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Simultaneous occurrence of two new myxosporean species infecting the central nervous system of *Hypopygus lepturus* from Brazil

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ABSTRACT: This paper describes 2 new myxosporean species, *Henneguya lepturus* sp. nov. and *Thelohanellus lepturus* sp. nov., simultaneously infecting the brain and spinal cord of *Hypopygus lepturus* Hoedeman, 1962 (Teleostei, Hypopomidae) from the Brazilian Amazon (Roraima State). Several spherical cysts of varying dimensions (up to 135 µm) were microscopically observed. The myxospores of *H. lepturus* sp. nov. measured 25.8 µm in total length, having an ellipsoidal body (12.4 × 6.4 × 2.2 µm) and 2 equal tapering tails (13.4 µm in length). Each of the 2 pyriform polar capsules measured 4.4 × 1.6 µm and possessed a polar filament coiled in 8–9 turns. The myxospores of *T. lepturus* sp. nov. were pyriform, formed by 2 equal valves (17.7 × 9.1 × 4.3 µm) surrounding a single polar capsule (10.9 × 3.5 µm) that had a coiled polar filament with 13–16 turns and a binucleated sporoplasm that contained several circular sporoplasmosomes. Molecular analysis of the small subunit (SSU) rRNA gene sequences of these 2 species were in agreement with the taxonomic classification derived from the ultrastructure of the myxospores. Histopathology of the host tissue showed degradation of the myelinated axons surrounding the cysts of both species, with the hosts displaying behavioural changes and erratic movements when observed in an aquarium.

KEY WORDS: *Henneguya lepturus* sp. nov. · *Thelohanellus lepturus* sp. nov. · Myxozoa · Brain · Spinal cord · Ultrastructure · Phylogeny · SSU rRNA gene · Histopathology

INTRODUCTION

The Amazon basin is the largest and most diverse freshwater ecoregion in the world (Abell et al. 2008, Reis et al. 2016) with a huge variety of fish species encompassing about 57 families, 515 genera and 2411 species (Reis et al. 2016). Numerous myxosporean species (phylum Cnidaria Hatschek, 1888; sub-phylum Myxozoa Grassé, 1970) have been reported infecting Brazilian ichthyofauna, mainly members of the genera *Henneguya* and *Myxobolus* (Pavanelli et al. 2013). Many of these descriptions, however, have only light micrograph and schematic illustrations available. In the last 2 decades, efforts

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have been made to provide ultrastructural data for developmental stages and mature myxospores (Azevedo & Matos 1996, Casal et al. 2003, Adriano et al. 2005, Azevedo et al. 2008, 2009, 2011a), while using molecular and phylogenetic tools to support the classification of the new species (Carriero et al. 2013, Azevedo et al. 2014, Rocha et al. 2014a,b).

Despite the large number of known myxosporean species (about 2180 assigned to a total of 62 genera), only a few have been reported from the nervous tissue (Lom & Dyková 1992, 2006, Moles & Heifetz 1998, Levsen et al. 2004, Eiras & Adriano 2012, Zhang et al. 2013, Scott et al. 2015). There are some occurrences in multivalvulids belonging to the genus *Kudoa* (Langdon, 1990, Grossel et al. 2003, Burger et al. 2007, Meng et al. 2011, Yokoyama 2017), as well as in some genera of myxobolids. Parasitosis of the brain caused by *Myxobolus* spp. has been reported in freshwater fish from different habitats, but mainly affect salmonid species (Kent & Hoffman 1984, Lorz et al. 1989, Langdon 1990, Moles & Heifetz 1998, Levsen et al. 2004, Hogge et al. 2008, Urawa et al. 2009, Scott et al. 2015). Few descriptions have also been made from *Henneguya* species infecting the nervous tissue, most from the Brazilian fauna (Azevedo et al. 2008, 2011a, Camus et al. 2017).

Henneguya species have been described infecting the nervous tissue, accounting for a total of 4 species reported (Eiras 2002, Eiras & Adriano 2012). In India, *H. thermalis* was reported in the brain of *Lepidocephalichthys thermalis* (Seenappa et al. 1981), and another 3 species have been reported in Brazilian freshwater fish. Specifically, the parasite *H. theca* has been described in *Eigenmannia virescens*, an electric glass knifefish that is spread across all of South America and intensively marketed as an ornamental fish (Kent & Hoffman 1984). From the Amazonian fauna, the myxospores of *H. rondoni* have been found in the peripheral lateral nerves located below the 2 lateral lines of *Gymnorhamphichthys rondoni* (Azevedo et al. 2008), and the myxospores of *H. torpedo* have been reported to infect the central nervous system (CNS) of *Brachyhypopomus pinnicaudatus* (Azevedo et al. 2011a).

Other than the genera *Henneguya* and *Myxobolus*, species of another 2 genera of the family Myxobolidae have been reported in freshwater Brazilian fauna: *Thelohanellus marginatus* in the gills of *Hypophthalmus marginatus* (Rocha et al. 2014b), *Thelohanellus* sp. in the liver of *Colossoma macropomum* (Videira et al. 2016), and *Tetrauronema desaequalis* in the ventral fins of *Hoplias malabaricus* (Azevedo & Matos 1996).

Histopathological damage of the nervous tissues and cartilage have has frequently correlated to parasitic infections by myxosporeans and microsporidians, with consequent behavioural alterations, especially in juvenile hosts (Lom & Dyková 1992, Matthews et al. 2001, Levsen et al. 2004, Scott et al. 2015). Parasites of other taxonomic groups, such as trematodes, have also been correlated to behavioural changes when parasitizing the CNS of fish (Lafferty & Morris 1996).

In the present study, information obtained from light and transmission electron microscopy, as well as from phylogenetic analyses, is presented for the myxospores of 2 new species of different genera (*Henneguya* and *Thelohanellus*) infecting the CNS of a teleost fish, which has commercial interest as an ornamental species (Wanderley Peixoto et al. 2013). This study represents the first report of a simultaneous infection of the CNS by 2 species of the phylum Cnidaria and sub-phylum Myxozoa, and further constitutes the first report of a myxoparasitosis in the aquatic fauna of the State of Roraima, Brazil. Behavioural alterations shown by the infected specimens are discussed and correlated to the histopathological damage induced by the parasite's development in the CNS.

MATERIALS AND METHODS

Fish and parasite sampling

Twenty-seven wild specimens of *Hypopygus lepturus* Hoedeman, 1962 (family Hypopomidae) (common name: sand knifefish, and Brazilian common name 'Sarapó'), with a total length varying between 53 and 75 mm were collected in the 'Wai Grande Igarapé' (02° 45' N, 60° 45' W), near the city of Boa Vista (capital of Roraima State), Brazil, and were transported and maintained alive for 3–5 d in the aquarium, with aeration, at the 'Laboratory of Applied Zoology of Agricultural Sciences Center' of the Federal University of Roraima. During this period, the behaviour of the fishes was carefully analysed, permitting the observation of 2 distinct situations: the majority of the infected fish, having been placed together in one aquarium, exhibited behavioural change, showing sudden and erratic movements, and sometimes colliding with the glass walls. The fish showing apparently normal behaviour were maintained in a separate aquarium. After being anaesthetized and euthanized by aqueous immersion in a 10% alcoholic solution of Eugenol, each fish was

microscopically analysed for parasitological study focused on the presence of cysts. The animal procedures and handling were performed in accordance with the European guidelines on animal welfare (Directive 2010/63/EU on the protection of animals used for scientific purposes; Brazilian guidelines are similar).

