Journal of Helminthology (2016) **90,** 712–718 doi:10.1017/S0022149X15001005

The nervous systems of *Tylodelphys* metacercariae (Digenea: Diplostomidae) from the catfish *Clarias gariepinus* (Clariidae) in freshwater habitats of Tanzania

F.D. Chibwana* and G. Nkwengulila

Department of Zoology and Wildlife Conservation, University of Dar es
Salaam, PO Box 35064, Dar es Salaam, Tanzania

(Received 29 July 2015; Accepted 27 October 2015; First Published Online 1 December 2015)

Abstract

The nervous systems of three *Tylodelphys* metacercariae (*T. mashonense*, *Tylodelphys* spp. 1 and 2) co-occurring in the cranial cavity of the catfish, *Clarias gariepinus,* were examined by the activity of acetylthiocholine iodide (AcThI), with the aim of better understanding the arrangement of sensillae on the body surface and the nerve trunks and commissures, for taxonomic purposes. Enzyme cytochemistry demonstrated a comparable orthogonal arrangement in the three metacercariae: the central nervous system (CNS) consisting of a pair of cerebral ganglia, from which anterior and posterior neuronal pathways arise and interlink by cross-connectives and commissures. However, the number of transverse nerves was significantly different in the three diplostomid metacercariae: *Tylodelphys* sp. 1 (30), *Tylodelphys* sp. 2 (21) and *T. mashonense* (15). The observed difference in the nervous system of the three metacercariae clearly separates them into three species. These findings suggest that consistent differences in the transverse nerves of digenean metacercariae could enable the differentiation of metacercariae to the species level in the absence of molecular techniques. This, however, might require further testing on a larger number of species of digenean metacercariae.

Introduction

Digenetic trematodes are implicated in several important parasitic diseases of humans and animals, such as schistosomiasis, clonorchiasis, opisthorchiasis, fascioliasis and diplostomiasis. This is due to the capability of their developmental stages to adapt to completely different environments or both poikilothermic and homeothermic hosts (Solis-Soto & De Jong-Brink, 1995). Trematodes also produce neurosubstances that enable them to evade the immune activities of the hosts (Duvaux-Miret *et al.*, 1992). Since the neurosubstances originate from the host in response to the parasitic stimulus (De Jong-Brink, 1995), and the release of neurosecretory material accords with the parasite's change from poikilothermic to homeothermic hosts (Gustafsson & Wilkgren, 1981), the study of the whole nervous system would contribute greatly to the knowledge of the phylogeny of trematodes (Grabda-Kazubska & Moczon, 1981).

The catfish, *Clarias gariepinus*, is the host to three *Tylodelphys* species metacercariae co-existing in the cranial cavities (Musiba & Nkwengulila, 2006; Chibwana & Nkwengulila, 2010). Morphologically, the three diplostomid metacercariae are easily separated. *Tylodelphys mashonense* has a flat, oval body with fore- and hindbody distinct, though not as distinct as in the European congeners, and well-developed pseudosuckers

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*E-mail: fredchibwana@udsm.ac.tz; fredchibwana@yahoo.com

(Beverley-Burton, 1963). Metacercariae of two other *Tylodelphys* species, designated as *Tylodelphys* spp. 1 and 2, are morphologically similar. They have a dorsoventrally flattened forebody and a small, conical and translucent hindbody, with no clear separation between them, and the pseudosuckers are lacking (Chibwana & Nkwengulila, 2010). They can be differentiated based on size and the presence of gonadal enlargen in *Tylodelphys* sp. 2. Furthermore, molecular analysis of the three morphotypes revealed the presence of three clearly distinct species (Chibwana *et al.*, 2013). Since the three *Tylodelphys* metacercariae types co-exist in the same locality within the fish host, it seems interesting to study whether their nervous system structures are similar with respect to the similar function they perform.

Studies of the nervous system of diplostomids are commonplace in Europe; those of *Diplostomum pseudospathaceum* in particular – its nervous system has been well documented in all stages of the life cycle (Niewiadomska & Moczon, 1982, 1984, 1987, 1990). In Africa, on the other hand, knowledge of the nervous system of diplostomid species is missing, with the exception of the partially studied *T. mashonense* (Beverley-Burton, 1963). Accordingly, the aim of the present study was to examine the nervous systems of the metacercariae of the three *Tylodelphys* species occurring in the cranial cavity of *C. gariepinus,* in order to better understand the biology of these parasites and thus provide information for taxonomic purposes.

