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doi: 10.12980/JCLM.2.201414J3

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Cholinergic neuromuscular junctions in *Brachionus calyciflorus* and *Lecane quadridentata* (Rotifera: Monogononta)

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PEER REVIEW

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Comments

The manuscript is dealing with an aspect for which not much information is available; the neurotransmission system of rotifers including *Brachionus calyciflorus*. This species along with other rotifers are being studied in order to be used in toxicity tests thus knowledge on the way the neuromuscular system functions is essential. Furthermore increasing the available information further adds to the comparative analysis elucidating ecological and evolutionary relationships.

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ABSTRACT

Objective: To identify the presence of joint muscular and cholinergic systems in two freshwater rotifer species, *Brachionus calyciflorus* and *Lecane quadridentata*.

Methods: The muscle actin fibers were stained with phalloidin–linked fluorescent dye, and acetylcholine was detected with Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit, and then confocal scanning laser microscopy was used.

Results: The musculature of *Brachionus calyciflorus* showed a pattern similar to other species of the same genus, while that of *Lecane quadridentata* was different from other rotifer genera described previously. The cholinergic system was determined by co–localization of both muscles and acetylcholine labels in the whole rotifer, suggesting the presence of neuromuscular junctions.

Conclusions: The distribution pattern of muscular and acetylcholine systems showed considerable differences between the two species that might be related to different adaptations to particular ecological niches. The confirmation of a cholinergic system in rotifers contributes to the development of potential neuro–pharmacological and toxicological studies using rotifers as model organism.

KEYWORDS

Acetylcholine, Co–localization, Confocal microscopy, F–actin, Rotifer

1. Introduction

Rotifers are cosmopolitan mainly aquatic or semiaquatic microscopic non–segmented, bilaterally symmetric and

pseudocoelomates invertebrates[1]. The body is typically divided into three regions: the apical end (head) forming a ciliated region known as corona, the trunk and the foot. In many species, the corona has two concentric rings of cilia

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Foundation Project: Supported by Consejo Nacional de Ciencia y Tecnología (Conacyt) through the scholarship (Grant No. 136453), and Sistema Nacional de Investigadores (Grant No. 49351).

Article history:

Received 12 Jan 2014

Received in revised form 1 Feb, 2nd revised form 8 Feb, 3rd revised form 18 Feb 2014

Accepted 23 Apr 2014

Available online 28 May 2014

that beat in a metachronous pattern creating enough flow to obtain food and engage in locomotion. Rotifers have a muscular pharynx (mastax) with a complex set of chitinous jaws (trophi) of high taxonomic importance used to classify rotifer species. Class Monogononta comprises 70% of rotifer species, including 95% of benthonic, sessile, and free swimming species[1,2]. The ultrastructural organization of rotifers has been studied by means of scanning and transmission electron microscopy[3]. The rotifer muscular system has been studied in whole individuals of *Philodina* sp. by specific F-actin fiber recognition with Alexa-488 phalloidin or labeled phalloidin-TRITC. Several works have studied the rotifer muscular system with confocal laser scanning microscope (CLSM) in *Euchlanis dilatata unisetata* and *Brachionus quadridentatus* (*B. quadridentatus*)[4], *Brachionus urceolaris*, *Floscularia ringens*, *Hexarthra mira*, and *Notommata glyphura*[5], three species of the genus *Proales*[6], *Filinia novaezealandiae*[7], *Encentrum mucronatum* and *Dicranophorus forcipatus*[8], *Hexarthra* and *Polyarthra*[9], *Bryceella stylata*[10], *Adineta ricciae* and *Macrotrachela quadricornifera*[11], *Brachionus manjavacas* and *Epiphanes senta*[12], *Squatinella rostrum*[13], and *Trichotria pocillum*[14]. The rotifer nervous system comprising a large cerebral ganglion known as the brain positioned dorsally below the corona, paired ventral neurons, which branches to the mastax, body and foot, and sensory organs (mechano, chemo, and photoreceptors); even a retrocerebral organ (paired subcerebral glands, unpaired retrocerebral sac, and paired ducts leading to the apical surface) is found behind the brain in many bdelloid and monogonont rotifers[1,2]. This nervous system has been described by showing the presence of neurotransmitters (acetylcholine) and enzymes (acetylcholinesterase), demonstrating the existence of a cholinergic system in rotifers by histochemical methods[1,15,16]. The studies did not include in their analysis individuals of the genera *Brachionus* or *Lecane*. Other studies have described catecholaminergic neurons in rotifers like *B. quadridentatus*[17,18]. Other types of neurotransmitters, such as FMRFamide and serotonin (5-hydroxytryptamine), have been reported in rotifers, using histochemical, immunocytochemical, and CLSM techniques, which indicate the presence of a serotonergic nervous system[19–23]. Otherwise, acetylcholinesterase receptors were recognized in *B. quadridentatus*, *Lecane luna*, *Lecane quadridentata* (*L. quadridentata*), *Plationus patulus* and *Rotaria neptunia* using epifluorescence microscopy and α -bungarotoxin fluorescein isothiocyanate (FITC)[24]. Also, the exocytotic membrane proteins known as SNARE (syntaxin-1, syntaxin-4, SNAP-23, and SNAP-25) involved in the vesicular release of secretory proteins were identified in three rotifer species by mean of immunohistochemical and immunoblot techniques[25].