Light microscopy (LM)

Several organs (muscles, gills, digestive tube, liver and gallbladder, kidney, urinary bladder, brain and spinal cord [i.e. the CNS]) were observed for microparasitological analysis, however, only the CNS appeared infected. Smears of small fresh fragments of brain and spinal cord collected from the periphery of the white matter tissues, containing cysts and free myxospores, were prepared for observation by LM using Nomarski differential interference contrast optics. The observed myxospores were morphologically identified by comparison with myxospores belonging to the genera *Henneguya* and *Thelohanellus*, and measured with an ocular micrometer adapted to the photomicroscope.

Transmission electron microscopy (TEM)

For TEM studies, small fragments of the peripherical tissues of the CNS (brain and spinal cord) containing cysts and free myxospores were isolated and fixed in 4–5% glutaraldehyde with a sodium cacodylate buffer 0.2 M (pH 7.2) for 20–24 h at 4°C, washed overnight in the same buffer at 4°C and postfixed in 2% OsO_4 buffered with the same solution for 3–4 h at the same temperature. The infected fragments of CNS and isolated myxospores were dehydrated in an ascending ethanol series, followed by propylene oxide, and embedded in Epon. Semithin sections were stained with methylene blue, and ultrathin sections were double-contrasted with uranyl acetate and lead citrate, and observed and photographed in a JEOL 100 CXII TEM (JEOL Optical), operated at 60 kV.

DNA extraction, amplification and sequencing

Prior to DNA extraction, cysts were isolated from the brain and spinal cord tissues, and observed one by one, in order to identify the genus of the parasite contained in each of the cysts. Myxospores extruded from identified cysts were fixed separately according to the genus, and preserved in absolute ethanol at 4°C. Genomic DNA extraction was performed using a GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich), following the manufacturer's instructions. The DNA was stored in 50 µl of TE buffer at −20°C until further use.

The SSU rRNA gene sequences were amplified using both universal primers and myxosporean-specific primers: the 5′-end with the pair of primers 18e / ACT3r; the 3′-end with ACT3f / 18r; and the overlapping sequence by pairing the primers MyxospecF / 18r (Table 1). PCRs were performed in 50 µl reactions using 10 pmol of each primer, 10 nmol of each dNTP, 2.5 mM $MgCl_2$, 5 µl 10× *Taq* polymerase buffer, 1.5 units of *Taq* DNA polymerase (Nzytech), and 3 µl (approximately 100–150 ng) of genomic DNA. The reactions were run on a Hybaid P×E Thermocycler (Thermo Electron), with initial denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 90 s. The final elongation step was performed at 72°C for 7 min. Aliquots of 5 µl of the PCR products were electrophoresed through a 1% agarose 1× Tris-acetate-EDTA buffer gel stained with ethidium bromide. PCR products were purified using a single-step enzymatic clean-up that eliminated unincorporated primers and dNTPs by means of the ExoFast method.

The PCR products from different regions of the SSU rRNA sequences were sequenced directly in the same condition as reported by Rocha et al. (2014a).

Table 1. Sequences of the primers used to amplify the SSU rRNA gene of *Henneguya lepturus* sp. nov. and *Thelohanellus lepturus* sp. nov.

Name	Sequence (5′–3′)	Paired with	Reference(s)
18e	CTG GTT GAT CCT GCC AGT	ACT3r	Hillis & Dixon (1991)
MyxospecF	TTC TGC CCT ATC AAC TTG TTG	18r	Fiala (2006)
ACT3f	CAT GGA ACG AAC AAT	18r	Hallett & Diamant (2001), Rocha et al. (2014a)
ACT3r	ATT GTT CGT TCC ATG	18e	Hallett & Diamant (2001)
18r	CTA CGG AAA CCT TGT TAC G	ACT3f, MyxospecF	Whipps et al. (2003)

Distance and phylogenetic analysis

To determine the phylogenetic position of the parasites amongst their closest relatives sequenced to date, namely myxobolids, 74 myxosporean SSU rRNA gene sequences were obtained from GenBank and analysed, including those with the highest similarity score. *Tetracapsuloides bryosalmonae* (U706-23) was selected as outgroup. Phylogenetic and molecular evolutionary analyses were conducted using MEGA 7.0.9 (Kumar et al. 2016).

Alignments were performed using the 'Multiple Alignment using Fast Fourier Transform' (MAFFT) with default parameters (Katoh & Standley 2013). The phylogenetic analysis was performed using maximum likelihood (ML) methodology in MEGA 7.0.9 software. For ML, the general time-reversible substitution model was performed with 4 gamma-distributed rate variations among sites. All positions with less than 75% site coverage were eliminated from the tree. The bootstrap consensus tree was inferred from 500 replicates.

Distance estimation was performed for a second alignment of the SSU rRNA gene sequences clustering together with the *H. lepturus* sp. nov. and for all *Henneguya* spp. that have South American freshwater fish as hosts. This analysis was also carried out in MEGA 7.0.9, using the *p*-distance model and all ambiguous positions were removed for each sequence pair.

RESULTS

Light and ultrastructural aspects

During captivity, observations showed 8 of the 27 specimens (~29.6%) displaying different grades of disorientation, including erratic and disturbed movements. The organs and tissues of the specimens showing abnormal behaviour were examined microscopically, but only the brain and spinal cord tissues were infected. Necropsy and parasitological survey of the specimens showing normal behaviour revealed the CNS apparently without parasitic infection. In cases of severe infections of the nervous tissues, several spherical cysts, up to ~135 µm, were observed randomly distributed in the peripheral tissues of the white matter of the brain and spinal cord (Fig. 1), with the infected specimens presenting conspicuous behavioural alterations. In cases of mild infections, the specimens exhibited slight behavioural changes, which could easily pass unnoticed. In semithin serial sections (Fig. 1C–E) it was observed that each cyst contained 1 of 2 types of myxospores; once the cysts ruptured, these were morphologically identified as belonging to the genera *Henneguya* (Fig. 2A) and *Thelohanellus* (Fig. 2B). It was observed that the smallest cysts measuring approximately 40 to 70 µm contained only myxospores of *Thelohanellus* sp., while the largest, measuring about 90 to 135 µm, contained only myxospores of *Henneguya* sp. Few disporic pansporoblasts and isolated myxospores were found among the CNS tissues, and were identified as belonging to 1 of these 2 genera (Fig. 2C). Five hosts were infected with only 1 of the 2 species, of which 2 fish were only infected with *Thelohanellus* sp. and 3 fish were infected by *Henneguya* sp.

