Amoebae isolated from organs of farmed tilapias, *Oreochromis niloticus*

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Abstract. Three amoeba species were isolated from 3 out of 193 farmed tilapias, *Oreochromis niloticus* (L.), screened for the presence of free-living amoebae in parenchymatous organs. *Hartmannella vermiformis* Page, 1967 and *Rosculus ithacus* Hawes, 1963 were isolated from the kidney tissue. The third strain isolated from the liver shared morphological features of *Mayorella* and *Platyamoeba* spp. and therefore its taxonomic position has not been determined as yet. Pathogenicity of cloned strain of *H. vermiformis* was proved in two fish hosts.

The Nile tilapia, *Oreochromis niloticus* (L.), the most widely distributed species among cichlids cultured in subtropical conditions (Natividad et al. 1986, Chen et al. 1994), was also introduced into the temperate zone. Attempts were made to establish intensive fish farms using thermally polluted water from electric power stations for maintaining optimal water temperature during the whole year.

Tilapias have recently received increased attention of parasitologists (see, e.g., review by Natividad et al. 1986), and several institutions have initiated a survey of parasites of wild and cultured Nile tilapia (Bondad-Reantaso and Arthur 1989), but the data on amoebic infections are very scarce (Rogers and Gaines 1975, Taylor 1977). Taylor (1977) cited two reported fish kills in tilapias that were attributed to amoebae found in the peritoneal fluid, intestinal mucosa, and on the gills.

Since thermally polluted waters were designated many times as the source of amoebic infections of humans and exceptionally of cultured fishes (Nash et al. 1988), there has been a good reason to screen for the presence of amoebae in organs of Nile tilapia cultured in mixture of riverine and thermally polluted water.
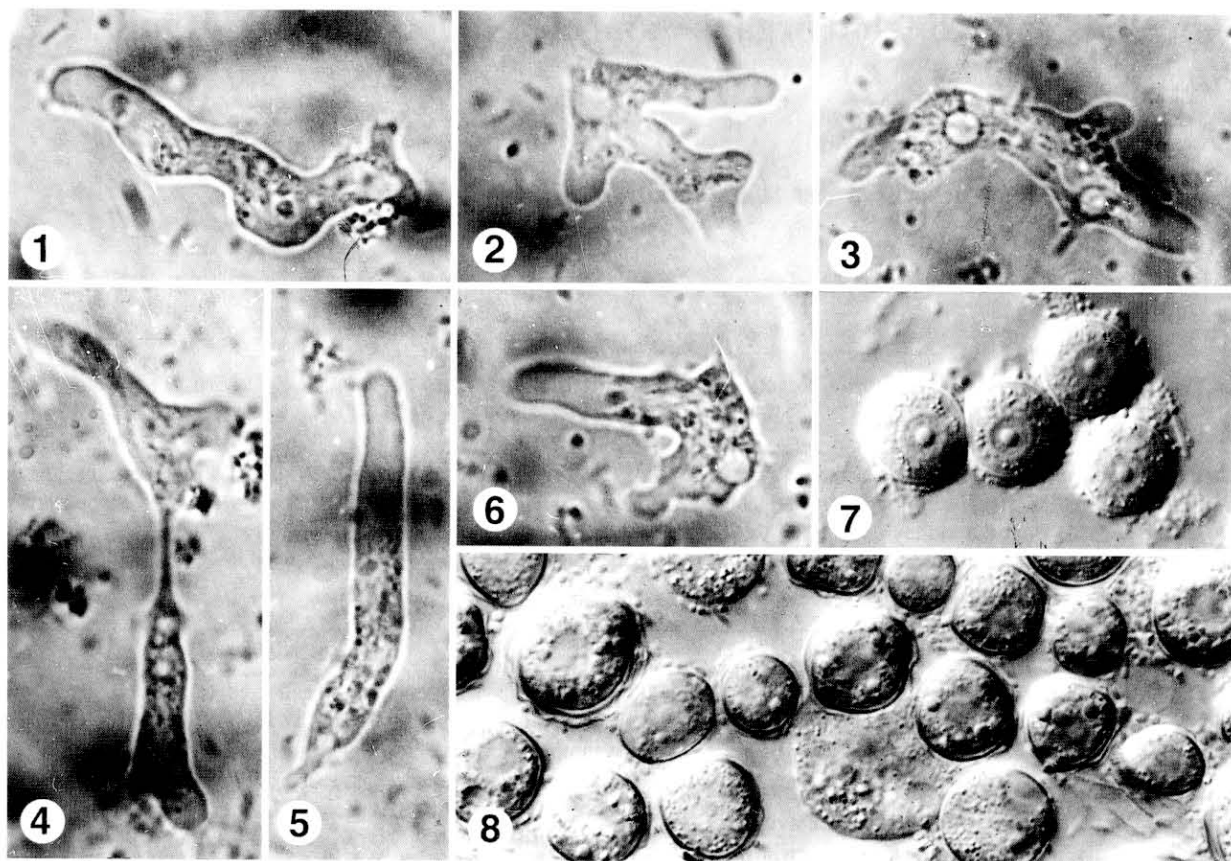
MATERIALS AND METHODS

Nile tilapias, *Oreochromis niloticus*, from two mutually distant farms were examined for the presence of amoebae in organs. Both farms were supplied with riverine water mixed with water from cooling system of electric power stations.

Thirty one tilapias were collected in a South Bohemian farm in January 1993. A North-west Bohemian farm was sampled six times throughout 1994. Since tilapias were cultured in tanks of the same size and transferred from one to another to adjust stock density, representatives of five size categories (4.5–6.9, 7.0–9.9, 10.0–14.9, 15.0–19.9 and longer than 20.0 cm) were selected in the second farm. In total 162 specimens were screened in 1994.

