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Myxobolus cuneus n. sp. (Myxosporea) infecting the connective tissue of *Piaractus mesopotamicus* (Pisces: Characidae) in Brazil: histopathology and ultrastructure

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Summary:

The characteristics of *Myxobolus cuneus* n. sp. and its relationship to the host *Piaractus mesopotamicus* are described based on light and electron microscopy and histological observations. Polysporic plasmodia measuring 20 µm to 2.1 mm in size were found in 63.3 % of the *P. mesopotamicus* examined. The parasite was found in the gall bladder, urinary bladder, gills, spleen, fins, head surface, liver and heart. Generative cells and disporoblastic pansporoblasts occurred along the periphery of the plasmodia, and mature spores were found in the internal region. The mature spores had a pear shaped body in frontal view, with a total length of 10.0 ± 0.6 µm and a width of 5.1 ± 0.3 µm (mean ± SD). The spore wall was smooth with sutural folds. The polar capsules were elongated, were pear shaped, and equal in size (length 5.7 ± 03 µm; width 1.7 ± 0.2 µm), with the anterior ends close to each other. The polar filaments were tightly coiled in 8-9 turns perpendicular to the axis of the capsule. The plasmodia were always found in connective tissue (wall of the arterioles of the gill filaments, serous capsule of the gall bladder, middle layer and subepithelial connective tissue of the urinary bladder, connective tissue between the rays of the fins, subcutaneous tissue of the head surface and fibrous capsule spleen). The parasite caused important damage in the gills, where development occurred in the wall of gill filament arterioles; a mild macrophage infiltrate was also observed. In advanced developmental stages, the plasmodia caused deformation of the arteriole structure, with a reduction and, in some cases, obstruction of the lumen. The parasite was found throughout the period studied and its prevalence was unaffected by host size, season or water properties.

KEY WORDS : Myxosporea, *Myxobolus cuneus* n. sp., *Piaractus mesopotamicus*, Characidae, connective tissue, histology, ultrastructure.

Résumé : *Myxobolus cuneus* n. sp. (Myxosporea) infectant le tissu conjonctif de *Piaractus mesopotamicus* (Pisces : Characidae) au Brésil : histopathologie et ultrastructure

Les caractéristiques de Myxobolus cuneus *n. sp. et ses relations avec l'hôte* Piaractus mesopotamicus *sont décrites d'après les données histologiques obtenues en microscopie photonique et en microscopie électronique. Les plasmodes polysporés dont les dimensions varient de 20 µm à 2,1 mm, ont été détectés chez 63,3 % des poissons examinés. Le parasite a été trouvé dans la vésicule biliaire, la vessie urinaire, les branchies, la rate, les nageoires, la surface de la tête, le foie et le cœur. Les cellules génératives et les pansporoblastes disporoblastiques sont produits en périphérie des plasmodes, les spores matures étant présentes à l'intérieur. Ces spores étaient piriformes en vue frontale, avec une longueur totale de 10 ± 6 µm et une largeur de 5,1 ± 0,3 µm (moyenne ± SD). La paroi sporale était lisse avec repli sutural. Les capsules polaires étaient oblongues, piriformes, de mêmes dimensions (longueur 5,7 ± 0,3 µm; largeur 1,7 ± 0,2 µm) et étroitement apposées à leur partie antérieure. Les filaments polaires étaient étroitement enroulés en huit à neuf tours de spire perpendiculaires à l'axe de la capsule. Les plasmodes ont toujours été trouvés dans le tissu conjonctif (paroi des artérioles des feuillets branchiaux, capsule séreuse de la vésicule biliaire, tissus conjonctif intermédiaire et sous épithélial de la vessie urinaire, tissu conjonctif entre les rayons des nageoires, tissus sous cutané de la tête et capsule fibreuse de la rate). Le parasite causait des dommages importants au niveau des branchies où son développement s'effectuait dans la paroi des artérioles du feuillet branchial ; un léger infiltrat macrophagique était également observé. Aux stades de développement plus tardifs, les plasmodes déformaient la structure artériolaire dont elles réduisaient la lumière et dans certains cas l'obstruaient. Le parasite a été retrouvé tout au long de la période étudiée et sa prévalence ne fut affectée ni par la taille de l'hôte, ni par l'environnement (saison, propriétés de l'eau).*

MOTS CLÉS : *Myxosporea,* Myxobolus cuneus *n. sp.,* Piaractus mesopotamicus, *Characidae, tissu conjonctif, histologie, ultrastructure.*

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INTRODUCTION

Piaractus mesopotamicus (Holmberg, 1887) is an omnivorous characid popularly known as pacu. This fish attains a large size (easily reaching 12 kg) and is economically one of the most important fish species in Brazil. The high reproductive capacity, rapid growth and widespread commercial acceptance of *P. mesopotamicus* have made it one of the most widely cultivated species by fish farms in Brazil.