TEM observations were congruent with the identification performed from LM (Figs. 2C,D & 3A). Some cysts were observed in close proximity, with their walls contacting and appearing to have intermingled cellular components (Figs. 1D–F & 2D). The walls of the cysts of both genera appeared similar, formed by a thick layer of fibroblasts externally surrounded by a light area of nervous tissues having several vacuoles and structures such as the axon and myelin sheaths, which appeared to present a certain degree of degradation (Fig. 3D,E). In serial semithin sections it was possible to see that the cysts containing myxospores of *Henneguya* sp. were bigger than the cysts containing *Thelohanellus* sp. No inflammatory response was apparent, but significant degradation and ultrastructural disorganization of the myelin sheaths was evident in the separation of the layers of myelin (Figs. 1C & 3D,E). These regions also showed numerous light areas with several vesicles and vacuoles among the axons (Figs. 1E, 2D & 3D,E). The myelinated axons located around the cysts also showed evident degradation of the layers of myelin membranes, characterized by disorganization and disarrangement of the myelin layer (Fig. 3D,E). Curiously, the transverse sections of the axons contained more mitochondria than the regions more distant from the cysts (Fig. 3E).

The schematic drawings in Fig. 4 obtained from the light microscopic observations and the serial ultrathin sections, show the typical morphological and ultrastructural aspects of the myxospores of *H. lepturus* sp. nov. (Fig. 4A) and *T. lepturus* sp. nov. (Fig. 4B).

Taxonomic placement

Phylum: Cnidaria Hatschek, 1888
Sub-phylum: Myxozoa Grassé, 1970
Class: Myxosporea Bütschli, 1881

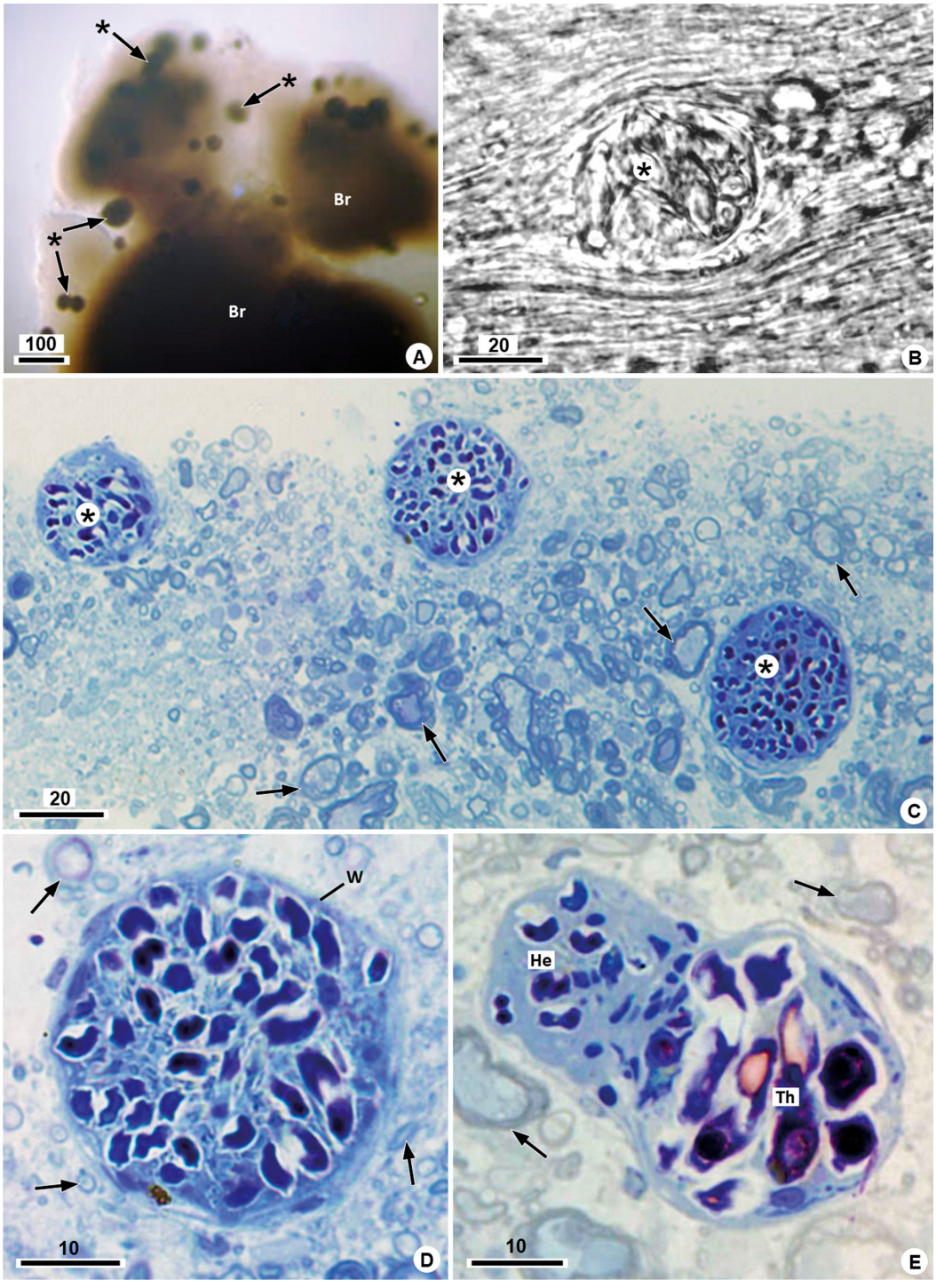


Fig. 1. Light micrographs of cysts of *Henneguya lepturus* sp. nov. and *Thelohanellus lepturus* sp. nov. found in the central nervous system (brain and spinal cord) of the teleostean *Hypopygus lepturus*. Scale bars in µm. (A) Small fresh fragment '*in toto*' of the brain (Br) showing several cysts located at the periphery (*). (B) Small fragment of fresh spinal cord showing a cyst of *Thelohanellus* (*) located among the periphery of the nervous tissues. (C) Semithin section of 3 cysts (*) located among the periphery of the myelin axons (arrows) of the spinal cord, each containing either myxospores of *T. lepturus* sp. nov. or *H. lepturus* sp. nov. (D) Semithin section of a cyst showing myxospores of *H. lepturus* sp. nov. sectioned at different levels. The cyst's wall (w) contacts directly with the nervous tissues (arrows). (E) Semithin section of 2 juxtaposed cysts, one containing myxospores of *T. lepturus* sp. nov. (Th), and the other one myxospores of *H. lepturus* sp. nov. (He). The area surrounding the cysts shows several axons (arrows)