Aseptically removed tissue samples of the liver, spleen, kidney and brain were inoculated on non-nutrient agar surface according to the method by De Jonckheere (1980). Isolated amoebae were grown on non-nutrient agar seeded with heat-killed *Bacillus subtilis* (Page 1967). Attempts were made to establish axenic cultures of isolated amoebae in three liquid media, BCS, SCGYEM and PPG (Červa 1969, Kalinina and Page 1992). The growth of cloned strains was tested also in the cell culture medium (MEM) and in fish cell cultures (EPC cell line). Characterization of isolated strains followed the morphological criteria by Page and Siemensma (1991). All strains of amoebae were tested for production of temporary flagellated forms (Page 1967).

Trophozoites and cysts cultured in liquid media and those from agar plate cultures were processed for transmission electron microscopy. They were fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer and embedded in Epon Araldite. Amoebae attached to adhesive coated cover slip (Polylysine hydrobromide, Polysciences, Inc., 0.1% aqueous solution) were fixed with 1% osmium tetroxide, dehydrated and critical point dried using CO_2, then sputter-coated with gold and examined with a Jeol 6300 scanning electron microscope.

All the specimens of fish screened for the presence of amoebae in organs which were apparently healthy were nevertheless examined histologically. Fixed tissues were processed routinely using paraffin technique.

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Figs. 1–6. *Hartmannella vermiformis*, trophozoites of clone TN 102/I, × 1475; photographed in hanging drop while moving and dividing. The smudges on the picture are due to other amoebae moving out of focus. **Fig. 7.** Cysts of *H. vermiformis* in the beginning of their formation, × 1530. **Fig. 8.** Mature cysts of *H. vermiformis*, × 1235.

Pathogenicity of clone TN 102/I was tested in 3 species of fishes and in Balb mice. Three groups of tilapias, *Oreochromis niloticus*, one of goldfishes, *Carassius auratus* (L.), and one of carps, *Cyprinus carpio* L., all from laboratory breeding, were inoculated with 1.6–1.9 × 10⁶ trophozoites and cysts which were introduced intraoesophageally by syringe with cannula. Mice were inoculated perorally by instillation of the same dose in the oral cavity. Each experimental group consisted of 6 animals. They were carefully observed while alive. The same type of screening for the presence of amoebae in organs used in farmed tilapias was applied in experimental animals in order to reisolate inoculated amoebae and detect disease signs and histopathological changes. Amoebae isolated from organs of experimentally infected animals were compared with inoculated clones.

Cloned strains TN 99/I and TN 900/I were not tested for pathogenicity because cultivation in the liquid media was not successful.
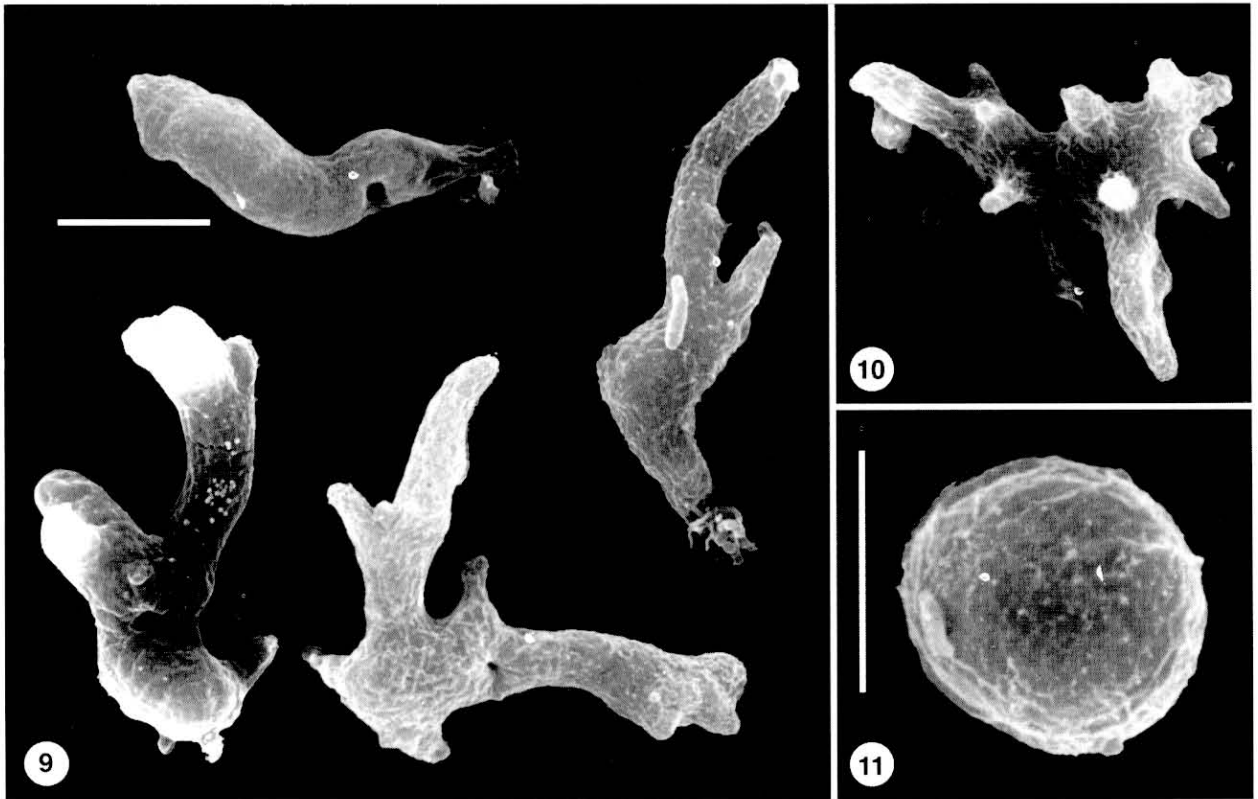
RESULTS

a) Amoebae isolated from organs of cultured tilapias

Three morphologically different strains of amoebae were isolated from organs of 3 young Nile tilapias,

