# AN ULTRASTRUCTURAL AND HISTOPATHOLOGICAL STUDY OF *HENNEGUYA PELLUCIDA* N. SP. (MYXOSPOREA: MYXOBOLIDAE) INFECTING *PIARACTUS MESOPOTAMICUS* (CHARACIDAE) CULTIVATED IN BRAZIL

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**Summary:**

During a study of myxosporean parasites of cultivated freshwater fish, a new myxosporean species, *Henneguya pellucida* n. sp., was discovered. Of the 120 *Piaractus mesopotamicus* sampled, only 10 specimens (8.3 %) were infected. Yellow, round plasmodia measuring 0.5-3 mm were found in the serous membrane of the visceral cavity and in the tunica externa of the swim bladder. Sporogenesis was asynchronous, with the earliest developmental stages aligned prevailingly along the endoplasmic periphery and mature spores in the central zone. The mature spores were pear shaped (total length: 33.3 ± 1.5 µm, mean ± SD; width: 4.1 ± 0.4 µm; body length: 11.4 ± 0.3 µm; caudal process length: 24.1 ± 1.5 µm). The polar capsules were elongated (length: 4.0 ± 0.4 µm; width: 1.6 ± 0.2 µm). The development of the parasite in the swim bladder produced thickening of the tunica externa and a granulomatous reaction. There was no correlation between the prevalence of the parasite and the chemical and physical characteristics of the water. Infection was recorded only in juvenile specimens ranging in size from 9.5 to 20 cm.

**KEY WORDS :** *Henneguya pellucida* n. sp., Myxosporea, Characidae, *Piaractus mesopotamicus*, ultrastructure, histopathology, Brazil.

***Résumé :*** ÉTUDE ULTRASTRUCTURALE ET HISTOPATHOLOGIQUE D'*HENNEGUYA PELLUCIDA* N. SP. (MYXOSPOREA : MYXOBOLIDAE) PARASITE DE *PIARACTUS MESOPOTAMICUS* (CHARACIDAE), POISSON D'ÉLEVAGE AU BRÉSIL

*Le contrôle des infections myxosporidiennnes dans un élevage de poissons d'eau douce a révélé l'existence de la nouvelle espèce,* Henneguya pellucida *n. sp., chez* Piaractus mesopotamicus. *Dix des 120 poissons examinés, soit 8,3 %, étaient infectés. Des plasmodes sphériques de couleur jaune, de 0,5 à 3 mm de diamètre, ont été trouvés dans la séreuse de la cavité viscérale et dans la tunique externe de la vessie natatoire. La sporogenèse est asynchrone avec localisation des stades précoces en bordure de l'endoplasme et des spores matures dans la partie centrale. Les spores matures sont piriformes (longueur totale : 33,3 ± 1,5 µm, moyenne ± SD; largeur : 4,1 ± 0,4 µm; longueur du corps : 11,4 ± 0,3 µm; longueur du processus caudal : 24 1 ± 1,5 µm). Les capsules polaires sont oblongues (longueur : 4,0 ± 0,4 µm; largeur : 1,6 ± 0,2 µm). Le développement du parasite dans la vésicule natatoire induit un épaississement de la tunica externe et une réaction granulomateuse. Aucune corrélation n'a été trouvée entre la prévalence du parasite et les caractéristiques chimiques et physiques de l'eau. L'infection n'affectait que des spécimens juvéniles mesurant de 9,5 à 20 cm.*

***MOTS CLÉS :*** Henneguya pellucida *n. sp., Myxosporea, Characidae,* Piaractus mesopotamicus, *ultrastructure, histopathologie, Brésil.*

## INTRODUCTION

Cultivated and wild fishes are infected by numerous myxosporean species of the genus *Henneguya* Thelohan, 1892, some of which are important pathological agents (Kalavati & Narasimhamurti 1985; Lom & Dyková 1995; Martins *et al.*, 1999). Eiras (2002) listed a total of 146 *Henneguya* species that parasitize fishes in different parts of the world. In South America, 32 *Henneguya* species have been reported in freshwater fishes (Barassa *et al.*, 2003), and are the most common myxosporean parasites of the fish, with the gills being the organ most frequently infected (Gioia & Cordeiro, 1996).