To date, *Henneguya lutzi* Cunha & Fonseca, 1918, *Myxobolus colossomatis* Molnár & Békési, 1993 and *Henneguya piaractus* Martins & Souza, 1997 have been found parasitising *P. mesopotamicus*. In this study, which is part of a survey of myxosporean parasites on fish farms, we describe an ultrastructural and histological analysis of a new *Myxobolus* parasite of pacu.

MATERIALS AND METHODS

Young specimens of four fish species obtained by artificial reproduction, namely pacu (*Piaractus mesopotamicus* (Holmberg, 1887); Characidae), curimba (*Prochilodus lineatus* (Valenciennes, 1836); Prochilodontidae), matrinxã (*Brycon cephalus* (Gunther, 1869); Characidae) and piauçu (*Leporinus macrocephalus*, Garavello & Britski, 1988; Anostomidae), were released in a pond at the Center for the Research and Management of Continental Fishing Resources-Cepta/Ibama Pirassununga, state of São Paulo, Brazil, and monitored for two years. Five specimens of each species were examined monthly for the presence of myxosporeans from March 2000 to February 2002. Immediately after collection, the fishes were transported alive to the laboratory where they were killed by transection of the spinal cord, and then measured and necropsied.

The parasite was identified according to Lom & Arthur (1989), and the measurements from 43 fresh mature spores of different plasmodia obtained from several specimens of *P. mesopotamicus* were performed with a micrometer incorporated into the microscope eyepiece. The dimensions were expressed as the mean ± standard deviation (SD). Smears containing spores free were stained with Giemsa's solution and mounted in low viscosity mounting medium (Cytoseal™) as permanent slides. For histological analysis, fragments of infected organs were fixed in 10 % buffered formalin for 24 h, embedded in paraffin, cut into sections 4 µm thick and stained with haematoxylin or eosin and Sirius Red (Adriano *et al*., 2002). For scanning electron microscopy, free spores were deposited on a coverslip coated with poly-L-lysine and fixed for 2 h at room temperature with glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After washing in the same buffer, the preparations were dehydrated in ethanol, critical point dried in CO_2, covered with metallic gold, and examined in a Joel JMS 35 microscope operated at 15 kV. For transmission electron microscopy, plasmodia were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h, washed in glucose-saline solution for 2 h, and post-fixed in OsO_4, all done at 4° C. After dehydration in an acetone series, the material was embedded in Epon-Araldite resin. Ultrathin sections, double stained with uranyl acetate and lead citrate, were examined in LEO 906 electron microscope operated at 60 kV.

The chemical and physical properties of the pond water, including dissolved oxygen levels and temperature, were measured daily. Other properties, such as alkalinity, pH, NH_3 and hardness, were measured weekly. Pearson's correlation was used to determine whether there was any correlation between the chemical and physical characteristics of the water and the prevalence of the parasite. The occurrence of the parasite throughout the study was examined by grouping the monthly samples according to the season of collection. The effect of season and host (fish) size on the prevalence of the parasite was assessed using the χ^2 test, with the level of significance set at $p < 0.05$.

RESULTS

Of the four fish species studied, only specimens of *P. mesopotamicus* had plasmodia of an unknown *Myxobolus* species (Figs 1-5). Of 120 pacu examined, 45 were 5-10 cm long, 41 were 10.1-20 cm long and 34 were 20.1-36 cm long. 76 fish (63.3 %) had the parasite. Parasite plasmodia were found in several organs, and the prevalence in each organ was: gall bladder, 41.6 %; urinary bladder, 26.6 %; gills, 25 %; spleen, 14.1 %; fin, 5.8 %; head surface, 5 %; liver, 2.5 % and heart, 2.5 %. Parasite spores were found in melano-macrophage centres in the kidney and, less frequently, in the spleen.

There was no correlation between the prevalence of the parasite and the chemical and physical characteristics of the water, such as dissolved oxygen levels ($r = 0.2660$, $p = 0.1986$), alkalinity ($r = 0.1001$, $p = 0.6339$), pH ($r = 0.0435$, $p = 0.8402$), hardness ($r = - 0.1001$, $p = 0.6417$), NH_3 ($r = - 0.0349$, $p = 0.8713$) and temperature ($r = - 0.1775$, $p = 0.4066$).