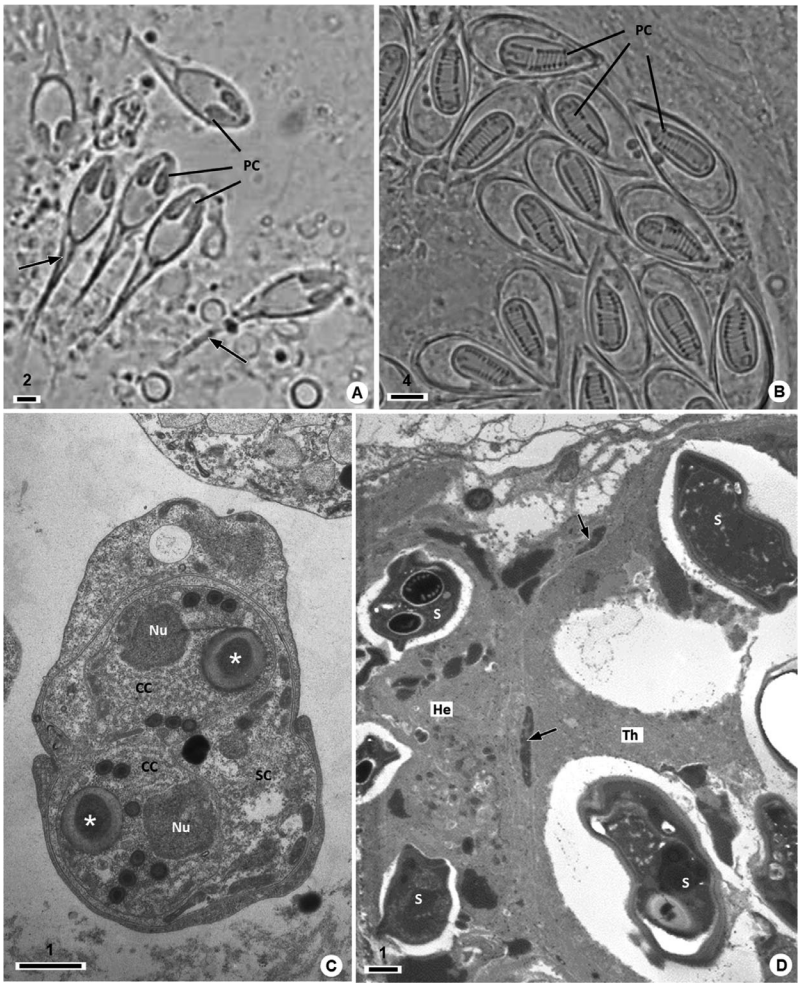


Fig. 2. Light and electron micrographs showing some aspects of the myxospores and cysts of *Henneguya lepturus* sp. nov. and *Thelohanellus lepturus* sp. nov. Scale bars in µm. (A,B) LM images of the myxospores of the reported species of *Henneguya* and *Thelohanellus*, respectively, showing their typical morphology, including the organization of the polar capsules (PC) and tails (arrows). (C) Ultrathin section of a sporoblast (SC: sporoplasm cell) of *Henneguya* displaying the capsulogenic cells (CC), each showing a capsular primordium (*) and the respective nucleus (Nu). (D) Ultrastructural image of the periphery of 2 juxtaposed cysts, one containing myxospores (S) of *Henneguya* (He), and the other myxospores of *Thelohanellus* (Th), and showing the presence of some fibroblasts between them (arrows)

Order: Bivalvulida Shulman, 1959
Family: Myxobolidae Thélohan, 1892
Genus: *Henneguya* Thélohan, 1892
Species: *H. lepturus* sp. nov.
Genus: *Thelohanellus* Kudo, 1933
Species: *T. lepturus* sp. nov.

Descriptions

H. lepturus sp. nov.

Myxospores of this new species were contained within spherical cysts measuring ~90–135 µm across, randomly distributed throughout the peripheral tissues of white matter of the supra-posterior portion of the brain, and along the outermost portion of the white matter of the spinal cord (Fig. 1A,C–E). The myxospore bodies were ellipsoidal, formed by 2 equal valves, each with a tapering tail and enclosing 2 ellipsoidal polar capsules and a binucleated sporoplasm with sporoplasmosomes. Mature fixed myxospores had a total length (mean ± SD, full range in brackets) of 25.8 ± 0.8 (25.1–26.7) µm, a body length of 12.4 ± 0.7 (11.2–12.1) µm, width 6.4 ± 0.4 (6.0–6.9) µm, and thickness 2.2 ± 0.2 µm (n = 30) (Figs. 2A & 4A). The polar capsules were ellipsoidal with a total length of 4.4 ± 0.5 (3.9–4.8) µm and a width of 1.6 ± 0.3 (1.3–2.0) µm, each displaying a polar filament coiled in 7–9 coils (Figs. 3A & 4A). Valves were thin and smooth, each with a caudal projection forming a tail with a total length of 13.6 ± 0.8 (13.0–14.5) µm (n = 30). Each tail was composed of an electron-dense material, similar to that of the valves (Figs. 2A, 3A & 4A).

T. lepturus sp. nov.

Myxospores with the morphological characters of the genus *Thelohanellus* Kudo, 1933 were observed within spherical cysts, measuring ~40–70 µm, located in the peripheral white matter tissue of the brain and