The CLSM studies have been limited to the musculature of individuals of some species of the genus *Brachionus*: *Brachionus plicatilis*, *B. quadridentatus*, *Brachionus urceolaris*, and *Brachionus manjavacas*[4,5,12,26]. *Brachionus calyciflorus* (*B. calyciflorus*) has not been studied by this method. Neither has the muscle system of the species

of *Lecane* been described although this genus is highly diverse with 163 species recorded so far[27]. Therefore, the information on Lecanidae would be valuable for comparison with other rotifer species and would increase knowledge about the diversity of rotifer muscular systems. The objective of this study was to reveal the joint muscular and cholinergic system in two rotifer species: *B. calyciflorus* and *L. quadridentata*.

2. Materials and methods

The rotifer species studied were *B. calyciflorus* (Pallas 1776) collected from the pond of the water treatment plant at Universidad Autónoma de Aguascalientes (21°55' N, 102°29' W), Aguascalientes, México, and *L. quadridentata* (Ehrenberg 1832) collected from Lake Chapala (20°12' N, 102°85' W), Jalisco, México. Rotifers were cultured at (25±2) °C in plastic Petri dishes with freshwater EPA medium[28], using a bioclimatic chamber with a 16: 8 Light: Dark period and fed with the green algae *Nannochloris oculata* (UTEX strain LB2194) grown in Bold's medium[29], following the method of Pérez-Legaspi and Rico-Martínez[30]. A hundred amictic females of each species were added randomly into microcentrifuge tube containing 500 μ L of EPA medium and incubated at 4 °C for one hour, to precipitate the rotifers. Each rotifer sample was centrifuged at 5000 r/min for 10 min at 4 °C to eliminate EPA medium, and then rested in 1% MgCl₂ for one hour to maintain the integrity of individuals. Centrifuged rotifer samples were fixed in 4% formalin in 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) for 30 min, centrifuged and rinsed with 0.01 mol/L PBS. Subsequently, rotifers were centrifuged to eliminate the medium and permeabilization solution 0.1% Triton X-100 in 0.01 mol/L PBS was added for one hour, following the protocol of Hochberg and Litvaitis[31].

2.1. Acetylcholine labeling

Acetylcholine was shown in rotifers using Amplex red reagent (400 μ mol/L) containing horseradish peroxidase, choline oxydase from *Alcaligenes* sp., and acetylcholinesterase from electric eel (Molecular Probes, Inc. Eugene, OR, USA); subsequently each rotifer sample was incubated in darkness for 30 min at room temperature. The method of Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit (A12217) was used to detect acetylcholine following hydrolysis to choline, and oxidation, by choline oxydase, to betaine and H₂O₂, which in contact with horseradish peroxidase, reacts with Amplex red reagent in a 1:1 stoichiometry to generate a fluorescent product (resorufin). After incubation, rotifers were washed in PBS and stained for F-actin fibers.