A few histological (Martins *et al.*, 1999; Adriano *et al.*, 2002; Barassa *et al.*, 2003) and ultrastructural (Rocha *et al.*, 1992; Azevedo *et al.*, 1997; Azevedo & Matos, 1989, 1995, 1996, 2002, 2003; Vita *et al.*, 2003) studies have been done on *Henneguya*, using Brazilian fish. The present study is part of an investigation into the ultrastructural and histopathological characteristics of myxosporean parasites of freshwater fish cultivated in Brazil. Using light, scanning electron and transmission electron microscopy, we describe a new species of *Henneguya* infecting *Piaractus mesopotamicus* (Holmberg, 1887), popularly known as "pacu", a South American fish species of considerable economic importance.

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## MATERIALS AND METHODS

During a survey of Myxozoa parasites done at Center for the Research and Management of continental fishing Resources CEPTA/IBAMA, located in the municipality of Pirassununga, in the state of São Paulo, Brazil, specimens of four fish species ("pacu", *P. mesopotamicus* (Characidae); "matrinxã", *Brycon cephalus* (Gunther, 1869) (Characidae); "curimbata", *Prochilodus lineatus* (Valenciennes, 1836) (Prochilodontidae) and "piauçu", *Leporinus macrocephalus*, Garavello & Britski, 1988 (Anostomidae)) four months old were stocked in a pond and monitored for two years.

Five specimens of each species were examined monthly (March 2000 to February 2002) for the presence of Myxozoa parasites. Immediately after collection, the fish were transported alive to the laboratory where they were killed by transection of the spinal cord, and then measured, weighed and necropsied. The parasite was identified according to Lom & Arthur (1989), and the measurements from 40 fresh mature spores of different cysts were obtained using a micrometer incorporated into the ocular. The dimensions were expressed as the mean ± standard deviation (SD).

Smears containing free spores were stained with Giemsa's solution and mounted in low viscosity mounting medium (Cytoseal$^{TM}$) to provide permanent slides (Adriano *et al.*, 2002). For histological analysis, the cysts were fixed in 10 % buffered formalin for 24 h, embedded in paraffin, cut into 4 µm thick sections and stained with hematoxylin and 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 two hours at room temperature with glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). After washing in the same buffer, the preparations were dehydrated in ethanol, dried by $CO_2$ critical point drying, covered with metallic gold and examined in a Joel JMS 35 scanning electron microscope operated at 15 kV.

For transmission electron microscopy, the cysts were fixed in 2.5 % glutaraldeyde in cacodylate buffer (2 h), post-fixed in 1 % $OsO_4$ (2 h.), dehydrated in increasing concentrations of acetone and embedded in Epon-Araldite resin. Ultrathin sections were double stained with uranyl acetate and lead citrate and examined in a LEO 906 transmission 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 analysis was used to determine whether there was any correlation between the characteristics of the water and the prevalence of the parasite.

The procedure Proc Catmod of the SAS (Statistical Analysis System) statistical package (SAS Institute, Inc. 1986) was used to test the effect of season on prevalence of the parasite. The independent variable was season and the response variable was the presence of parasites. The frequency of parasitism was the weight variable.

## RESULTS

Of the fish species studied, only specimens of "pacu" had the parasite. Of the 120 "pacu" specimens examined, 45 were 5-10 cm long, 41 were 10.1-20 cm long and 34 were 20.1-36 cm long. Ten fish (8.3 %) had plasmodia of an unknown *Henneguya* species. Infection was recorded only in specimens ranging in size from 9.5 to 20 cm.