Oreochromis niloticus (L.). Two of them from 7.0–9.9 cm fish taken at the North-west Bohemian farm were identified as *Hartmannella vermiformis* Page, 1967 (clone TN 102/I) and *Rosculus ithacus* Hawes, 1963 (clone TN 99/I). The third amoeba from a 4.5–6.9 cm fish from the South Bohemian farm (clone TN 900/I) shared light microscopical features described for *Mayorella* and ultrastructural features for *Platyamoeba* and its specific identity has not been determined. Descriptions of the amoebae are as follows:

Hartmannella vermiformis (clone TN 102/I) was derived from the strain isolated from the kidney tissue of Nile tilapia, June 1994. Microscopic studies of amoebae grown on agar plates and in liquid media (PPG, BCS, and MEM) yielded both monopodial nonflagellated trophozoites and round cysts characteristic of the genus *Hartmannella* (Figs. 1–11). Trophozoites were elongated and cylindrical, and they exhibited a characteristic limax type of motion. Monopodial trophozoites of the limax type (Fig. 5) predominated in cultures on agar plates. When observed in hanging drops, trophozoites washed off agar plates often changed direction of movement, forming lateral branches of the same breadth as monopodial forms (Figs. 1–3,9). The

Figs. 9,10. *Hartmannella vermiformis*, trophozoites of clone TN 102/I, SEM, bar = 5 µm. **Fig. 9.** The most common monopodial trophozoite of limax type (left over) and trophozoites with lateral branches. **Fig. 10.** Exceptional finding of extensively branched trophozoite. **Fig. 11.** Mature cyst of *H. vermiformis*, SEM, bar = 5 µm.

hyaline cap was distinct in the locomotory end; the posterior end (uroid region), was slightly constricted and persisted during locomotion as a relatively stable structure. The length (L) of live trophozoites was 34.1 (22.5–42.5) µm, breadth (B) 4.1 (2.5–5.0) µm, L/B ratio 8.32. The diameter of nucleus was 2.4 (2.0–3.0) µm. The average rate of locomotion of trophozoites observed in water under the cover slip was 36.4 µm/min. Floating forms of trophozoites were rounded with thick short projections; 4 to 5 long pseudopodia were observed only exceptionally.
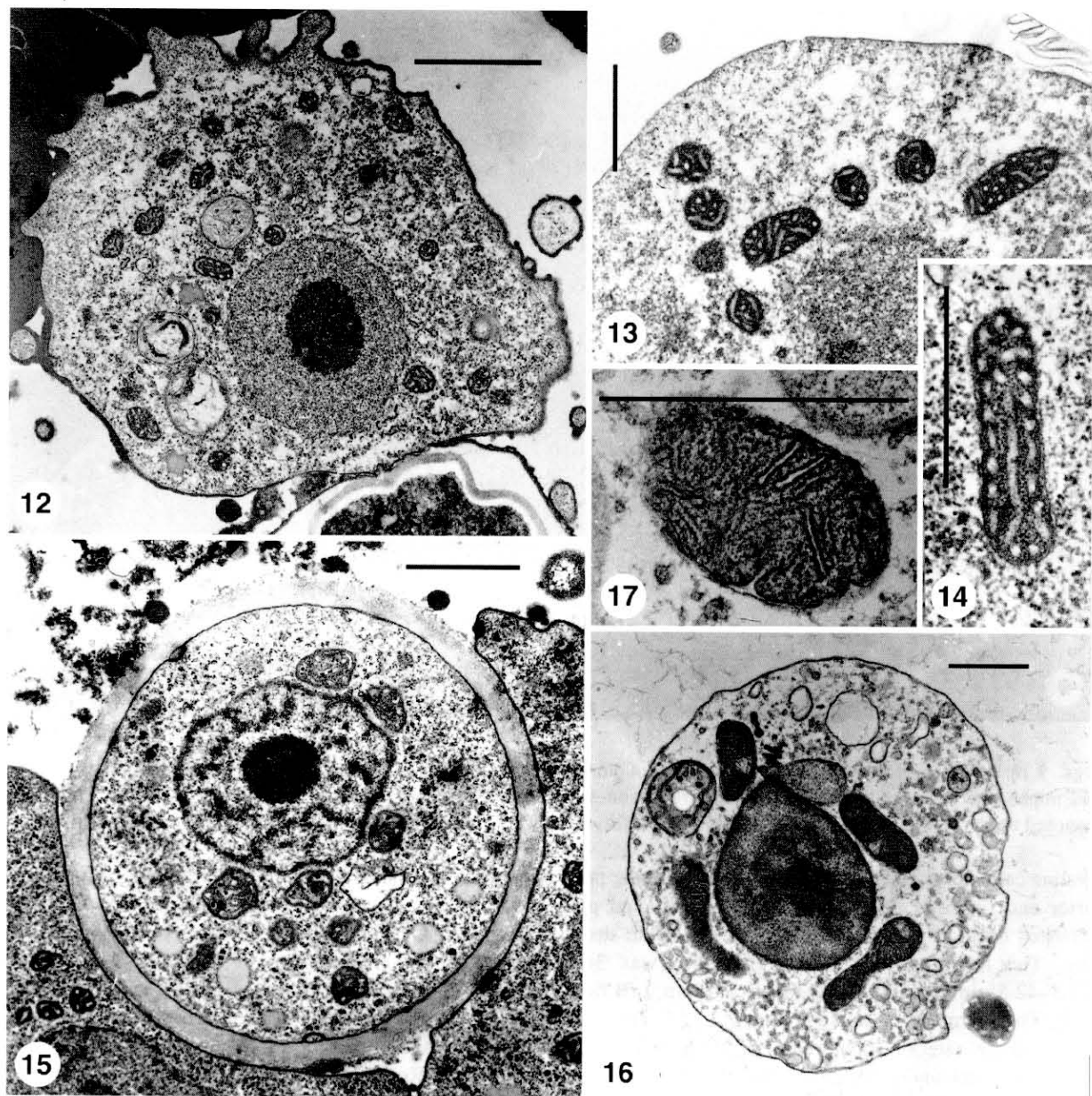
Cysts were spherical or slightly oval with a smooth surface (Figs. 7,8,11); their diameter was 7.6 (6.0–9.5) µm.