2.2. F-actin labeling

FITC-labeled (SIGMA-Aldrich) was added to rotifers to show mainly the muscular system following the protocol of Hochberg and Litvaitis with slight modifications[31]. The

sample was incubated overnight at room temperature in the dark. Subsequently, rotifer samples were rinsed in 0.01 mol/L PBS, mounted on microscopic slides and examined on the Confocal/two photons Microscope Leica Systems TCS SP5-MO equipped with laser Argon/HeNe 543, using excitation spectrum in 530–560 nm, and emission detection at ~590 nm. The scanning step size was about 0.5 μ m. The max-projection (flat projections of sections) was obtained by scanning from series of optical sections in order to make reconstructions of the whole animal by using the Leica LAS AF software.

3. Results

3.1. *L. quadridentata*

Rotifer CSLM images obtained from the entire specimen ($n=6$) showed various muscles stained by phalloidin (Figure 1A); in the lateral view it is possible to observe a pair of thin circular muscles (cm) in the medial region of the trunk, and two wide pairs of superior and inferior dorso-ventral muscles (dv_{1-2}). Longitudinal musculature showed different strong and wide retractor muscles observed in the dorso-ventral medial region as the medial retractor muscles of corona (rcm) inserted from upper ventral-medial region and connected into the muscles of mastax (m); also the wide pyramid shape lateral retractor muscles of the corona (rcl) connected to the upper sides of the superior mastax (sm), while thin ventral retractor muscles of the corona (rcv) were observed in the middle and connected into the lateral sides of mastax region. Muscles of the mastax (m) were present between the insertions of retractor muscles of corona suggesting that they are connected to the trophi (either ramus or fulcrum). A superior mastax muscle (sm) was observed above the insertions of retractor muscles of the corona. In the lower ventral-medial region the strong and broad retractor muscles of the foot (rf), extended from the ventral middle toward the foot, was present in the medial region. Several stronger and conspicuous ventral retractor muscles of the foot (rfv_{1-3}) were easily observed, while at the sides narrow lateral retractor muscles of foot (rfl_{1-3}) were seen; it was clear that the muscles of the foot engrossment, from the middle to inferior of the trunk, are attached to the lower foot. A CSLM picture obtained from a whole specimen showed diverse sites marked with acetylcholine (Figure 1B). All mastax (m) regions and some dots near the corona region were observed, while the conspicuous stomach (st) region showed the greatest amount of acetylcholine dye in rotifers. In Figure 1C a whole mount of rotifer reveals double-staining of both fluorescent markers, one for muscles (phalloidin in green color) and one for acetylcholine (rhodamine in red color) using CSLM. A photograph of a complete rotifer using light microscopy showed several structures such as stomach (st), and a conspicuous trophi (tr) by optical light microscopy (Figure 1D). Double-labeling revealed co-localization (white dots) of many specific features (neuromuscular junctions) concentrated in the stomach region of *L. quadridentata* (Figure

3A). The neuromuscular junctions indicate the co-localization of both muscles (green dots) and acetylcholine (red dots).

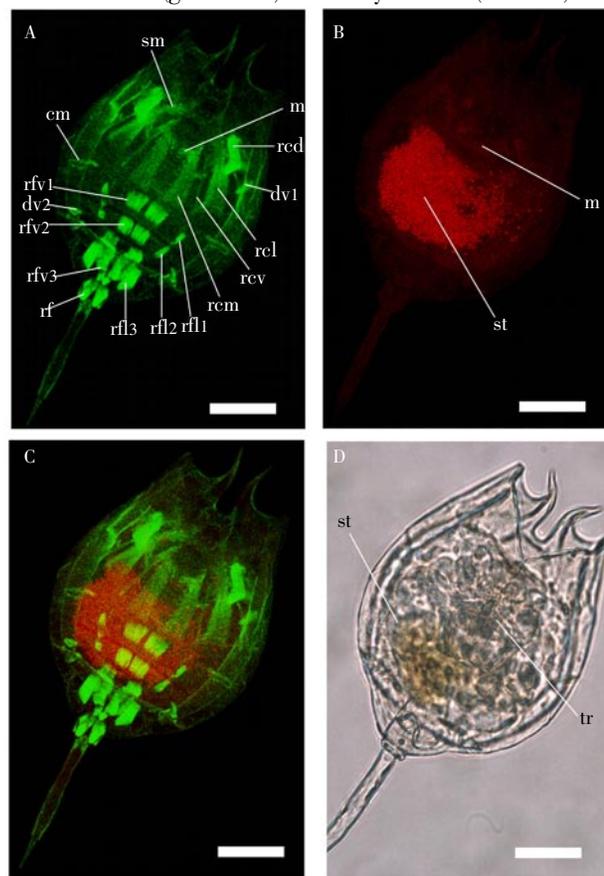


Figure 1. Whole mounts of *L. quadridentata* at dorsoventral examination (20 \times , scale bar: 30 μ m).