There was no correlation between the prevalence of the parasite and the chemical and physical characteristics of the water such as dissolved oxygen levels ($r = -0.1147$; $p = 0.5934$), alkalinity ($r = -0.3051$; $p = 0.1471$), pH ($r = 0.0217$; $p = 0.9197$), hardness ($r = -0.3085$; $p = 0.1424$), $NH_3$ ($r = 0.3415$; $p = 0.1024$) and temperature ($r = -0.0651$; $p = 0.7623$). The presence of the parasite was observed only between the spring of 2000 and the winter of 2001. The prevalence in the autumn was of only 9.1 % but reached 25 % in the winter. In spring and summer, the prevalences were 13.3 % and 21.4 %, respectively. The differences of prevalence did not vary significantly ($\chi^2 = 0.40$, df = 3, ns: non significant). The intensity of the parasite was low, and ranged from one to five plasmodia per fish. The specimen with the highest parasitemia was collected in February 2001 and measured 14 cm. No fish mortality was observed during the study.

### Description of plasmodia

Plasmodial forms were yellow, round and measured 0.5-3 mm. The parasite occurred in the serous membrane of the visceral cavity and in the tunica externa of the swim bladder. The development was asynchronous and the earliest stages were aligned at the periphery of the plasmodium while the mature spores appeared in the central zone (Fig. 1B, C).

### Description of spores

Mature spores had a pear shaped body in face view (Figs 1A, 3E), with a total length of 33.3 ± 1.5 µm; a width of 4.1 ± 0.4 µm, and a body length of 11.4 ± 0.3 µm. In lateral view, the body spores were fusiform, symmetrical and with a thin suture line in the junction of two thin valves. The valve surfaces were smooth and prolonged by a caudal process 24.1 ± 1.5 µm long