Of the liquid media found suitable for cultivation, MEM was the medium of choice. Clone TN 102/I was successfully cultured at 37°C (three subsequent passages were observed).

The trophozoite and cyst morphology observed in light microscope was consistent with the original description of *Hartmannella vermiformis*. Also the size range fitted in the known data. Ultrastructural details observed on trophozoites, encysting cells and cysts from stationary culture in liquid medium (Figs. 12–15), were in favour of this determination. Chromatin bodies between nucleolus and nuclear membrane were present in all stages, most pronounced being in encysting cells (Fig. 15). Oval or slightly elongate mitochondria possessed tubular cristae (Figs. 13,14) with the diameter of 42 nm corresponding to the original description. Obviously, not all the tubular cristae were straight. In several longitudinal sections they gave the impression of being branched. Rough endoplasmic reticulum occurred as long flattened cisternae. Discrete sucker-like structures were not clearly discernible on the surface of trophozoites. Contrary to the lack of discernible details in the fine structure of mature cysts, encysting cells contained more ultrastructural details. They were uninucleate, smooth walled, with mitochondria surrounding the nucleus (Fig. 15).

***Rosculus ithacus* (clone TN 99/I)** isolated from the kidney tissue of Nile tilapia, June 1994: Trophozoites (Fig. 18) were flat, mostly elongate oval, or less frequently spatulate. Due to attachment of their posterior end to the substratum trophozoites were sometimes flabelliform. In locomotion, a thicker granuloplasmic mass extended in the flattened hyaloplasm. Length (L) of trophozoites in locomotion was 7.3 (5.0–11.0) µm, breadth (B) 6.0 (4.0–9.0) µm, L/B 1.21, diameter of nucleus 1.3 (1.0–2.0) µm. The average rate

Figs. 12–15. Ultrastructure of *Hartmannella vermiformis*, clone TN 102/I. Trophozoite (**Fig. 12**), part of trophozoite with mitochondria (**Fig. 13**) and detailed view of mitochondrion containing tubular cristae (**Fig. 14**). **Figs. 16,17.** Ultrastructure of *Rosculus ithacus*, clone TN 99/I. Trophozoite (**Fig. 16**) and detailed view of mitochondrion containing discoid cristae (**Fig. 17**). Bar for Fig. 12 = 2 µm. bars for Figs. 13–17 = 1 µm.
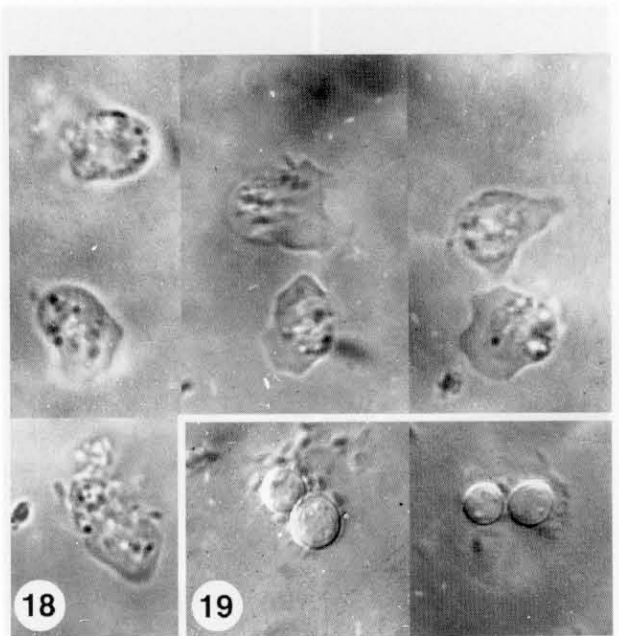
of locomotion of trophozoites was 36.4 µm/min. Cysts (Fig. 19) were spherical or slightly oval with the diameter 4.1 (3.0–5.5) µm. Trophozoites suspended in liquid medium were rounded, and they did not form a special floating phase. Flagellate phase could not be induced. The strain is currently maintained on agar plates, subcultures failed to grow in liquid media. Co-cultivation with cell culture (EPC cell line) was successful for one passage only. The strain did not grow at 37°C. Comparisons of parallel cultures grown on modified agar media

proved the positive effect of malt and yeast extract. Unfortunately, this complement also stimulated bacterial growth. On the basis of light and electron microscopy, clone TN 99/I could be identified with *Rosculus ithacus*, previously isolated also from *Carassius auratus* (Dyková et al. 1996). Ultrastructural observations confirmed the presence of discoid cristae in mitochondria (Figs. 16,17).