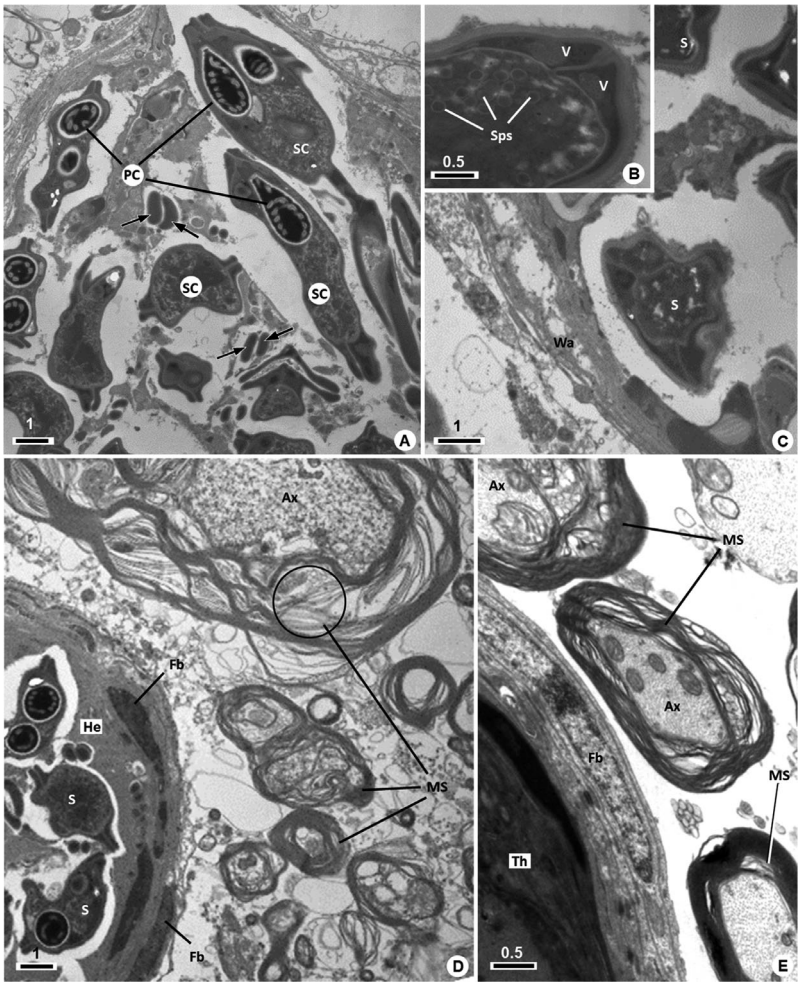


Fig. 3. TEM micrographs showing some features of the cysts containing myxospores of *Henneguya lepturus* sp. nov. and *Thelohanellus lepturus* sp. nov. Scale bars in µm. (A) Periphery of a cyst of *H. lepturus* sp. nov. containing myxospores sectioned at different levels and showing the polar capsules (PC) and the sporoplasm cell (SC). (B) Part of a myxospore of *T. lepturus* sp. nov. showing its valves (V) and the sporoplasm cell with several sporoplasmosomes (Sps). (C) Section of a cyst showing its wall (Wa) and containing myxospores (S) of *T. lepturus* sp. nov. (D) Periphery of a cyst of *H. lepturus* sp. nov. (He) showing some myxospores (S) and its wall formed by some layers of fibroblasts (Fb). Near the cyst wall some axons (Ax) show evident disorganization and degradation of the myelin sheaths (MS). (E) Detail of the interface between the cyst wall of *T. lepturus* sp. nov. formed by fibroblasts (Fb), and the surrounding axons (Ax), which myelin sheaths (MS) display alterations and degradation of its concentric layer membranes

spinal cord (Fig. 1A,B,E). Fixed myxospores presented 2 equal valves without extensions (without tails), having a total length of 17.7 ± 0.6 (17.2–18.3) µm, width 9.1 ± 0.6 (8.6–9.7) µm (n = 30), and thickness 2.3 ± 0.4 µm (n = 15). The single polar capsule was 10.9 ± 0.5 (2.2–2.7) µm long and 4.3 ± 0.4 (3.0–4.0) µm wide (n = 30), its wall was ~0.3 nm thick, and the polar filament coiled forming a single row of 13–16 turns (Figs. 2B & 4B). The binucleated sporoplasm was located in the posterior pole of the myxospore and contained 2 nuclei and several sporoplasmosomes. These were globular electron-dense vesicles, with a circular section ~0.2 µm in diameter (Fig. 3B).

Characters common to these two myxosporean species

Type host: the freshwater fish *Hypopygus lepturus* Hoedeman, 1962 (Teleostei, Gymnotiformes, Hypopomidae) (Brazilian common name: 'Sarapó').

Type locality: 'Wai Grande Igarapé' (02° 45' N, 60° 45' W), near the city of 'Boa Vista' (capital of Roraima State), Brazil.

Type of infection: cysts located in the CNS (brain and spinal cord) tissues, randomly distributed throughout the peripheral white matter tissues of these organs.

Prevalence: eight in a total of 27 specimens (~29.6%) presented infected. Two specimens only infected with *Thelohanellus lepturus* sp. nov. (~7.4%) and 3 infected with *Henneguya lepturus* sp. nov. (~11.1%).

Etymology: the specific epithet (*lepturus*) of these two new species is derived from the specific epithet of the type host.

Type material: two glass slides with fixed and lightly stained myxospores (syntype), each containing myxospores of *H. lepturus* sp. nov. and *T. lepturus* sp. nov., were deposited in the International Protozoan Type Slide Collection of the Museum at the 'Instituto Nacional de Pesquisa da Amazônia' (INPA), Manaus, Brazil (INPA access no. 35 for *Henneguya* and no. 36 for *Thelohanellus*). The SSU rDNA sequences were

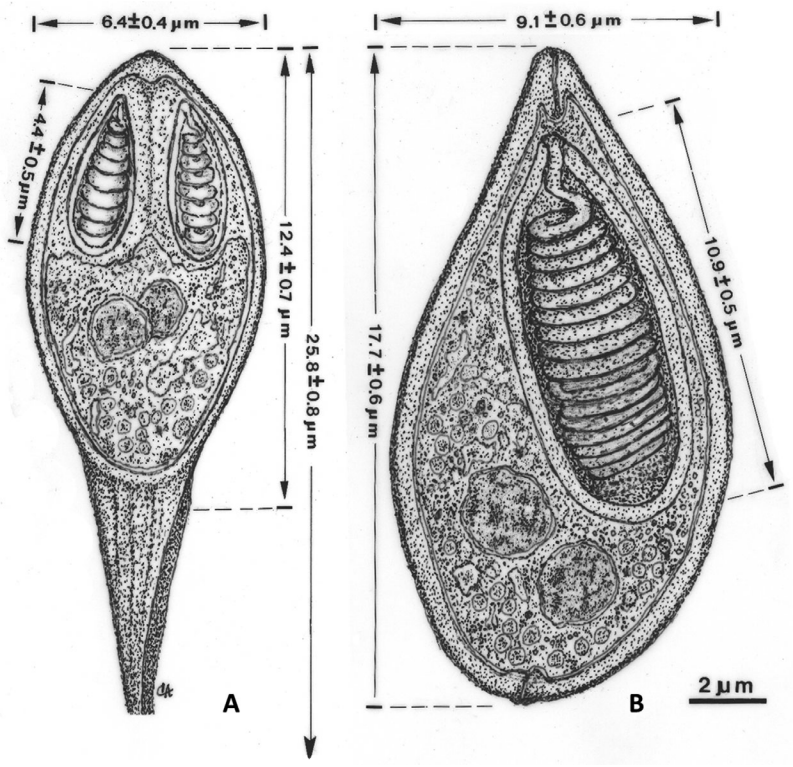


Fig. 4. Comparative semi-schematic drawings of the myxospore morphology of (A) *Henneguya lepturus* sp. nov. and (B) *Thelohanellus lepturus* sp. nov., as observed from light and serial ultrathin section data. Both presented in valvular view and with the same magnification

deposited in GenBank (accession no. MF765752 for *H. lepturus* sp. nov. and MF765753 for *T. lepturus* sp. nov.).

Histopathology: the external periphery of the cyst walls contained several lysed fibroblasts surrounded by an external light area containing several vesicles and vacuoles. The myelin sheaths of the axons located near the cysts showed evident disaggregation and disorganization of the myelin sheath layers.

Behaviour: in aquaria, most of the infected fish exhibited behavioural changes, showing sudden and erratic movements, sometimes colliding with the glass walls of the aquarium, followed by lethargy and congregation at the bottom of the aquaria. These behavioural alterations were not present in the specimens without parasitic infection.