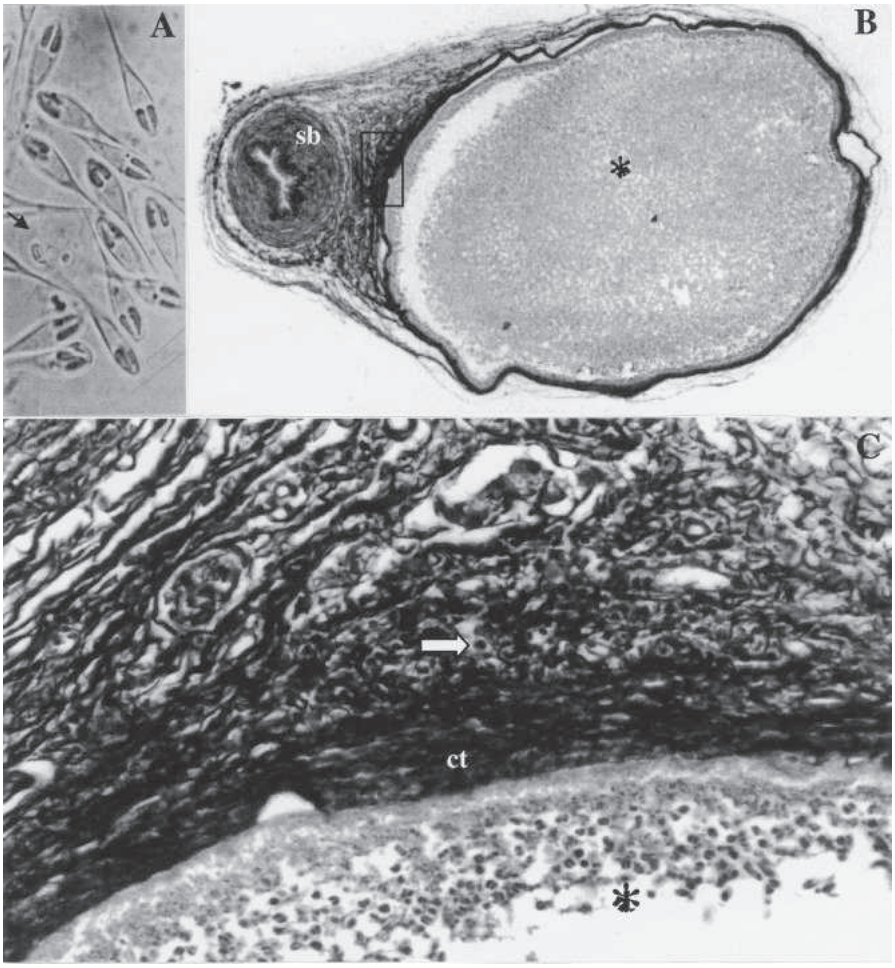


Fig. 1. – Light photomicrographs of *Henneguya pellucida* n. sp. A - Fresh preparation showing mature and one immature (arrow) spores (× 3,800). (B-C) Histological sections of a *plasmodium* (*) found in the anterior end of the swim bladder (sb). B - Panel shows thickening of the tunica externa (arrow) (× 200). C - Amplified part of B: note the capsule of connective tissue (ct) with collagenous fibres in the region nearest to the parasite and cellular elements typical of a granulomatous reaction in the outermost face (arrow) (× 1,680).

(Fig. 3E). These caudal processes were linked to each other by an adhesive material surrounding the spores (a sheath-like membrane), which gave the impression that there was only one caudal process (Figs 1A, 3E). The polar capsules were elongated, (length: 4.0 ± 0.4 µm; width: 1.6 ± 0.2 µm). The polar filaments coiled in six or seven turns, were arranged perpendicularly to the long axis (Fig. 3D).

Type host: *Piaractus mesopotamicus* (Pisces: Characidae).
Site of infection: serous membrane of the visceral cavity and tunica externa of the swim bladder.
Prevalence : 10/120 (8.3 %) of *P. mesopotamicus* were infected.
Locality: Center for the Research and Management of continental fishing Resources CEPTA/IBAMA located in the municipality of Pirassununga, the state of São Paulo, Brazil.
Type material: slides with stained spores of *H. pellucida* n. sp. (Syntipe) were deposited in the Museum of Natural History of the Institute of Biology at the State University of Campinas (UNICAMP), state of São Paulo, Brazil (accession n$^{os}$: ZUEC 20, 21 and 22).

Etymology: the specific name originates from the Latin word *pellucida* (= covered with a transparent skin) which describes the thin outer layer surrounding the spores.

Histopathological analysis showed that the development of the plasmodia led to the formation of a thick capsule of connective tissue, which contained cellular elements and collagen fibers (Figs 1B, C). The latter were more frequent in the region closest to the parasite, whereas the cellular elements were more common in the outermost face (Fig. 1C). The development of the parasite in the swim bladder produced thickening of the tunica externa and a granulomatous reaction was observed around the plasmodia. This reaction occurred all around of the plasmodium, but was more evident on the surface adjacent to the tunica interna of the swim bladder (Fig. 1C).

Ultrastructural study revealed a thin layer of fibrils close to the plasmodial wall and an outer capsule of connective tissue composed of cross-linked collagen fibres surrounding the plasmodia (Fig. 2A). The wall of the plasmodia consisted of a single membrane. A thick