The unidentified amoeba (clone TN 900/I) isolated from the liver of Nile tilapia, January 1993:

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Trophozoites (Figs. 20,21,23) were flattened on the perimeter, and the hyaloplasm, not always well defined, formed a narrow rim with blunt, short, transitory subpseudopodia. Individual long projections and rhizoidlike short filaments at the posterior end were only exceptionally observed in hanging drop preparation, while under the pressure of cover slip their presence was very common. Locomotive forms were longer than wide. Their length (L) was 13.3 (7.0–20.0) μm, breath (B) 7.1 (5.0–13.0) μm, L/B ratio 1.87. The rate of locomotion of trophozoites was calculated as 34.8 μm/min. Normal locomotive forms mostly changed direction of the motion at a right angle. Eruptive activity was never observed. Individual inconspicuous folds were observed quite frequently. The diameter of nucleus was 2.3 (2.0–3.0) μm. Cysts (Figs. 22,24) were spherical and smooth with a diameter 5.5 (5.0–7.0) μm. Spherical floating forms were characterized by pointless and several (usually 3) needle-like projections whose length slightly exceeded the diameter of the cell. In hanging drop preparations observed in the light microscope, rounded forms resembling precystic stages transformed into irregular trophozoites very quickly. Flagellate transformation was not achieved. Amoebae grew at room temperature, but a trial at 37°C was negative. A cloned strain is currently subcultured on agar plates streaked with heat-killed *Bacillus subtilis*. Results of repeated attempts at growing the strain in liquid media have so far been inconsistent. Patterns of morphology which were observed in trophic stages in the light microscope resembled diagnostic features of mayorellid amoebae.
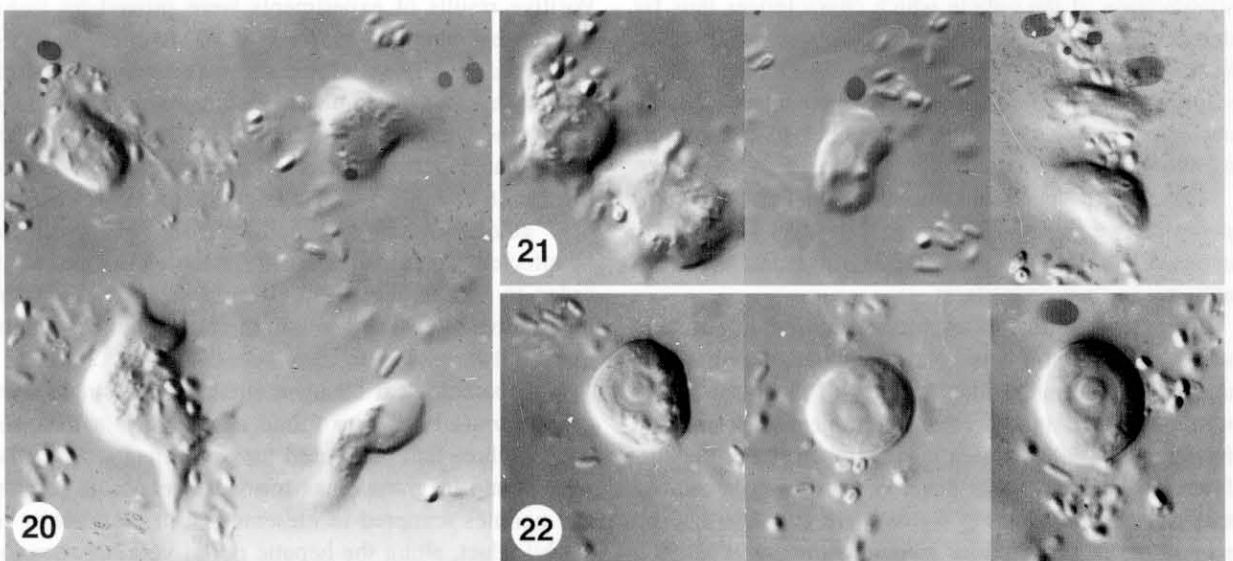
In the transmission electron microscope, trophozoites with the plasma membrane covered by a fuzzy filament-
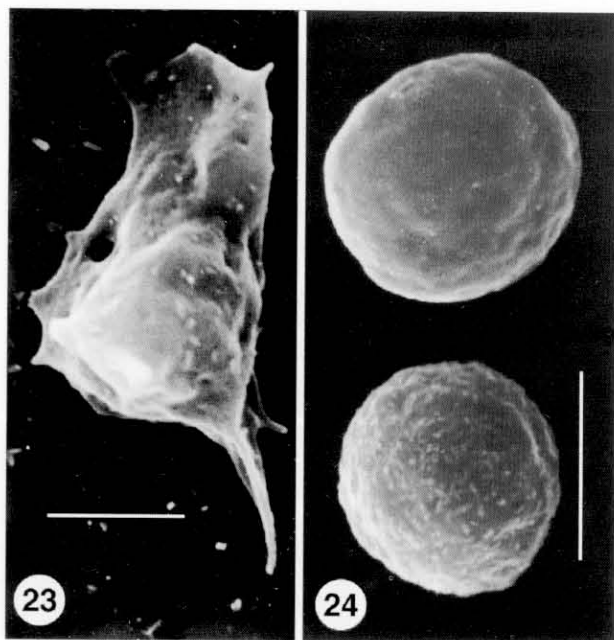

Figs. 18,19. *Rosculus ithacus*, clone TN 99/I. **Fig. 18.** Trophozoites observed in hanging drop, × 1700. **Fig. 19.** Two doublets of cysts observed in Nomarski differential interference contrast, × 1600.

ous material contained ovoid, bean shaped or slightly constricted mitochondria with tubular anastomosing cristae (Figs. 25–27). The arrangement of surface coat (Figs. 28,29) resembled the type demonstrated in *Platyamoeba* Page, 1969 (Page 1988). Trophozoites of this type were found only among cells fixed for TEM *in situ*, on the surface of agar plate cultures.