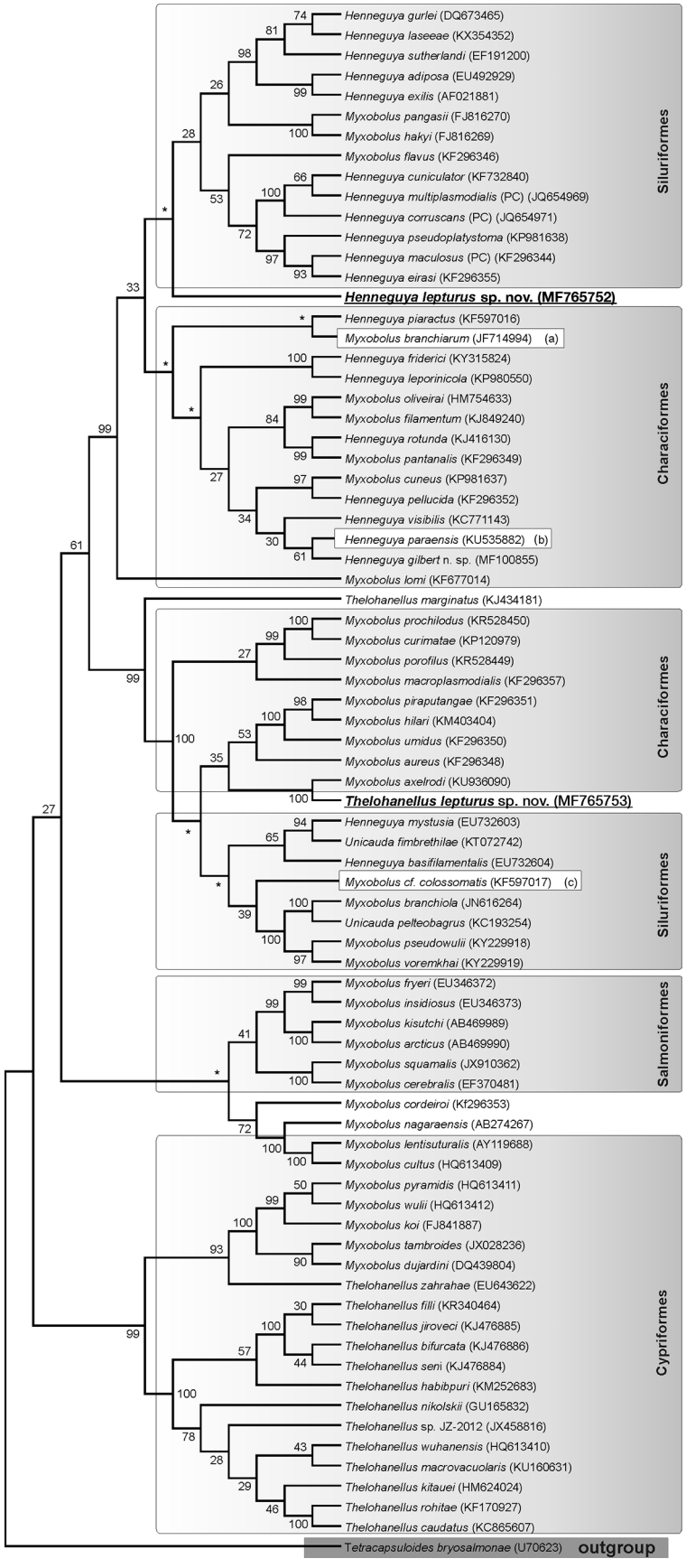
Phylogenetic analysis

Partial SSU rRNA gene sequences of each myxosporean species were obtained from the analysis of several cysts, resulting in a consensus DNA sequence of 1968 bp for *H. lepturus* sp. nov and 1970 bp for *T. lepturus* sp. nov. These sequences were deposited in GenBank with the accession numbers MF765752 and MF765753, respectively.

A BLAST search for similar sequences confirmed relationships to other myxospore sequences that parasitize freshwater fish from the Brazilian fauna. *H. lepturus* sp. nov. presents a high degree of similarity with *Myxobolus oliveirai* (HM754633), *H. maculosus* (KF296344), *H. friderici* (KY315824), and *H. pseudoplatystoma* (KP981638); while *T. lepturus* sp. nov. has a high degree of similarity with *M. axelrodi* (KU936091). In total, 74 SSU rRNA sequences of myxobolid parasites with freshwater teleost fish hosts from the Brazilian fauna and other geographic areas were aligned with *H. lepturus* sp. nov. and *T. lepturus* sp. nov., resulting in an alignment consisting of 2593 positions. For the phylogenetic tree, a total of 1437 positions were used in the final dataset.

The phylogenetic analysis showed 6 large clades with *H. lepturus* sp. nov. and *T. lepturus* sp. nov. positioned in distinct clusters (Fig. 5). In the majority of the phylogenetic trees obtained, *H. lepturus* sp. nov. occupied a basal position to the Siluriformes clade, which contains several *Henneguya* spp. that infect South American freshwater fish from the family Pimelodidae. However, in some phylogenetic trees, *H. lepturus* sp. nov. appeared closely related to *M. lomi* (KF677014), both being located in a basal position to the larger clade composed of myxobolids (*Myxobolus*/*Henneguya*) that infect fish belonging to the orders Characiformes and Siluriformes, with a bootstrap of 99%. On the other hand, *T. lepturus* sp. nov. was inserted within a clade composed of *Myxobolus* sp. that parasitize Characiformes species of the Brazilian fauna, displaying strong phylogenetic affinity (bootstrap 100%) to another parasite of the nervous tissue, *M. axelrodi* (KU936090). With the exception of *T. marginatus* (KJ434181) and *T. lepturus* sp. nov., all other *Thelohanellus* spp. have cyprinid hosts from North America and Eurasia and together constitute a well-supported clade.

For pairwise comparisons between the SSU rRNA sequences, a second alignment was performed containing only the *Henneguya* spp. from Brazilian fauna. All ambiguous positions were removed for



each sequence pair. The minimum genetic distance (*p*-distance) obtained was 19.1% to *H. friderici* (KY315824), which had only 1050 nt. All other analyzed sequences presented genetic distances greater than 20.0% (Table 2).

DISCUSSION

Microparasites belonging to different genera of the sub-phylum Myxozoa have been described occurring in almost all organs and tissues of fish. Considering the high number of described species, however, relatively few have been described infecting the nervous system (NS), and there have been even fewer cases of 2 different species of myxosporeans simultaneously occurring in the brain and spinal cord, as is described in the present work. Reports of myxosporean infection in the NS have been performed for species of the genera *Myxobolus*, *Henneguya* and *Kudoa* (Lom & Dyková 2006). The morphological differences observed between the myxospores found in the brain and spinal cord of *Hypopygus lepturus* easily identified them as belonging to 2 distinct genera: *Henneguya* and *Thelohanellus*. The latter has never before been reported infecting the central or peripheral NS (Zhang et al. 2013).

When comparing the characteristics of the 2 types of myxospores in this study with similar myxospores infecting the NS of fishes (i.e. morphology, measurements of spores and polar capsules, the number of coils and other details), it is clear that the parasites described herein could be considered as 2 new species.