Materials and methods

Fish collection and parasite recovery

Fish were bought from artisanal fishermen from the Ruvu River. The metacercariae were recovered from the brain cavity of *C. gariepinus* by opening the cranial cavity using a strong and sharp scalpel. Metacercariae were removed from their sites in the front of the brain and along the nasal cavity by squirting tap water using a pipette. They were left for 3 h in water in a watch glass in order that the immature ones died and were removed before subsequent treatments (Shigin, 1986, cited in Musiba & Nkwengulila, 2006). All subsequent examinations were carried out on mature metacercariae as described below.

Examination of the nervous system

For histochemical investigation of the nervous systems, the metacercariae were placed in physiological saline to which a trace amount of 4% formaldehyde (formic acid

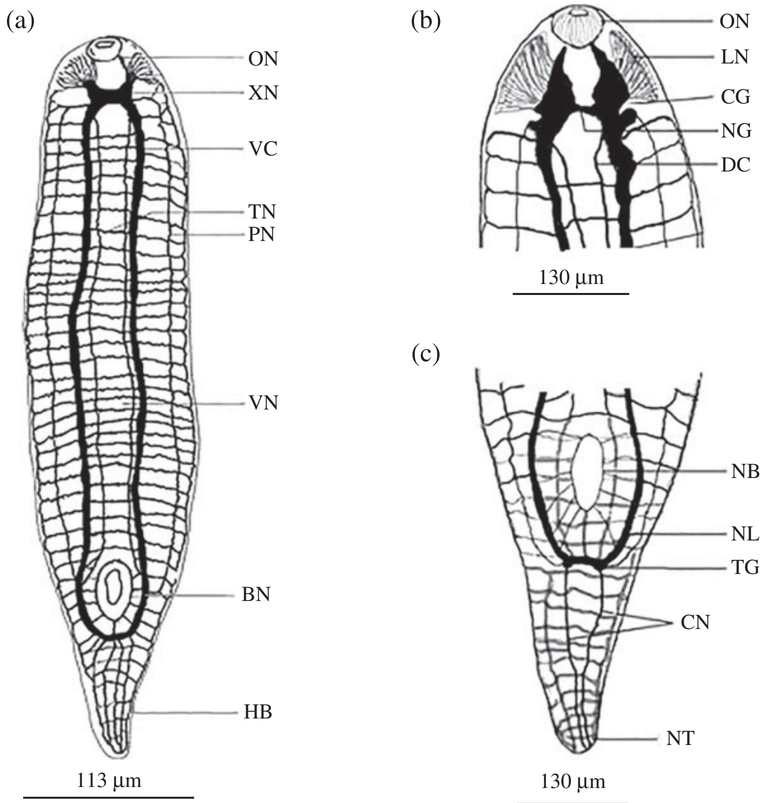

Fig. 1. The nervous system of the metacercaria of *Tylodelphys* sp. 1 from *Clarias gariepinus* to show (a) the entire body, (b) the forebody and (c) the hindbody; BN, nerves supplying Brandes organ; CG, cerebral ganglion; CN, caudal ventral nerves; DC, dorsal nerve cord; HB, hindbody; LN, nerves supplying pseudosucker region; NB, Brandes organ nerves; NG, nerve connecting cerebral ganglia; NL, nerve loop; NT, nerves supplying excretory pore; ON, nerves supplying oral region; TG, caudal ganglion; PN, peripheral longitudinal nerve cord; TN, transverse nerve; VC, longitudinal ventral nerve cord; VN, nerves supplying ventral sucker; XN, suprapharyngial nerves.

and methanol free) was added (0.01 ml per 1 ml saline) in order to paralyse the muscle system, thereby preventing rapid and irreversible contraction of the body caused by the higher concentration of formaldehyde added subsequently. This pre-fixation was controlled visually. Immediately after the relaxation of the muscles, the saline medium was replaced by 4% formaldehyde buffered to pH 7.0 with 0.05 M cacodylate buffer (Niewiadomska & Moczon, 1982).