The parasite was found throughout the study and its occurrence did not vary significantly with the seasons ($\chi^2 = 2.86$, df = 7, ns: non significant). The highest prevalence (73.3 %) was in the summers of 2000 and 2001, while the lowest (53.3 %) was in the autumn and spring

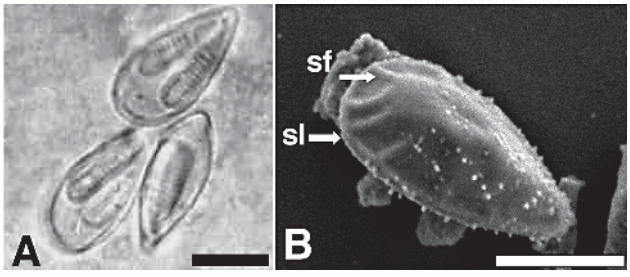

Fig. 1. – Mature spores of *Myxobolus cuneus* n. sp. A - light photomicrographs. Scale bar = 5 µm. B - scanning electron image showing the sutural folds (sf) and the suture line (sl). Scale bar = 5 µm.

of 2001. In the autumn and spring of 2000, the prevalence was 66.6 %, and in the winter of 2000 and 2001, the prevalence was 60 %. The prevalence was of 62.2 % in fish up to 10 cm long, 55.6 % in fish 10.1-20 cm long, and 61.9 % in fish 20.1-36 cm long. These differences were not significant (χ^2 = 1.64, df = 2, ns).

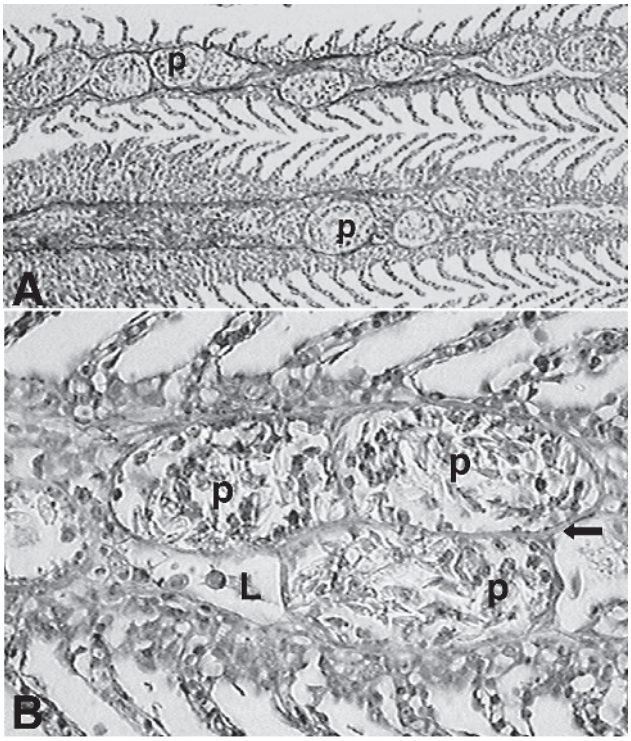

Fig. 2. – Light photomicrographs of histological sections of gills of *Piaractus mesopotamicus* infected with *Myxobolus cuneus* n. sp. Sirius red staining. A - plasmodium in the arterioles wall of the gill filament (p). 260 ×. B - agglomerate of plasmodia (p) producing obstruction of the arteriole lumen (L). Note the capsule of connective tissue (arrow). 1960 ×.

Description of plasmodia

The polysporic plasmodia were 20 µm to 2.1 mm in size (Figs 2A-B, 3A-D). Ultrastructural analysis revealed the presence of different sporogenic stages, such as generative cells and disporoblastic pansporoblasts, along the periphery of the plasmodia and mature spores and in different developmental stages in the internal region (Fig. 4A-E).

Description of spores

Fresh, mature spores had a pear shaped body in frontal view, with the anterior end more slender than the posterior end (Figs 1A-B, 5A-B), and had a total length of 10.0 ± 0.6 µm and a width of 5.1 ± 0.3 µm. The spore wall was smooth with sutural folds (Fig. 1B). In lateral view, the spores were symmetric, convex and had a conspicuous sutural line (Fig. 1B). The polar capsules were elongated, were pear shaped, and equal in size (length 5.7 ± 03 µm; width 1.7 ± 0.2 µm), with the anterior ends close to each other. The polar filaments were tightly coiled in 8-9 turns perpendicular to the axis of the capsule (Figs 4D-E, 5A). The sporoplasm was binucleated (Fig. 4B).