A: Max projection showed musculature by phalloidin green fluorescent dye FITC by means of CSLM; B: Max projection display acetylcholine stained red by Amplex red assay using CSLM; C: Max projection revealed both musculature (green) and acetylcholine (rhodamine) by the two pictures using CSLM; D: Optical light microscopy picture showed. rcm: Medial retractor muscles of corona; rcv: Ventral retractor muscles of corona; rcl: Lateral retractor muscles of corona; m: Mastax; sm: Superior mastax muscle; dv_{1-2} : Dorso-ventral muscles; rfv_{1-3} : Ventral retractor muscles of foot; rfl_{1-3} : Lateral retractor muscles of foot; rcd: Dorsal retractile muscle of corona. st: Stomach; tr: Trophi.

3.2. *B. calyciflorus*

The CLSM picture obtained from whole specimens ($n=42$) showed the musculature stained by phalloidin green fluorescence (Figure 2A); in the upper lateral side it was possible to recognize three pairs of dorso-ventral muscles (dv_{1-3}). Longitudinal musculature was represented by long, wide retractor muscles of the corona (rc) originating near the mastax and in the middle part of the body extending anteriorly and connected to the corona region. These muscles were present in the contracted state (the animal retracted the corona before dying); and they include pairs of ventral (rcv), lateral (rcl) and dorsal (rcd) retractor muscles of the corona. The largest mastax muscle corresponded to the superior mastax (sm), also under and at both sides of this muscle, there are medial mastax muscles (mm_{1-2}). Below them a pair of inferior mastax muscles (im) formed a

“V” shape. In the lower ventral–medial region conspicuous large and strong contracted muscles were evident such as retractor muscles of the foot (rfv₁₋₂). At the sides thin and larger narrow longitudinal muscles (lm) were observed that extended towards the lower body. A pair of thinner and longitudinal muscles corresponded to dorsal tubular muscles (dt). Acetylcholine was present in diverse areas within the rotifer body (Figure 2B). The corona region (cr) showed a conspicuous presence of acetylcholine. A whole specimen double–marked in the muscular system (by phalloidin in green color) and cholinergic system (acetylcholine stained by rhodamine in red color) showed some of the red dots corresponding to the localization of acetylcholine masked by phalloidin (Figure 2C). Double–labeling revealed co–localization (white spots) of the specific points (neuromuscular junctions) of *B. calyciflorus* indicating the proximity or co–localization of both muscles (green dots) and acetylcholine (red dots), mainly in the stomach region and the corona with slight points near the mastax region (Figure 3B).

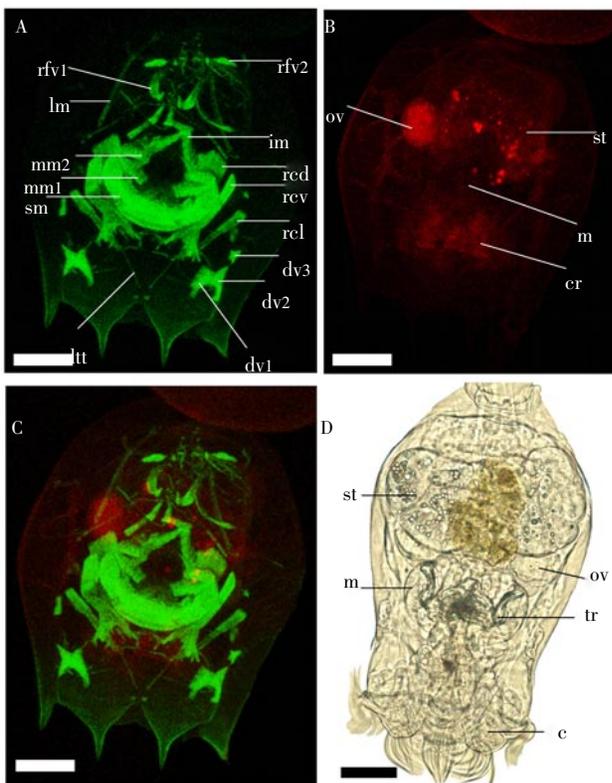


Figure 2. Entire specimens of *B. calyciflorus* with foot retracted viewed at dorsoventral examination (20X, scale bar: 30 μm).