Fig. 5. Maximum likelihood tree of the SSU rRNA gene sequences of *Henneguya lepturus* sp. nov., *Thelohanellus lepturus* sp. nov. and other selected myxobolid species. The numbers on the branches are bootstrap confidence levels for 500 replicates. GenBank accession numbers are in parentheses after the species names. Asterisks are shown for values under 25%. The order of the fish are indicated. In some clades, there are exceptions: (a) Centrarchiformes; (b) Perciformes; (c) Characiformes

Table 2. Comparison of some SSU rRNA gene sequences: pairwise distance obtained by *p*-distance using MEGA 7 software. The number of base differences per site between sequences is shown. All ambiguous positions removed for each sequence pair

Henneguya sp. (GenBank acc. no.)	nt	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
(1) *H. lepturus* sp. nov. (MF765752)	1968	–														
(2) *H. friderici* (KY315824)	1050	0.191	–													
(3) *H. leporinicola* (KP980550)	1954	0.207	0.070	–												
(4) *H. cuniculator* (KF732840)	1214	0.201	0.182	0.162	–											
(5) *H. paraensis* (KU535882)	888	0.211	0.179	0.147	0.172	–										
(6) *H. gilbert* (MF100855)	2012	0.211	0.163	0.162	0.152	0.142	–									
(7) *H. pseudoplatystoma* (KP981638)	1946	0.212	0.174	0.170	0.144	0.170	0.167	–								
(8) *H. piaractus* (KF597016)	1913	0.212	0.152	0.152	0.168	0.134	0.179	0.177	–							
(9) *H. corruscans* (JQ654971)	1913	0.214	0.180	0.172	0.048	0.185	0.187	0.140	0.184	–						
(10) *H. maculosus* (KF296344)	1930	0.215	0.155	0.157	0.133	0.152	0.170	0.103	0.164	0.153	–					
(11) *H. rotunda* (KJ416130)	1856	0.216	0.163	0.158	0.173	0.147	0.152	0.168	0.172	0.175	0.172	–				
(12) *H. visibilis* (KC771143)	1710	0.225	0.176	0.166	0.174	0.151	0.169	0.189	0.169	0.191	0.186	0.169	–			
(13) *H. pellucida* (KF296352)	1574	0.235	0.167	0.179	0.184	0.147	0.187	0.211	0.196	0.215	0.208	0.182	0.197	–		
(14) *H. multiplasmodialis* (JQ654969)	1560	0.238	0.184	0.193	0.018	0.181	0.192	0.168	0.208	0.075	0.172	0.202	0.214	0.213	–	
(15) *H. eirasi* (KF2963355)	1204	0.246	0.189	0.188	0.135	0.160	0.187	0.127	0.206	0.175	0.114	0.194	0.205	0.205	0.174	–

Morphological and ultrastructural comparisons

Comparison between the myxospores of *H. lepturus* sp. nov. and other *Henneguya* species, namely the 4 species previously reported infecting the NS, showed differences (Table 3). *Henneguya thermalis* was the first of its genus to be described from the CNS, more specifically occurring in the brain of the common spiny loach *Lepidocephalichthys thermalis* in India. Despite having similar dimensions to *H. lepturus* sp. nov., it possesses 2 asymmetric PCs (Seenappa et al. 1981). The other 3 species all parasitize fish belonging to the order Gymnotiformes from the Amazonian fauna. Two of them, *H. theca* (Kent & Hoffman 1984) and *H. torpedo* (Azevedo et al. 2011a), parasitize the CNS but differ significantly in their total size, being twice as long as the species presented in this work. Furthermore, these myxospores are externally surrounded by an electron-dense and a hyaline homogenous sheath, respectively. In addition, *H. rondoni*, which has been found infecting the peripheral NS of *Gymnorhamphichthys rondoni*, presents smaller myxospores than *H. lepturus* sp. nov., also being surrounded by a homogeneous hyaline layer (Azevedo et al. 2008).

According to the literature, there are more than 108 nominal species belonging to the genus *Thelohanellus*, occurring in different infection locations, habitats and geographic areas, although they infect predominantly the gills of freshwater cyprinid hosts in Asia (China and India) (Azevedo et al. 2011b, Zhang et al. 2013, Rocha et al. 2014b, Lewisch et al. 2015). Comparison between *T. lepturus* sp. nov. and other *Thelohanellus* spp. showed significant dissimilarities, either in terms of myxospore dimensions, host or locality of infection. In the Brazilian hydrographic network only 2 *Thelohanellus* spp. have been reported: *T. marginatus* in the gills of *Hypophthalmus marginatus* (Rocha et al. 2014b) (Table 4), and *Thelohanellus* sp. in the hepatic parenchyma of *Colossoma macropomum* (Videira et al. 2016). Morphologically, these myxospores have similar dimensions to *T. lepturus* sp. nov., but the organization of the polar filament differs considerably, as well as the host and locality of infection.

Normally, infections by *Henneguya* sp. and *Thelohanellus* sp. do not cause severe diseases and mortalities. Nonetheless, some are harmful, such as *H. ictaluri*, which is associated with proliferative gill disease, causing mortalities of up to 50% in fish stocks, e.g. the catfish *Ictalurus punctatus* in North America (Lovy et al. 2011). Furthermore, *T. hovorkai* can cause haemorrhagic thelohanellosis disease in

Table 3. Morphologic characteristics of the myxospores of *Henneguya lepturus* sp. nov. and other species of the same genera infecting the nervous system. All measurements in µm; mean ± SD, full range in brackets where available. SpL: myxospore length; SpBL: myxospore body length; SpBW: myxospore width; SpT: myxospore thickness; PCL × W: polar capsule length × width; PFc: polar filament coils; TaL: tail length; Sh: sheath surrounding the myxospores; L: larger; S: smaller; NS: nervous system

Henneguya sp.	Host (order; family)	Infection location	Country	SpL	SpBL	SpBW	SpT	PCL × W	PFc	TaL	Sh
H. thermalis Seenappa et al., 1981	*Lepidocephalithys thermalis* (Cypriniformes; Cobitidae)	Brain	India	~24.5	12.5 (1.0–13.0)	7.0 (0.0–8.0)	–	L – 4.5 × 2.5 S – 3.5 × 1.7	–	12 (1.0–13.0)	No
H. theca Kent & Hoffman, 1984	*Eigemannia viriscens* (Gymnotiformes; Sternopygidae)	Brain	Brazil	48.0 (40.6–52.0)	24.8	3.5 (3.0–4.1)	–	L – 11.1 × 1.4 S – 10.4 × 1.4	–	23.2 (20.3–24.2)	Yes
H. rondoni Azevedo et al., 2008	*Gymnorhamphichthys rondoni* (Gymnotiformes; Rhamphichthyidae)	Peripheral NS	Brazil	17.7 (16.9–18.1)	7 (6.8–7.3)	3.6 (3.0–3.6)	–	2.5 × 0.8	6–7	10.7 (10.3–11.0)	Yes
H. torpedo Azevedo et al., 2011a	*Brachyhypopomus pinnicaudatus* (Gymnotiformes; Hypopomidae)	Brain and spinal cord	Brazil	48.6 ± 0.5 (48.3–48.9)	28.5 ±0.4 (28.3–30.1)	7.2 ± 0.3 (7.0–7.5)	3.1 ± 0.3	6.4 × 1.8	5–6, rarely 7	19.6 ± 0.4 (19.2–19.9)	Yes
H. lepturus sp. nov.	*Hypopygus lepturus* (Gymnotiformes; Hypopomidae)	Brain and spinal cord	Brazil	25.8 (25.1–26.7)	12.4 (11.2–12.1)	6.4 (6.0–6.9)	2.2	4.4 × 1.6	7 – 9	13.6 (13.0–14.5)	No

common carp *Cyprinus carpio* (Yokoyama et al. 1998), and *T. kitauei* is the causative agent of intestinal giant-cystic disease (Egusa & Nakajima 1981).