Cells covered with a layer composed of fine fibrils running parallel to the plasma membrane (Fig. 30) were

Figs. 20–22. Unidentified amoeba, clone TN 900/I. **Figs. 20,21.** Trophozoites observed in hanging drop, × 2300. **Fig. 22.** Sphaerical, resting or cyst forming stages, × 2300, Nomarski differential interference contrast.

Figs. 23,24. Unidentified amoeba, scanning electron micrographs of clone TN 900/I. **Fig. 23.** Trophozoite, bar = 5 μm. **Fig. 24.** Cysts, bar = 5 μm.

the most numerous in culture of the same passage which were fixed for TEM after being washed from the agar surface. The shape of such cells was mostly irregular, their cytoplasm increased in electron density. These cells contained vacuoles and vesicles, but the dense content (food particles) were not discernible in the digestive vacuoles. Because of irregular shape and character of the surface layer which did not follow in detail the contour of the plasma membrane, the cells were similar to cuticle bearing trophozoites of mayorellid amoebae. No stratification of the cuticle which characterizes thus far described trophozoites of *Mayorella* spp. was observed in the cloned strain TN 900/I. In the more advanced stage, cyst forming cells possessed filamentous material adjacent to the layer running parallel to the plasma membrane.

Cysts were spherical, smooth, with the wall composed of two layers. Thin outer wall layer was closely apposed to the inner one (Fig. 31) or separated, probably due to fixation artifacts.

Since morphology of trophozoites differed noticeably while observed on agar plates, in hanging drops or under the cover slip, other features of diagnostic value were compared in order to determine the strain. The facts that TN 900/I was a cyst forming clone, the surface of trophozoites was covered with fine filamentous material and the floating forms were characterized by relatively short projections, excluded the genus *Vannella* from consideration. Despite similarities in trophozoite morphology, the size of trophozoites and the

absence of stratification in the layer coating irregular cells of the strain TN 900/I eliminated all species of *Mayorella (sensu stricto)* from consideration.

Because light microscopy of the cloned strain TN 900/I revealed characters attributable to several genera, we have tried to classify it according to the ultrastructural features of trophozoites which were fixed *in situ* on agar plates. These features did not contravene the assignment to the genus *Platyamoeba*. However, the exact determination has to be postponed until inconsistencies existing in earlier descriptions are clarified.
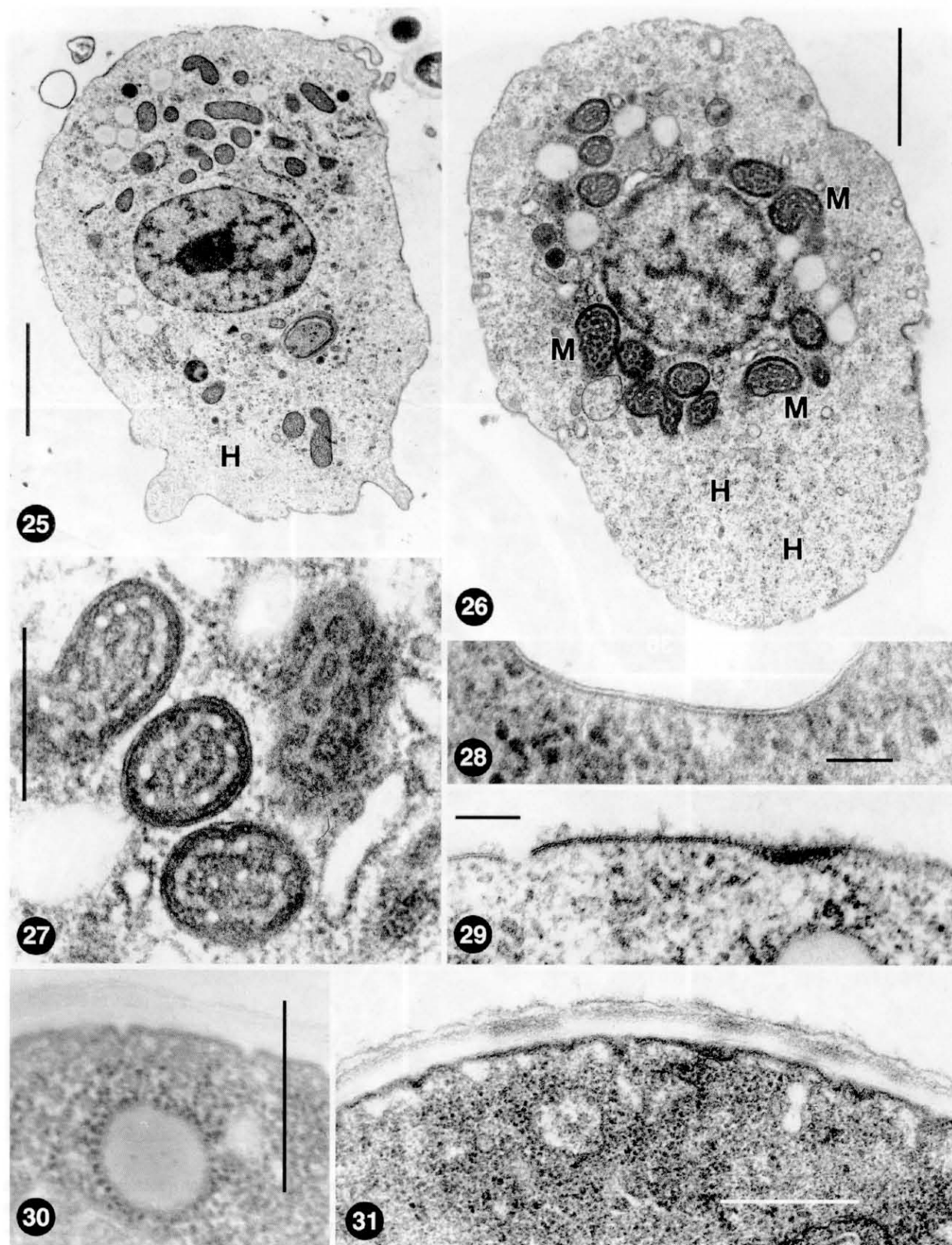
b) Histological examination of farmed tilapias