Materials were left in the fixative for about 1 h at room temperature, then washed several times with distilled water to remove all traces of fixative, and incubated in a medium consisting of 20 mg acetylthiocholine iodide (AcThI) dissolved in 1 ml of water and added to 10 ml of stock solution containing 0.3 g copper sulphate ($CuSO_4$), 0.38 g glycerine, 1 g magnesium chloride ($MgCl_2$), 1.75 g maleic acid, 30 ml of 4% aqueous sodium hydroxide (NaOH) and 170 ml of 40% aqueous (saturated) sodium sulphate (Na_2SO_4). Material was then washed in three changes of 40% Na_2SO_4, followed by dilute yellow ammonium sulphide $(NH_4)_2S$ for 2 min, and then thoroughly washed in distilled water. The material was then counterstained in 0.5% aqueous eosin. The counterstained metacercariae were mounted in glycerine jelly and line drawings were made with the help of a camera lucida. Photographs were taken with the aid of a Motic microscope camera with Motic Image Plus 2 software (Motic®, Xiamen, China). Terminologies were adopted from Niewiadomska & Moczon (1982, 1984, 1987, 1990).

Results

The central nervous system (CNS) of the three metacercariae from *C. gariepinus* consists of a pair of cerebral ganglia (CG), from which anterior and posterior neuronal pathways arise and inter-link by cross-connectives and commissures. The peripheral nervous system (PN) includes innervations of the alimentary tract, reproductive organs, attachment and digestive organs (ventral sucker, oral sucker, pseudosuckers and Brandes organ) and the sub-tegumental muscles. Both the CNS and PN are bi-laterally symmetrical and better developed ventrally than laterally and dorsally. However, cholinesterase enzyme activity (ChE) revealed remarkable anatomical differences of the nervous system among the three metacercariae.

Metacercariae of Tylodelphys *spp. 1 and 2*

The nervous system of metacercariae of *Tylodelphys* spp. 1 and 2 (Figs 1, 2 and 3) is composed of a pair of cerebral ganglia, as in most digenetic trematodes, from which two large ventral longitudinal nerve cords (VC) run

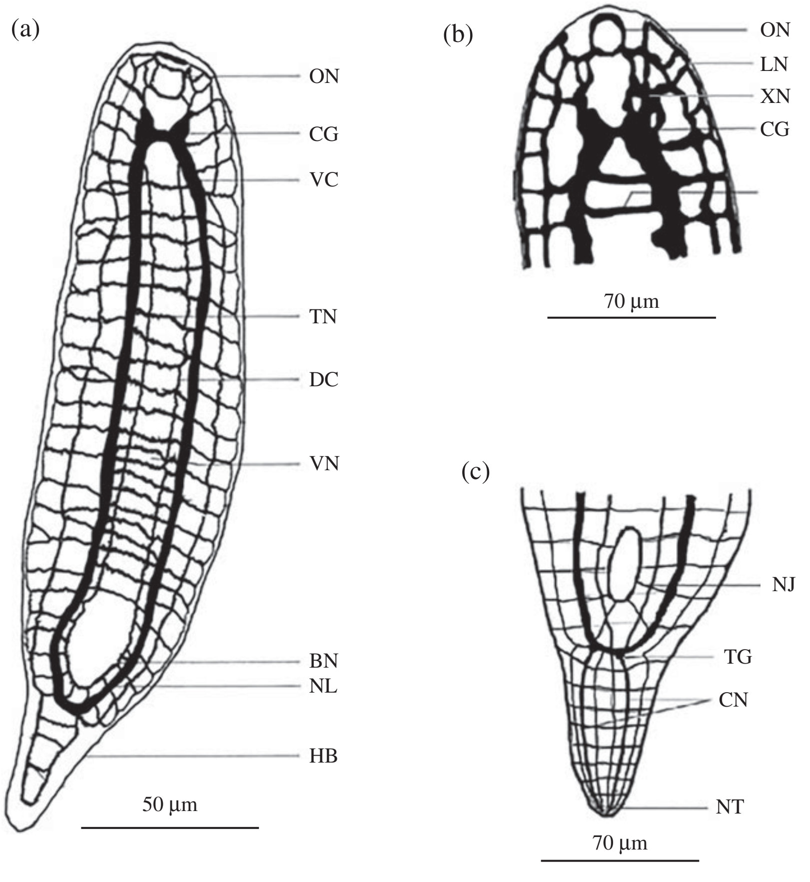

Fig. 2. The nervous system of the metacercaria of *Tylodelphys* sp. 2 from *Clarias gariepinus* to show (a) the entire body, (b) the forebody and (c) the hindbody. NJ, nerves connecting Brandes organ. See Fig. 1 for the key to the rest of the lettering.