Behavioural alterations have been correlated to myxozoan infection in the hosts' CNS, even in the absence of inflammation response (Khoo et al. 2010, Camus et al. 2017). In salmonids, infections with *Myxobolus articus* have been associated with a decrease in swimming speed (Moles & Heifetz 1998); infections of *M. neurophius* in *Perca flavescens* apparently cause abnormal swimming behaviour and bouts of hyperexcitability (Guilford 1963, Khoo et al. 2010); and infections with *M. balantiocheili* are considered to be at the origin of severe neurological symptoms in the tropical fish tricolor sharkminnow *Balantiocheilos melanopterus* (Levsen et al. 2004). However, these behavioural alterations are not always subsequent to infections that specifically target the CNS. For instance, the anatomic alterations induced by the proliferation of *M. cerebralis* in cartilage can lead to compression of the brain and vertebral column in juvenile salmonids, causing the condition known as whirling disease. Furthermore, its migratory route from the skin to the cartilage involves dissemination in the peripheral and central nervous tissues, which causes damage to the nervous system (Lorz et al. 1989, Lom & Dyková 1992, El-Matbouli et al. 1999, Rose et al. 2000). Similar behavioural alterations have also been associated with microsporidian infections established in the CNS. In zebrafish *Danio rerio,* an important aquarium fish widely used as vertebrate model organism in scientific research, the microsporidium *Pseudoloma neurophilia* infects the motor neurons and ventral spinal cord, causing myositis and muscle atrophy that lead to spinal deformities and emaciation (Matthews et al. 2001, Spagnoli et al. 2015, 2017).

In this study, the presence of a high number of cysts in contact with the myelin sheaths of white matter of the axon of the brain and spinal cord, combined with the observation of alterations in the behaviour of infected fish, suggest that the described parasites have a pathogenic effect. This correlation was similarly found by Frasca et al. (1998), who explained the behavioural alterations observed as probably resulting from degradation of the myelin sheaths of the axons located near the cysts, as well as the consequence of a compressing of the NS. In the present study the infected specimens presented an erratic swimming behaviour and evident ultrastructural disorganization of the classic concentric layers of membranes of the myelinated axons located close to the cysts, which is consistent with findings reported in

Table 4. Morphologic characteristics of *Thelohanellus* sp. from the Brazilian fauna. All measurements in µm; mean ± SD, full range in brackets where available. SpBL: myxospore length; SpBW: myxospore width; SpT: myxospore thickness; PCL × W: polar capsule length × width; PFc: polar filament coils

Thelohanellus sp.	Host (order; family)	Infection location	Country	SpBL	SpBW	SpT	PCL × W	PFc
T. marginatus Rocha et al., 2014b	*Hypophthalmus marginatus* (Siluriformes; Pimelodidae)	Gill	Brazil	17.1 ± 0.6	6.9 ± 0.4	5.1 ± 0.5	9.0 × 6.1	4–5
T. lepturus sp. nov.	*Hypopygus lepturus* (Gymnotiformes; Hypopomidae)	Brain and spinal cord	Brazil	17.7 ± 0.6 (17.2–18.3)	9.1 ± 0.6 (8.6–9.7)	2.3 ± 0.4	10.9 × 4.3	13–16

other studies (Scott et al. 2015). The structural alteration of the myelin sheaths has been reported as having a major influence on the behaviour of specimens with their CNS infected by myxosporeans. Considering that the main purpose of these structures is to increase the speed of impulses propagated along the myelinated fibres, interferences in their organization can be expected to influence the movements of the infected host (Scott et al. 2015).

Molecular and phylogenetic analysis

A large number of myxosporean SSU rRNA gene sequences are now available, for example for myxobolids parasitizing Brazilian freshwater fish hosts (Fiala 2006, Bartošová et al. 2009, Carriero et al. 2013, Rocha et al. 2014a, Camus et al. 2017). Thus, the molecular analyses performed in this study aimed to establish phylogenetic correlations between *H. lepturus* sp. nov., *T. lepturus* sp. nov. and the myxosporeans most similar to them.

Comparison of the SSU rRNA gene sequences of *H. lepturus* sp. nov. and *T. lepturus* sp. nov. to all other known sequences from myxobolid infections showed that, despite them being most similar to other species from South America, they are not identical. The basal phylogenetic position of *H. lepturus* sp. nov. in relation to several myxobolids from the same region could be explained by the fact that there are no available sequences for the other Brazilian *Henneguya* species that infect the NS. Also, no others parasitize fish hosts of the family Hypopomidae and order Gymnotiformes. In addition, *T. lepturus* sp. nov. did not cluster with *T. marginatus* (KJ434181) (Rocha et al. 2014b), despite the latter being the only other *Thelohanellus* species molecularly described from the Amazonian fauna. Overall, no other *Thelohanellus* spp. has been reported from the NS, so that *T. lepturus* sp. nov. clustered with the parasite *Myxobolus axelrodi*, which has been described infecting the brain and retina of the cardinal tetra *Paracheirodon axelrodi*, a popular ornamental neotropical freshwater fish native to South America (Camus et al. 2017). Furthermore, *M. axelrodi* presents a rudimental polar capsule, which may reinforce its phylogenetic relationship to the myxobolids comprising 2 valves and 1 polar capsule. In a recent revision of Myxozoa character evolution, phylogenetic evidences suggested that *Thelohanellus* spp. possibly evolved from the freshwater lineage of the *Myxobolus* morphotype by progressive reduction and consequent loss of 1 of its polar capsules (Fiala & Bartošová 2010). The current and previous studies seem to suggest these features evolved multiple times, hence the lack of monophyly in the trees and, consequently, the divisions between the myxobolid genera are largely artificial.

The results obtained in this study contribute to a better understanding of the myxosporean parasites of the Brazilian aquatic fauna. The parasites here described are the first to be described from fish hosts of the family Hypopomidae and order Gymnotiformes, with *T. lepturus* sp. nov. further constituting the first species of its genus described from the CNS. As this study did not aim to provide a long-term assessment of the histopathological damages caused by the parasitic infection in the CNS, further studies should be performed in order to understand the evolution of this parasitic disease.

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