; Tukey test; n = numbers of cells observed.

Table 2b. Nucleo-cytoplasmic Index of A- and B cells in ventral nerve cord ganglia of *E. fetida* before, during, and after conjugation (n=20).

NPI*	Control	Conjugating	2 hrs post conjugation	4 hrs post conjugation
A cells	0.06 ± 0.002 ^a 0.052 – 0.074	0.099 ± 0.002 ^b 0.09 – 0.11	0.065 ± 0.002 ^a 0.053 – 0.07	0.063 ± 0.004 ^a 0.05 – 0.083
B cells	0.083 ± 0.004 ^a 0.064 – 0.11	0.139 ± 0.007 ^b 0.103 – 0.18	0.101 ± 0.003 ^a 0.083 – 0.126	0.08 ± 0.005 ^a 0.068 – 0.096

Values represent Range; Mean ± (SE); Dissimilar letters indicating significant difference at 5% level of significance;

* One way ANOVA; Tukey test; n = numbers of cells observed.

Thus accumulation of neurosecretion in both A cells and B cells, “zone of accumulation” enriched with neurosecretion prior to conjugation, cellular hypertrophy with acute depletion and appearance of cytoplasmic vacuoles in the neurosecretory cells with disappearance of neurosecretion from “zone of accumulation” during conjugation and reappearance of neurosecretion in both A and B cells, as well as, in the “zone of accumulation” during 2 hours and 4 hours post conjugating periods indicates clearly the role of both A and B NSCs in cerebral and ventral nerve cord ganglia during conjugation in earthworms. Distinct secretory rhythm with synthesis, transport and release of neurosecretion from A and B cells, more specifically A cells during active reproductive phase in *E. fetida* and *E. eugeniae* was reported by Lattaud [28], Gunashekharan [29] and Parthasarathi and Ranganathan [30]. The occurrence of gonadotropins in neurosecretion and their influence on gametogenesis has been reported in earthworm *Dendrobaena veneta* by Siekierska [31].

4. CONCLUSION

In spite of hermaphroditism most of the earthworm species undergo cross fertilization between two partners by the process of conjugation. Presence of cerebral ganglia is essential for conjugation. Copulatory behaviour such as reciprocal touch by prostomium is exhibited prior to conjugation in *Eisenia fetida* where copulation lasts for 50 minutes. Neurosecretion from cerebral neurosecretory type A and type B cells are probably involved in the copulatory process in earthworm.

Authors’ Contributions: PSC: provided the guidance for designing the scheme of work; DB: executed the research work as per the scheme through discussion; AB: helped in the drafting of manuscript and provided technical support. All authors read and approved the final manuscript.

Conflict of Interest: The authors declare no conflict of interest of any type for this article.

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