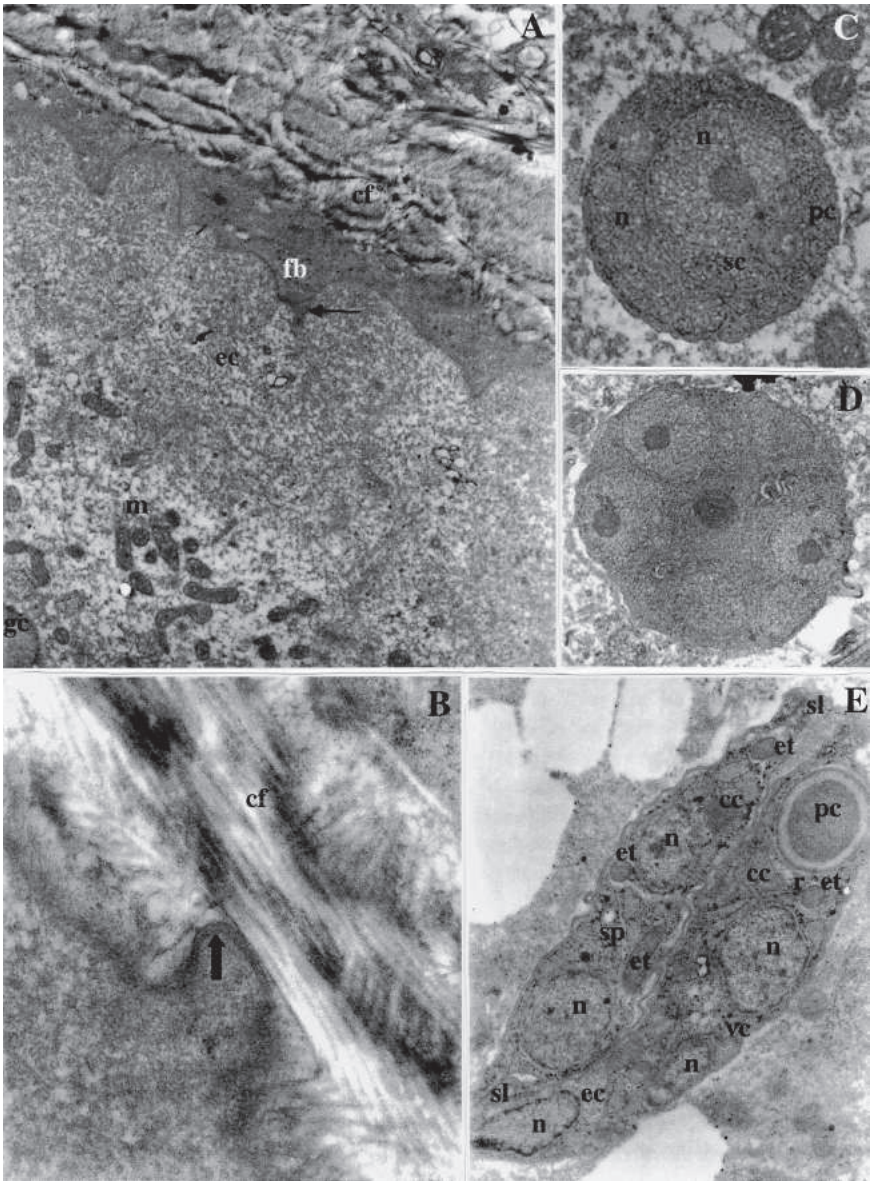


Fig. 2. – Electron micrographs of *Henneguya pellucida* n. sp. (A-B) section of the host-parasite interface. A - *Plasmodium* showing the granular ectoplasm (ec), numerous mitochondria (m), a generative cell (gc), a folded single limiting membrane (arrows) surrounded by a thin layer of fibrils (fb) and network of collagen fibres (cf) (× 7,430). B - Collagen fibres (cf) of the connective tissue capsule showing a characteristic orientation. Note the plasmodial wall with a single limiting membrane (arrow) (× 34,500). (C-E) Sporogonic developmental stages. C - Secondary cell (sc) inside of a primary cell (pc). (× 20,862). D - Pansporoblast consisting of undifferentiated cells. (× 10,431). E - Pansporoblast with two sporoblasts. Note the envelope cell (ec), the valvogenic cells (vc), the sporoplasm cells (sp), the capsulogenic cells (cc), the nucleus (n), the polar capsule (pc), the external tubule (et) the sutural line (sl), and numerous scattered ribosomes (r) (× 13,060).

ectoplasm was present on the internal surface adjacent to the wall. At the interface with the membrane, this ectoplasm consisted of thin granules and no pinocytotic canals were observed. In the subsequent layer, there were large numbers of mitochondria, numerous generative cells and young disporoblastic pansporoblasts in different developmental stages (Fig. 2A, C-E). In advanced stages of maturation, the spores contained valvogenic cells with a large accumulation of electron-dense "valve-forming material" (Fig. 3C, D). The binucleated sporoplasm cell had numerous randomly scattered ribosomes and a few sporoplasmosomes. The polar capsules consisted of a zone dark outer, an intermediate electron-lucent layer and a internal granular content, containing the polar filament with six or seven turns (Fig. 3D). The spores were surrounded by a thin "sheath-like" membrane that involved the valve walls and formed the junction of the two tails (Fig. 3A-C).