Type host: *Piaractus mesopotamicus* Holmberg, 1887 (Characidae).

Site of infection: gall bladder, urinary bladder, gills, spleen, fins, head surface, liver and heart.

Prevalence: 76/120 (63.3 %) of *P. mesopotamicus* were infected.

Locality: Center for the Research and Management of Continental Fishing Resources (Cepta/Ibama), Pirassununga, state of São Paulo, Brazil.

Type material: slides with stained spores (syntype) have been deposited in the collection of the Museum of Natural History, Institute of Biology, State University of Campinas (Unicamp), State of São Paulo, Brazil (accession numbers ZUEC 18 and 19).

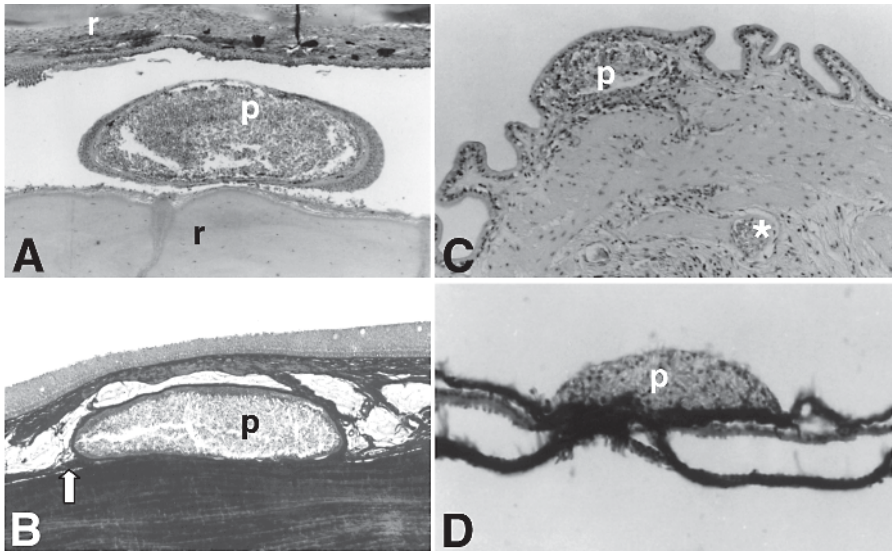

Fig. 3. – Histological sections of several organs infected by *Myxobolus cuneus* n. sp. A - longitudinal section of fin showing a plasmodium (p) in connective tissue between the rays (r). H & E staining. 200 ×. B - transversal section of operculum showing a plasmodium (p) deep within the subcutaneous tissue, near the periosteum (arrow). Sirius red staining. 200 ×. C - sections of urinary bladder showing plasmodia (p) in the subepithelial connective tissue and in the middle layer (*). H & E staining. 260 ×. D - section of gall bladder showing a plasmodium (p) in the serous capsule. Sirius red staining. 700 ×.