(A) Max projection illustrated musculature by phalloidin green fluorescent dye FITC by mean of CSLM; B: Max projection display acetylcholine showed in red color by Amplex red assay by CSLM; C: Whole specimen double–marked in their muscular system (by phalloidin in green color) and cholinergic system (acetylcholine stained by rhodamine in red color) using CSLM; D: Optical light microscopy image. red: Dorsal retractor muscles of corona; rev: Ventral retractor muscles of corona; rcl: Lateral retractor muscles of corona; sm: Superior muscles of mastax; mm₁₋₂: Medial muscles of mastax; im: Inferior muscles of mastax; dv₁₋₃: Dorso–ventral muscles; rfv₁₋₂: Retractor muscles of foot; dt: Dorsal tubular muscle of corona; lm: Longitudinal muscle; ov: Ovary; st: Stomach; cr: Corona region; st: Stomach; tr: Trophi; m: Mastax; c: Corona.

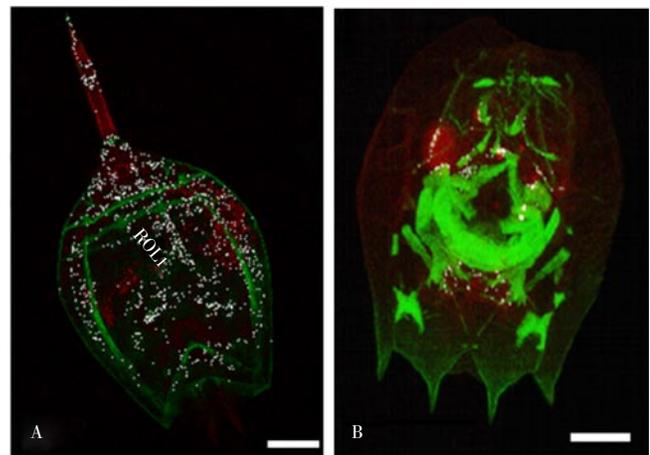


Figure 3. Max projections showing muscular system (green dots), cholinergic system (red dots), and co–localization of muscular and cholinergic systems (white dots) (scale bar: 30 μm).

A: The area (green circle) corresponds to the region of interest in *L. quadridentata*. (B) *B. calyciflorus*.

4. Discussion

The co–localization of muscular (phalloidin–stained points) and cholinergic systems (rhodamine–stained points) in both *B. calyciflorus* and *L. quadridentata*, indicate the existence of cholinergic neuromuscular junctions where acetylcholine is released from presynaptic vesicles to muscles to control most movements of the rotifer. Nerve endings were previously reported using transmission electron microscopy by Clément and Wurdak[3]. These authors observed synaptic vesicles suggesting that they might contain the acetylcholine neurotransmitter. Our CLSM study confirms the presence of acetylcholine as a potential marker of neuromuscular junctions, and is useful for demonstrating their location within the rotifer body.

The Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit makes it possible to detect acetylcholine by rhodamine stain using CLSM to demonstrate fluorescence signals in both rotifers studied *L. quadridentata* and *B. calyciflorus*. Clearly *L. quadridentata* shows a remarkable concentration of neuromuscular junctions than *B. calyciflorus*. A greater number of neuromuscular junctions results in a finer control of muscular contractions, probably *L. quadridentata* requires a greater effort coordinated than *B. calyciflorus* to move into the sediment such as benthic environment. Phalloidin stain revealed skeletal muscles in several orientations (longitudinal, circular, and visceral) in both rotifer species studied. Kotikova *et al.*[4] considered that the visceral musculature was represented by several structures: 1) mastax muscles; 2) cutaneo–pharyngeal muscles; 3) the network of muscles that envelop internal organs of the posterior part; 4) the muscles near both foot and dorsal groove of the lorica. The rotifer species studied did not show coronal muscles (par coronalis or coronal ring muscles). This result agrees with reports of musculature in rotifers of the genus