## DISCUSSION

*Henneguya pellucida* was compared with other *Henneguya* species previously reported in South American fishes (Gioia & Cordeiro, 1996; Eiras, 2002; Eiras *et al.*, 2004). Of the 32 *Henneguya* species recorded so far in the continent, only *Henneguya lutzi* (Cunha & Fonseca, 1918) and *Henneguya piaractus* (Martins & Souza, 1997) have been found in *P. mesopotamicus* and the spores of both differ in shape and size from those of *H. pellucida*. Of the spe-

cies described in other South American fish, *Henneguya santae* (Guimarães & Bergamin, 1934) found in *Tetragnopterus santae* and *Henneguya leporinicola* (Martins *et al*., 1999) found in *Leporinus macrocephalus* share some morphological similarities with *H. pellucida*, although the first has a smaller caudal process (11.8 µm) and the latter a smaller body spore (7.6 µm) than *H. pellucida* (body spore length: 11.4 ± 0.3 µm; caudal process length: 24.1 ± 1.5 µm). In *Henneguya travassosi* (Guimarães & Bergamin, 1933) described in *Leporinus copelandi*, the spores are approximately equal in size to those of *H. pellucida*, but are oval in shape, with white plasmodia occurring in the muscles. *Henneguya schizodon* (Eiras *et al*., 2004) found in *Schizodon fasciatus* differes of *H. pellucida* by ellipsoidal shape of the spores, smaller caudal process (16.3 µm) and by plasmodia white. *Henneguya malabaricus* (Azevedo & Matos, 1996) described in *Hoplias malabaricus* and *Henneguya adherens* (Azevedo & Matos, 1995) found in *Acestrorhynchus falcatus* resemble *H. pellucida* in spore size and by the presence of a sheath surrounding the spores. However, in both species the spores are ellipsoidal and the plasmodial forms occur in the gills.

The plasmodia of *H. pellucida* occurred in the serous membrane of the visceral cavity and in the tunica externa of the terminal region of the swim bladder. The presence of myxosporeans in these tissues has also been reported by others. Molnár *et al.* (1998) suggested that the site of development of *Myxobolus macroplasmodialis* (Molnár *et al*., 1998) was the serous membrane of the visceral cavity. According to these authors,