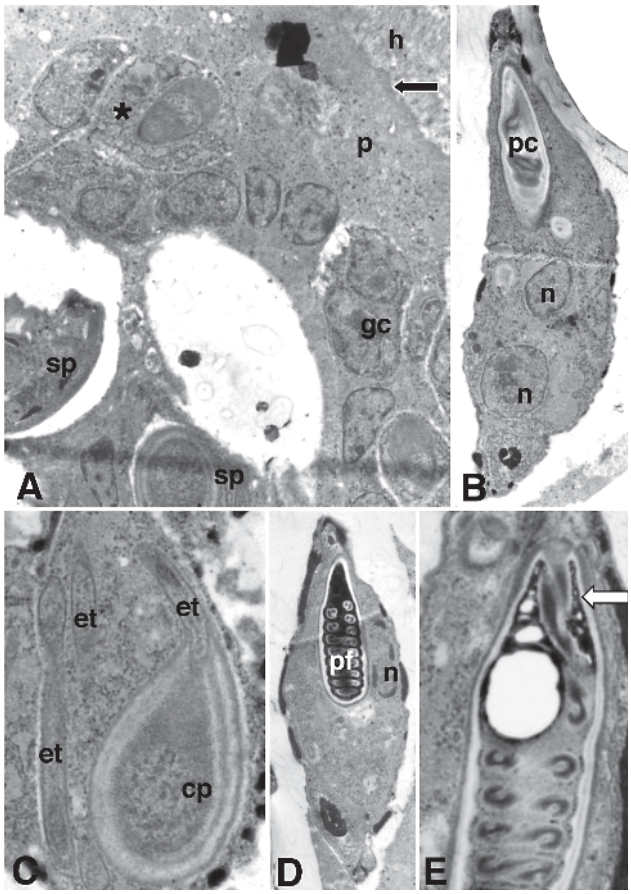

Fig. 4. – Electron micrographs of *Myxobolus cuneus* n. sp. A - section of a plasmodium (p) showing the plasmodial wall (arrow), host tissue-connective tissue capsule (h), disporoblastic pansporoblast (*), generative cells (gc) and fragments of young spores (sp). 7970 ×. B - longitudinal section of a young spore showing the polar capsule (pc) and binucleated sporoplasm cell (n). 10133 ×. C - early capsulogenic stage with the capsular primordium (cp) attached to the external tube (et). Note also two longitudinal sections of the external tube. 12926 ×. D - longitudinal section of a nearly mature spore showing the polar filament (pf) within the polar capsule and the nucleus (n) of the capsulogenic cell. 7546 ×. E - longitudinal section showing details of the anterior end of a polar capsule (arrow). 15920 ×.

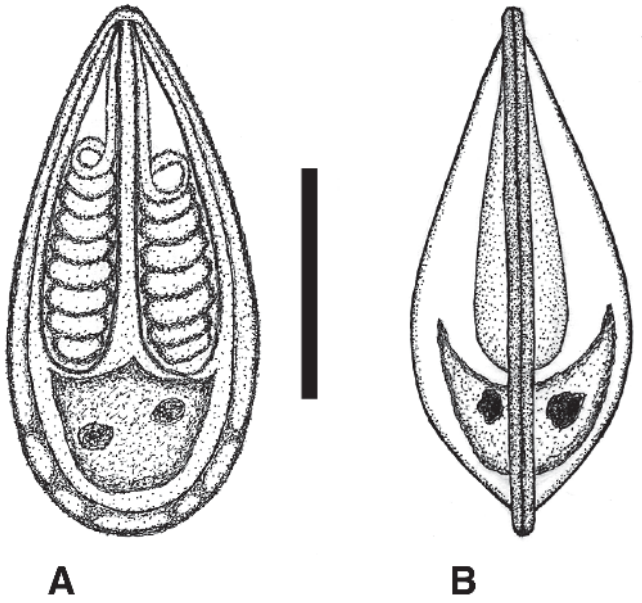

Fig. 5. – Schematic representation of mature spores of *Myxobolus cuneus* n. sp. A - frontal view. B - lateral view. Scale bar = 5 µm.

Etymoloy: the species name is based on the shape of the spores (wedge-shaped, from the Latin = *cuneus*).

Histological analysis showed that at all sites of infection the plasmodia were surrounded by a collagen capsule (Fig. 2B). In the gills, the plasmodia developed in the adventitia of arterioles in the gill filaments, and a mild macrophage infiltrate was observed. In advanced developmental stages, the plasmodia deformed the wall of the arterioles, compressing them in the direction of the lumen, thereby diminishing and, in some cases, obstructing the lumen of the arterioles (Figs. 2A-B). In the gall bladder, the plasmodia appeared externally in the serous capsule (Fig. 3D), while in the urinary bladder, the parasite developed in the middle layer and in the subepithelial connective tissue (Fig. 3C). In the fins, the parasite developed in connective tissue between the rays (Fig. 3A). In the head, the plasmodia were located deep within the subcutaneous tissue, near the periosteum (Fig. 3B), and in the spleen they occurred in the fibrous capsule. The prevalence of the parasite in the heart and in the liver was low (2.5 %) and plasmodia were seen only in fresh preparations.