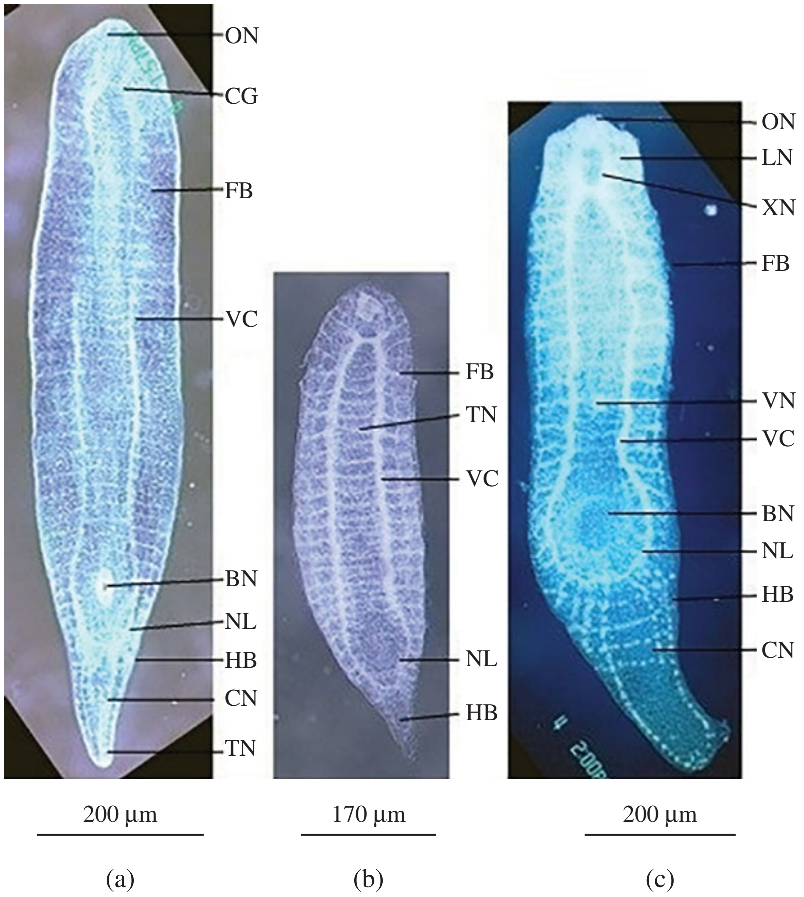

Fig. 3. Inverted photographs of entire (a) *Tylodelphys* sp. 1, (b) *Tylodelphys* sp. 2 and (c) *Tylodelphys mashonense* from *Clarias gariepinus* to show the cephalic and caudal nervous systems. FB, forebody. See Fig. 1 for the key to the rest of the lettering.

posteriorly to form a loop around the Brandes organ. The ChE activity also showed two minor dorsal longitudinal nerve cords (DC) and two peripheral longitudinal nerves (PN). The considerably thin dorsal nerve cords are connected to the lateral nerve cords via dorsolateral connectives, while they are linked to the ventral nerve cords via dorsoventral connectives. The longitudinal nerve cords are joined by transverse commissures (TN). However, there are more transverse nerves in *Tylodelphys* sp. 1 (30) than in *Tylodelphys* sp. 2 (21). In both species the central transverse nerves (TN) are more concentrated on the ventral sucker region. The ChE activity showed more nerves in the hindbody of *Tylodelphys* sp. 1 than in the *Tylodelphys* sp. 2. Both species have caudal ganglia (TG) that coordinate the hind nerve to the nerve loops. The anterior and suprapharyngeal nerves supplying the oral region also vary: in *Tylodelphys* sp. 2 ChE activity showed a network of nerves supplying the oral region while in *Tylodelphys* sp. 1 there are nerves from the cerebral ganglia supplying the oral region, as in *T. mashonense*.