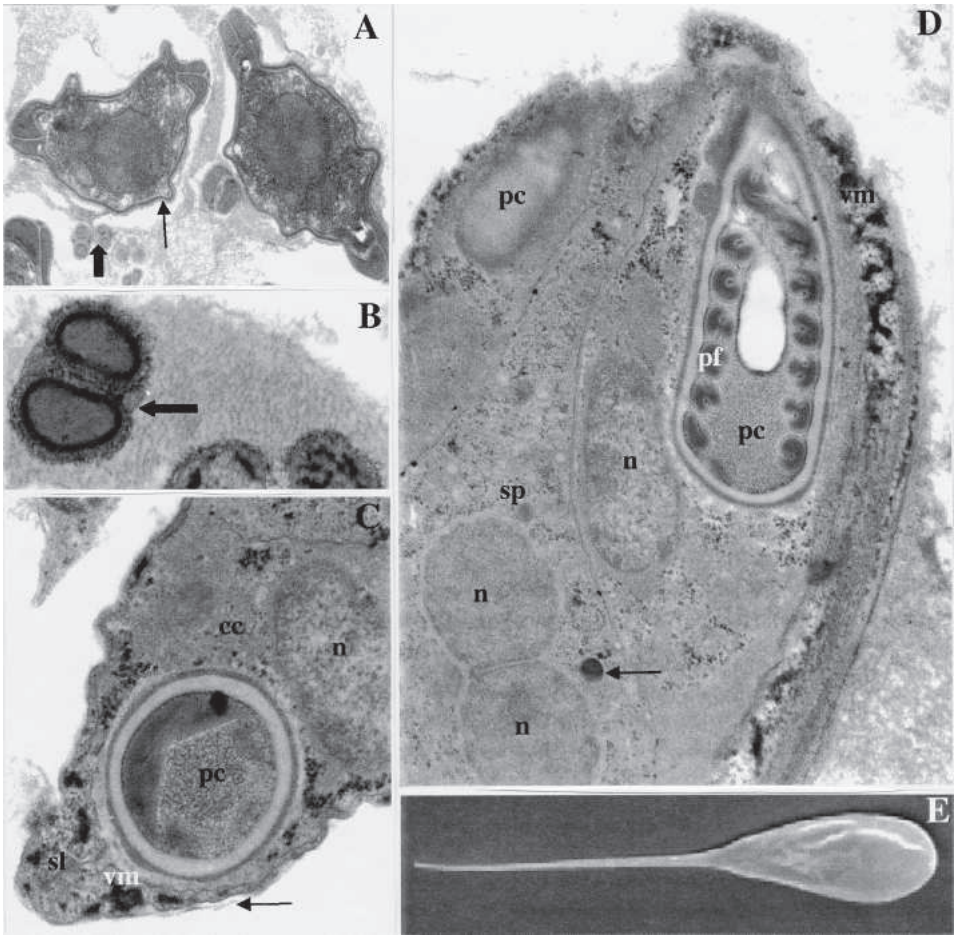


Fig. 3. – Electron micrographs of spores of *Henneguya pellucida* n. sp. (A-D) section of young spores.
A - Transversal section at the level of the sporoplasm cells (sp). Note the thin, sheath-like membrane surrounding the spores (arrows) (× 12,230).
B - Detail of transversal sections at the level of the caudal process showing the two tail projections united by a sheath-like membrane (arrows) (× 60,370).
C - Transversal section at the level of the capsulogenic cell (cc) showing the polar capsule (pc), sutral line (sl), valve-forming material (vm) and the sheath-like membrane surrounding the spores (arrows) (× 32,340).
D - Longitudinal section of the anterior portion of a young spore showing the capsulogenic cell (cc), polar filament (pf) within the polar capsule (pc), a sporoplasm cell (sp) with two paired nuclei (n) and a few sporoplasmosomes (arrow) (× 24,570).
E - Scanning electron image of a mature spore showing the junction of the tail projections. (× 9,400).

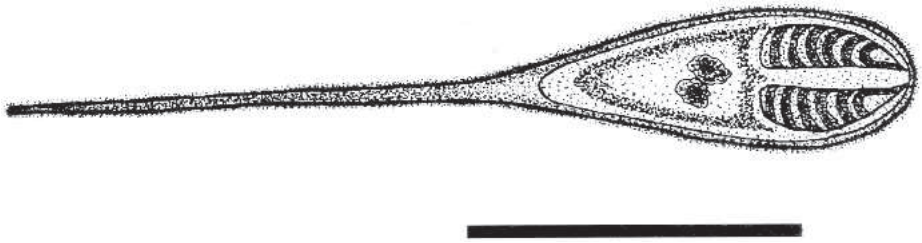

Fig. 4. – Schematic representation of mature spores of *Henneguya pellucida* n. sp. Bar = 10 µm.

the plasmodia occurred free in the visceral cavity of *Salminus maxillosus* (Characidae), but the site of development could have been the serous membrane of the visceral cavity or organs, from which the parasite became detached at an advanced stage of development. A similar localization was suggested by Adriano *et al.* (2002) for *Myxobolus porofilus* (Adriano *et al.*, 2002) found in the visceral cavity of *Prochilodus lineatus*.

The swim bladder was the site of sporogonic plasmodia or of the extra-sporogonic proliferative stages of myxosporeans whose sporogonic stages occur in other organs (Lom & Dyková, 1995). The extra-sporogonic stage of *Sphaerospora renicola* (Dyková & Lom, 1982) has been implicated as the etiologic agent of swim-bladder inflammation (SBI) in European carp (Dyková & Lom, 1988). To date, the presence of *Henneguya* species infecting the swim bladder is restricted to *Henneguya rhomboides* (Ma *et al.*, 1982), found in the swim bladder of *Carassius auratus auratus* in China (Eiras, 2002).