DISCUSSION

Myxobolus cuneus was compared with other *Myxobolus* spp. parasites of South American fish. The spores of *M. cuneus* resembled those of other South American *Myxobolus* (*M. inaequus* Kent & Hoffman, 1984, *M. cunhai* Penido *et al.*, 1927, microspores of *M. serrasalmi* Walliker, 1969, and *M. maculatus* Casal *et al.*, 2002) in shape, but only the spores of *M. cunhai* and the microspores of *M. serrasalmi* were similar in size. However, *M. cunhai* was related in a host of the family Pimelodidae and their spores differed from those of *M. cuneus* by unequal size of the polar capsules (Gioia & Cordeiro, 1996). In contrast to *M. cuneus*, the plasmodia of *M. serrasalmi* had spores of two distinct shapes and sizes – macrospores with an oval shape and microspores with a pyriform shape. The existence of macro and microspores in the plasmodia of *M. serrasalmi* may have resulted from the fusion of two neighbouring plasmodia of different species (Molnár & Békési, 1992). However, since plasmodia of *M. serrasalmi* were found in the spleen and

kidney (Walliker, 1969), it seems improbable that this fusion of plasmodia involved different species in these two organs simultaneously. According to Walliker (1969), all the plasmodia of *M. serrasalmi* contained dense patches of dark brown pigment. However, similar patches were observed scattered throughout uninfected spleen tissue, and macro and microspores were frequently present in and around immature plasmodia. In addition, plasmodia in the kidney lacked a wall. Based on this description, we believe that the structures reported by Walliker (1969) were not myxosporean plasmodia, but were agglomerations of spores from two different species of *Myxobolus* within melanomacrophage centres.

The melanomacrophage centres, also known as macrophage aggregates, are distinctive groupings of pigment-containing cells in the tissues of heterothermic vertebrates. In fish, these centres are normally located in the stroma of the haemopoietic tissue of the spleen and kidney (Agius & Roberts, 2003), and play an important part in the host's defense reactions (Dyková, 1984; Agius & Roberts, 2003). Infections by different species of the genus *Myxobolus* manifested themselves by the appearance of spores in melanomacrophage centres or in aggregates of melanomacrophage in the kidney, spleen and hepatopancreas (Dyková, 1984). Melanomacrophages can attach to large myxosporean spores and transport them to melanomacrophage centres where the spores are encapsulated by fibroblasts and eventually destroyed (Dyková, 1984). Thus, spores of different species can accumulate in these centres. If the description by Walliker (1969) represents melanomacrophage centres, then the macro and microspores represent the spores of two species distinct whose site of development is unknown. In this context, the spores of *M. cuneus* are very similar in size and shape to the microspores of *M. serrasalmi*. However, the anterior end of the spores of *M. serrasalmi* is more pointed that in *M. cuneus*. In addition, the spores of *M. cuneus* are slightly larger than those of *M. serrasalmi* and the polar capsules are proportionally larger in *M. serrasalmi*. Thus, we suggest that *M. cuneus* is a new myxosporean species.

Myxobolus cuneus occurred throughout the period of this study and there was no correlation between the chemical and physical properties of the water and the prevalence of the parasite. Likewise, the prevalence of the parasite did not vary significantly with the seasons or the host size. Thus, it seems that the life cycle of this parasite was unaffected by environmental conditions and the development of the host, in contrast to *Myxobolus muelleri* Bütschli, 1881 and *Myxobolus dujardini* Thelohan, 1892, parasites of *Psychochelus oregonenseis*, *P. caurinus* and *Richardsonius blateatus*, for which the prevalence is greater in larger specimens (Mitchell, 1988), and *Myxobolus porofilus* Adriano, Aranas, Ceccarelli & Cordeiro, 2002, a parasite of *P. lineatus*, which was reported only in young specimens (Adriano *et al.*, 2002).

In this study, the specimens of *P. mesopotamicus* examined were confined to a pond with three other fish species, but *M. cuneus* was found only in pacu, which suggested host specificity. Similar host specificity has been reported for *M. porofilus* infecting *P. lineatus* maintained under the same conditions (Adriano *et al.*, 2002). According to Molnár *et al.* (1998), although little is known about the host specificity of *Myxobolus* species, the number of species with a large host range is low and most species appear to be strictly host-specific or capable of developing only in closely related fishes.

The histological analysis showed that the development of the parasite was not organ-specific, but the plasmodia of *M. cuneus* were always found in connective tissue (wall of the arterioles of the gill filaments, serous capsule of the gall bladder, middle layer and subepithelial connective tissue of the urinary bladder, connective tissue between the rays of the fins, subcutaneous tissue of the head surface and fibrous capsule spleen). A similar non-specificity of organs was reported for *M. colossomatis* parasitizing *Colossoma macropomum,* a large characid from the Amazon river basin (Molnár & Békési, 1992).

Of the organs parasitized by *M. cuneus*, the parasite caused greatest damage in the gills since the development of the plasmodia reduced the vessel lumen and, in some cases completely obstructed the lumen of the gill filament arterioles. Thus, a high parasite load could compromise the blood circulation and, consequently gill functions.

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