Histological examination of organs of farmed tilapias confirmed the good health condition of the whole stock. No correlation was found between positive results of isolation and tissue alterations. Considering the high density of tilapia population per tank, spectrum of the organ lesions observed was surprisingly poor. Small xenomas filled with mature spores of microsporidia were found in the kidney tissue of two specimens, and granulomatous lesions of unknown etiology were found in the liver parenchyma of three specimens. Dystrophic changes in renal tubules resembling initial stages of nephrocalcinosis were the most common lesions.
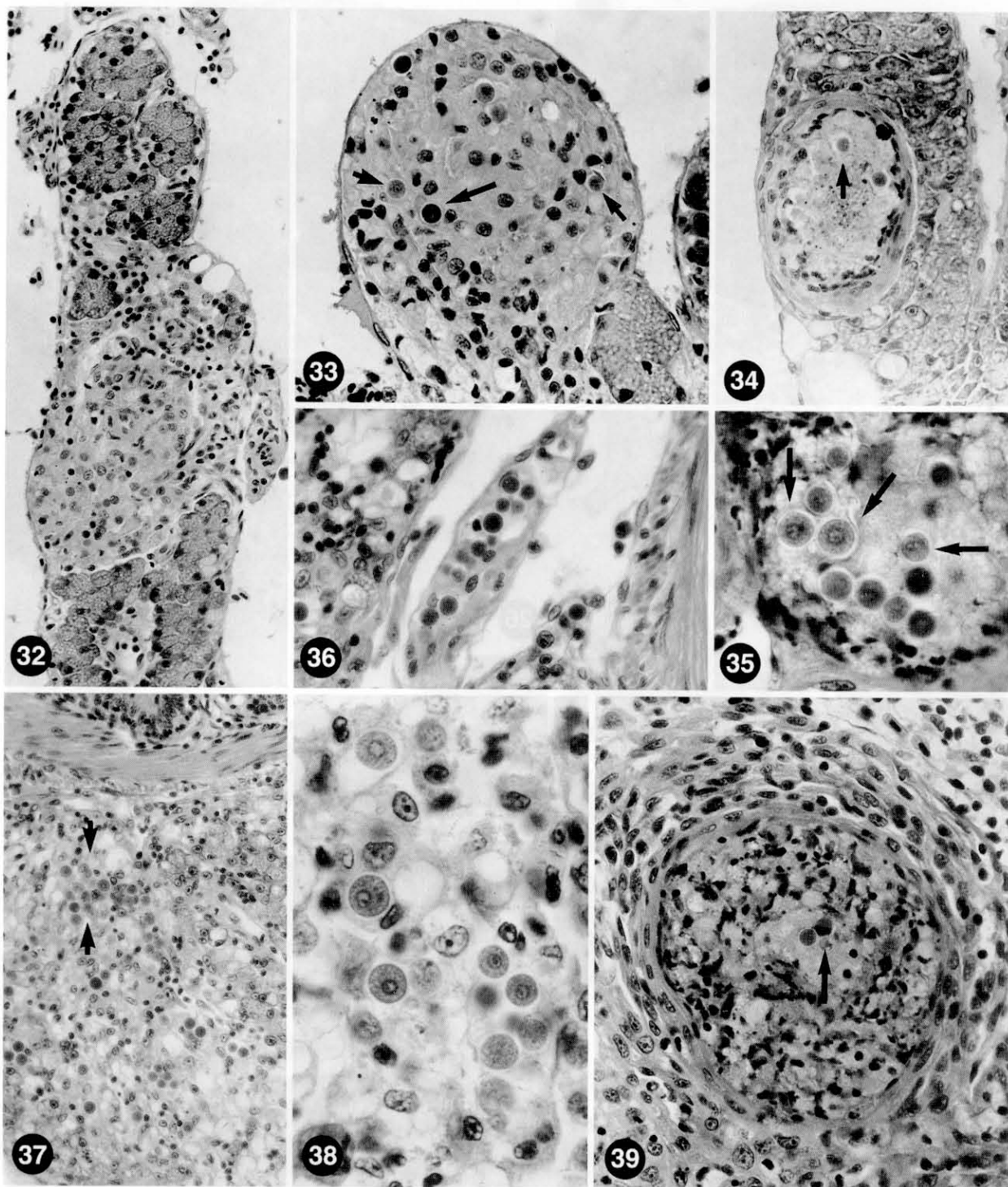
c) Experimental infections

Experimental infection with *Hartmannella vermiformis* (cloned strain TN 102/I) succeeded in two fish host species selected for preliminary experiments. The attempts to infect Balb mice with the same agent failed. Positive results of experiments were proved by histological examination in 7 out of 10 tilapias and in 4 out of 6 carps. The etiology of granulomatous lesions found in 1 out of 6 infected goldfishes was not clear. By the method of de Jonckheere (1980) *H. vermiformis* was isolated from 6 out of 10 experimentally infected tilapias 16 and 30 days post infection (dpi), from 1 out of 6 goldfishes (39 dpi) and from all (6) specimens of infected carps (40 dpi). Of the organs sampled in order to isolate the agent, liver and spleen had the highest positivity rate in all three species of experimental fish.