The prevalence and intensity of *H. pellucida* were low, with only 8.3 % of the specimens of *P. mesopotamicus* being infected and the specimen with the highest parasitaemia had only five plasmodia. Other workers have also reported the low intensity and prevalence of myxosporeans in visceral organs. Molnár *et al.* (1998) recorded a prevalence of 9.7 % in *S. maxillosus* parasitized by *M. macroplasmodialis*. Most of the fish examined by these authors had only one plasmodium, with only one specimen harbouring 28 plasmodia. Molnár (2002) found plasmodia of *Myxobolus cyprinicola* (Reuss, 1906) in 14.6 % of the specimens of *Cyprinus carpio* examined in Europe and the intensity ranged from one to 11 plasmodia per specimens. Adriano *et al.* (2002) reported a prevalence of 3 % for *P. lineatus* infected by *M. porofilus* and the fish with the highest parasitemia had only two plasmodia.

There was no correlation between the chemical and physical properties of the water and the prevalence of *H. pellucida*. Likewise, there were no significant seasonal differences in the prevalence. However, the occurrence of the parasite was related to the host's size, since only specimens ranging in size from 9.5 to 20 cm were infected. These finding are similar to those of Adriano *et al.* (2002), who also reported *M. porofilus* only in juveniles of *P. lineatus,* but differ from those of Mitchell (1988) who reported that infection by *Myxobolus muelleri* (Buetschli, 1882) and *Myxobolus dujardini* (Thélohan, 1892), parasites of *Psychochelus oregonenseis*, *P. caurinus* and *Richardsonius blateatus*, was higher in adult fish. Molnár (1998) also recorded the highest prevalence of *Henneguya creplini* (Gurley, 1894) in specimens of pikeperch (*Stizostedion lucioperca*) larger than 40 cm in length. The absence of *H. pellucida* in very young *P. mesopotamicus* (< 9.5 cm) can be explained by the time required for the appearance of the plasmodia after the initial contact of the fish with the parasite, while the absence of the parasite in adults (> 20 cm) may indicate that the fish acquire some type of resistance that prevents infection.

The specimens of *P. mesopotamicus* examined were confined to a pond with three other fish species: *B. cephalus* (Characidae), *P. lineatus* (Prochilodontidae) and *L. macrocephalus* (Anostomidae), but *H. pellucida* was found only in the pacu, suggesting host specificity.

Histopathological analysis of *H. pellucida* showed that development of the parasite in the swim bladder caused thickening of the tunica externa and a granulomatous reaction around the plasmodia. This reaction, which occurred around all of the plasmodium and was more evident on the surface adjacent to the tunica interna of the swim bladder, is common in myxosporeans. According to Dyková & Lom (1978), the response of fish soft tissues to myxosporidian infections involves displacement, atrophy or hyperplasia of the tissue surrounding the plasmodium during growth and maturation. In more advanced stages, when the cysts are full of mature spores, an inflammatory reaction occurs, resulting in the rapid replacement of the cyst by granulomatous tissue.

Ultrastructural analysis of the capsule of connective tissue surrounding the plasmodia of *H. pellucida* revealed a thin layer of fibrils close to the plasmodial wall and an outer layer of cross-liked collagen fibres with a peculiar net-like organization. The capsule of collagen fibres prevented direct contact between the plasmodium wall of *H. pellucida* and the host cells. Current (1979) also observed a capsule of collagen fibres surrounding the plasmodia of *Henneguya adiposa* (Minchew, 1977), a parasite of the adipose fin of *Ictularus punctatus*. According to this author, the capsule of collagen fibres allow it to take up only interstitial material.

Thus, despite the presence of the capsule of collagen fibres, that allow *H. pellucida* incorporate only interstitial material, the histopathological analysis show that this species was able to induce a granulomatous reaction around the plasmodia, indicating that it was pathogenic.

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