Metacercariae of Tylodelphys mashonense

The nervous system of metacercariae of *T. mashonense* (Figs 3 and 4) is composed of two thick and prominent longitudinal ventral nerve cords (VC) originating from the cerebral ganglia, running posteriorly, one on each side of the body, and joining to form a loop around the Brandes organ. The longitudinal nerve cords are connected by 16 transverse commissures (TN). The ventral nerve cords also communicate with the lateral nerve cords by means of ventrolateral connectives. Two considerably thick nerve cords (LN and ON) arise from the cerebral ganglia and run anteriorly, to innervate the pseudosuckers and the oral region, respectively. The ventral sucker is innervated by nerves branching from the 11th transverse nerve (VN). In the hind body, two considerably thin branches of nerves arise, one from each ventral nerve cord, at the holdfast region, and re-connect at the level of the excretory pore. Two other thin branches originate dorsally from the posterior region of the ventral nerve cord loop (NL) and join the ventral thin cord at the excretory pore. Seven ventral transverse nerves connect the ventral and dorsal nerve cords.

Discussion

The application of the s-acetylcholine iodide staining technique was crucial for determining the arrangement of

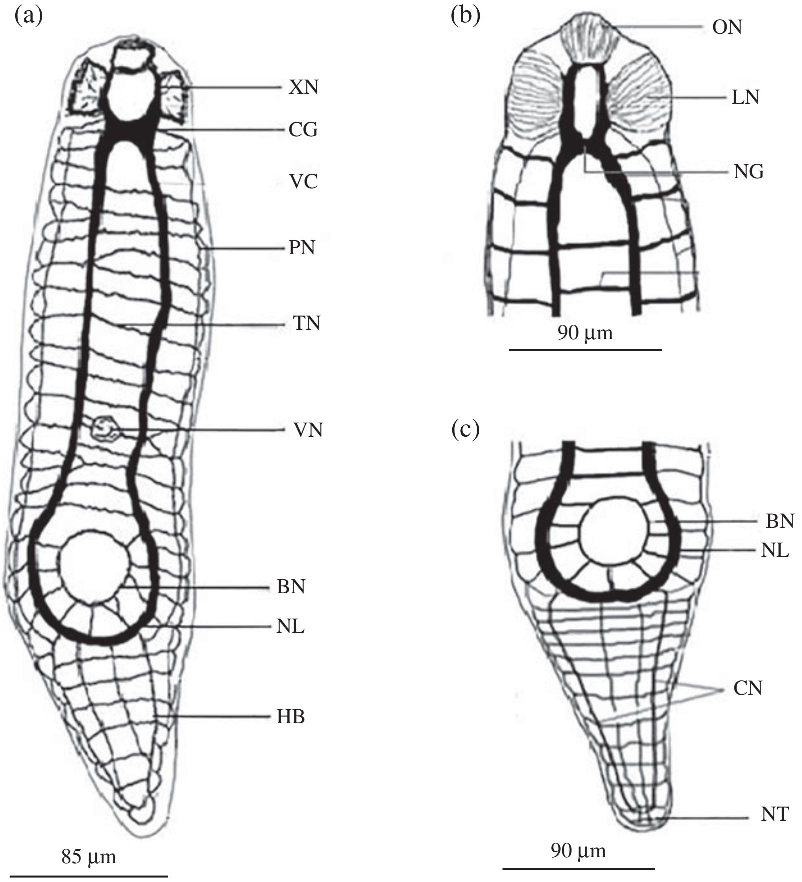

Fig. 4. The nervous system of the metacercaria of *Tylodelphys mashonense* from *Clarias gariepinus* to show (a) the entire body, (b) the forebody and (c) the hindbody. See Fig. 1 for the key to the lettering.