Brachionus^[4,5,12,26]. The species where these muscles have been reported are *Filinia novaezealandiae*^[7], *Squatinella rostrum*^[13], and *Encentrum mucronatum* and *Dicranophorus forcipatus*^[8]. The muscular arrangement pattern reflects morpho–ecological adaptations^[12]. Therefore, rotifers might vary in the muscle arrangement depending on their adaptations to each ecological niche such as their swimming behavior through the water column for planktonic rotifer or littoral zone for epi–benthic rotifer.

The two rotifer species examined in this study revealed similar patterns of muscular system with regard to coronal retractor muscles and circular incomplete muscles. However, there are differences in the proportions and arrangement of the muscles around the trophi and foot. These differences might be due to adaptations for a particular habitat. The epi–benthic rotifer *L. quadridentata* showed stronger retractor muscles of the corona (rc) and foot (rf) and this may be an adaptation to move in a more dense benthic habitat. The planktonic rotifer *B. calyciflorus* showed stronger muscles in the retractors of the corona (rc), shorter dorso–ventral muscles (dv_{1–2}) connected to corona, and conspicuous strong muscles of mastax (m) particularly the superior mastax (sm). This muscular arrangement is similar to that of other members of the genus *Brachionus*^[4,5,12,26], suggesting a conservative feature; therefore, the description of the *B. calyciflorus* muscular system is useful to increase our knowledge about interspecific variation. In the case of *L. quadridentata* a unique pattern among rotifers was observed, different from other rotifers reported. Nevertheless, more studies of the muscular system are needed to elucidate phylogenetic relationship in the family Lecanidae, which is one of the most species of the phylum Rotifera^[27]. It is probable that this type of musculature might be common in most Lecanidae, since they live mainly in littoral habitats. In conclusion, this study reveals cholinergic neuromuscular junctions in two rotifer species that have been identified previously. The confirmation of a cholinergic system in rotifers contributes to the development of model organisms to assess the neuro–pharmacological effects related to agonist or antagonist receptors in rotifers, to perform further toxicity test to assess adverse effects on anticholinesterase substances (like the ones produced by organophosphate and carbamate pesticides) in laboratory conditions, or to monitor aquatic ecosystems susceptible to pesticide pollution.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We thanks to Kalman Kovacs and Jhon Telford for reviewing the language of the manuscript. Gustavo Santos–Medrano and Araceli Adabache–Ortiz by optical microscopy

pictures of *L. quadridentata* and *B. calyciflorus*, respectively. Financial support was provided by Consejo Nacional de Ciencia y Tecnología (Conacyt) through the scholarship (Grant No. 136453), and the support of Sistema Nacional de Investigadores (Grant No. 49351).

Comments

Background

Rotifers are exquisite small metazoan invertebrates highly sensitive to pollutants, with life characteristics (e.g. short generation time, reproduce parthenogenetically, produce resting eggs) which make them good test organisms. On that basis among other aspects of their ecology, biology and systematics their muscular and nervous system is being explored.

Research frontiers

B. calyciflorus has not been studied by CSLM, neither has the muscle system of the genus *Lecane* been described, despite the fact that this genus is considered one of the most numerous with 163 species recorded so far.

Related reports

The authors applied the methodology that has proven appropriate to reveal the muscular and nervous system of the rotifers.

Innovations and breakthroughs

The research was applied to rotifers that have not been previously studied accordingly, increasing the relative knowledge for the phylum rotifera.

Applications

This kind of research is mainly basic research which is necessary to understand the function of rotifera in their environment as well as to further exploit their unique characteristics and use them in neuropharmacological studies and toxicity tests.

Peer review

The manuscript is dealing with an aspect for which not much information is available; the neurotransmission system of rotifers including *B. calyciflorus*. This species along with other rotifers are being studied in order to be used in toxicity tests thus knowledge on the way the neuromuscular system functions is essential. Furthermore increasing the available information further adds to the comparative analysis elucidating ecological and evolutionary relationships.

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