the nervous system of the diplostomid metacercariae infecting the catfish, *C. gariepinus*. As in other studies on digeneans (see Niewiadomska & Moczon, 1982, 1984, 1987, 1990; Halton *et al.*, 1992; Pax & Bennett, 1992; Gustafsson *et al.*, 2002; Arafa *et al.*, 2007), it was shown to be an 'orthogon', a rectilinear, ladder-like configuration of longitudinal nerve cords connected at intervals by transverse ring commissures. The structure and functions of the nervous system in *Diplostomum* species have been documented in detail by Niewiadomska & Moczon (1982, 1984, 1987, 1990). Niewiadomska & Moczon (1982, 1984), studying the nervous system of *D. pseudospathaceum*, concluded that the structure of the nervous system in the metacercaria was modified from that of the cercaria, which consisted of the cerebral ganglia and three pairs of stems joined by eight commissures, and that it grew with the increasing body dimensions of the metacercaria after penetration of the intermediate host. The nervous system differentiates and forms numerous commissures simultaneously with differentiation of the metacercarial body. This suggests that the observed differences in the nervous system of the three metacercariae *Tylodelphys* spp. 1 and 2 and *T. mashonense* described here reflect three systems of three different cercariae yet to be described.

The nervous system of the metacercaria of *T. mashonense*, as described here, closely resembles that of *D. pseudospathaceum* studied by Niewiadomska & Moczon (1984). Nerves are more distributed to the vital organs such as the oral and ventral suckers (ventral surface), to enable the metacercariae to exercise the needs of a parasitic life (attachment). The significance of the concentration of nerves on the ventral surface is better explained by the features revealed by chaetotaxy and scanning electron microscopy (SEM), i.e. the papillae on *Diplostomum* species (Niewiadomska & Moczon, 1984; Field & Irwin, 1995; McKeown & Irwin, 1995). The SEM of *T. mashonense* was undertaken by Nkwengulila (1995), who reported an increased number of papillae on the ventral surface and around vital organs such as the Brandes organ, oral and ventral suckers. The findings of the present study are consistent with Nkwengulila's (1995) findings. Both chaetotaxy and SEM studies have shown that in *Diplostomum* species a greater number of sensillae (papillae), which are thought to be tango- or chemoreceptors, occurs on the ventral surface, most likely due to the need to have close contact with the host.

However, *Tylodelphys* spp. 1 and 2 and *T. mashonense* show some differences in the number and the compactness of transverse commissures in comparison to the descriptions of *D. pseudospathaceum* by Niewiadomska & Moczon (1984). In the material under study, the transverse commissures are more compacted, especially in *Tylodelphys* spp. 1 and 2, and possess a large nerve loop joining the ventral longitudinal cords. It is from this loop that the nerves innervating the hind bodies originate. However, in both *T. mashonense* (present study) and *D. pseudospathaceum* (Niewiadomska & Moczon, 1984) nerves also innervate the pseudosuckers.

There are too few studies on the nervous system of *Diplostomum* species and other flatworms in general for one to make comparisons. This has been attributed to the difficulty in studying flatworms (Halton *et al.*, 1992; Pax & Bennett, 1992). The small sizes of most parasitic flatworms, the physiological problems of maintaining them *in vitro* and difficulty in maintaining the histochemical reactions are the reasons for the difficulty in detailing the nervous system using light microscopy (Halton *et al.*, 1992; Pax & Bennett, 1992). Therefore, increased efforts need to be made to study the nervous system of more diplostomid species and other digeneans.

In conclusion, although the studies on the nervous systems of trematodes are difficult to perform under light microscopy, the present study has shown their usefulness in the delineation of species. This proposition is corroborated by the fact that the observed differences in the nervous system of the three metacercariae infecting *C. gariepinus* were enough to clearly separate them into three species, i.e. *T. mashonense*, *Tylodelphys* spp. 1 and 2, as reported using DNA methods (Mwita & Nkwengulila, 2010; Chibwana *et al.*, 2013). In addition, the presence of nerve networks around the pseudosucker regions of both *Tylodelphys* sp. 1 and *Tylodelphys* sp. 2, although these species lack pseudosuckers, is an indication that the absence of pseudosuckers could be an advanced evolutionary state in the family Diplostomidae.

Acknowledgements

We are indebted to the Departments of Chemistry and Microbiology and Biotechnology of the University of Dar es Salaam for permission to use some of their chemicals for preparation of cacodylate buffer.

Financial support

This work was supported financially by the Swedish International Development Cooperation Agency (SIDA) – Department for Research Cooperation (SAREC) through the Faculty of Science, University of Dar es Salaam, Tanzania.

Conflict of interest

None.

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