Histopathological changes elicited by a single dose of *H. vermiformis* were found exclusively in the pancreas in three fish hosts used for experiments. Amoebae were found in granulomas formed in exocrine pancreatic nodules scattered in mesenteries, in the vicinity of the bile duct, along the hepatic portal vein between the spleen and liver, and within the liver (Figs. 32–39). While 16 dpi early stages of the epithelioid granulomas

Figs. 25–31. Unidentified amoeba, ultrastructure of clone TN 900/1. Fig. 25,26. Trophozoites with a broad hyaloplasmic lobe (H) and ovoid mitochondria (M) around the nucleus. Bar for Fig. 25 = 2 μm, bar for Fig. 26 = 1 μm. Fig. 27. Detail view of mitochondrial cristae, bar = 500 nm. Figs. 28,29. Surface of trophozoites. Filamentous material more pronounced in Fig. 29, bars = 100 nm. Fig. 30. Surface of encysting cells, bar = 500 nm. Fig. 31. Cyst wall with a thin outer layer closely apposed to inner one, bar = 500 nm.

Figs. 32–39. Lesions caused by *Hartmannella vermiformis* in organs of experimentally infected fish (sections stained with hematoxylin-eosin). **Figs. 32,33.** Early stage of granuloma formation in the extrahepatic exocrine pancreas of carp (40 dpi) with amoebae (arrows) scattered among inflammatory cells, (× 312 and × 552). **Figs. 34,35,39**. Advanced stage of granuloma formation in the exocrine pancreas of tilapia (30 dpi), (× 360), and of carps (40 dpi), (× 1026) and (× 520). Amoebae (arrows) are discernible in the necrotic material of granulomas. **Fig. 36.** The branch of portal vein of tilapia (16 dpi) with intra- and perivascularly localized amoebae (× 556). **Figs. 37,38.** Extensive inflammatory changes developed among intestine and other organs of tilapia (16 dpi). Amoebae accumulated in small groups (arrows), pancretic tissue almost disappeared (× 345 and × 1026).

associated with amoebae were observed in 2 out of 4 specimens of tilapias (Figs. 37,38), 30 dpi the granulomas reached the productive stage (Fig. 34). Most advanced stages of granuloma formation were observed in experimentally infected carps (40 dpi). Also the extent of lesions was the largest in carps.

The morphology of amoebae found in histological sections was identical in all fish regardless of the interval post infection and/or phenotype used as an infective dose (trophozoites/cysts). The amoebae were globular with distinct nuclei and nucleoli, resembling resting stages or the early phase of cyst formation (Figs. 35, 38). This is why multiplication of amoebae in the host could not be conclusively proved. However, it was indicated by the different size of amoebae and their perivascular accumulation in the portal system (Fig. 36) as late as 40 dpi.

DISCUSSION

The screening of 193 farmed tilapias for the presence of free-living amoebae in organs resulted in a relatively low number of isolated strains compared to cyprinid fishes from ponds (Dyková et al., unpublished data) and to tilapias screened by Taylor (1977). He isolated several strains of *Acanthamoeba* and *Vahlkampfia* but none from any parenchymatous organ. Low number of isolated strains, along with the good health condition proved by histological examination, was indicative of good environmental conditions for tilapia in both farms. Contrary to the findings in a heated recirculation system (Nash et al. 1988), neither systemic amoebiasis nor losses were observed in the two, North-west and South Bohemian farms, where water quality, constantly deteriorated due to high stock density, was probably counterbalanced by the volume of water supply.

Taking into account that some variation in the measurements is inevitable, the morphology of cloned strain TN 102/I was consistent with the original description of *Hartmannella vermiformis*. Identifying the clone we faced the same problem which was mentioned by Nerad et al. (1995) for *Acanthamoeba pearcei*: different appearance of trophozoites of the same population on the agar surface versus in hanging drops where contracted due to influence of light and/or heat from the microscope. Except for one tiny free-hand sketch in the original description of *H. vermiformis* by Page (1967), trophozoites rapidly changing direction of locomotion have not been portrayed in the descriptive literature.

While Page (1985), who considered strains of *H. vermiformis* the most difficult to fix in a quality appropriate for TEM, recommended a mixture of glutaraldehyde and osmium tetroxide, we achieved the best results with osmium tetroxide fixation.

Trying to determine the cloned strain TN 900/I we have experienced the situation mentioned by Page (1988). Our strain had characters attributable to more than one genus. Despite the ultrastructural details available we have decided to postpone a definite classification.

The rapid transformation of trophic stages growing on agar plates into cyst forming stages under the influence of liquid overlay presented an unwelcomed practical problem: amoebae of irregular shape which were in the early stage of exocyst synthesis were at the beginning misinterpreted as cuticule bearing trophozoites. To reveal the ultrastructure of young trophozoites we have to fix them *in situ* on the agar plate surface.

Cell cuticle which did not follow exactly the contour of the cell surface was depicted by Page (1988) for *M. cantabrigiensis* Page, 1983, *M. penardi* Page, 1972 and *M. vespertilioides* Page, 1983, i.e., for those species of the genus *Mayorella* which are much bigger than the species in question. It could hardly be identified with *Mayorella* species of the similar size, i.e., with *M. microeruca* Bovee, 1970 or *M. spatula* Bovee, 1970, because they belong to the group of *Mayorella* species *sensu lato* still awaiting an exact generic diagnosis by means of TEM. Yet another point makes the diagnosis rather difficult and uncertain, namely that cysts have not been observed in the majority of described/named species of the genus *Mayorella*. Smooth, elliptical cysts have been mentioned only for *M. cultura* Bovee, 1961. Thus we could not compare the cyst wall with the cell cuticle, and because the inner structure of cysts is unknown, we also could not distinguish spherical resting stages of trophozoites from cysts.

Speculations on the pancreatic localization of lesions caused by *Hartmannella vermiformis* took into account the possible role of a pancreatic duct which opens into the anterior part of the intestine as well as the rather improbable injury of the anterior part of intestine due to inoculation. Since the same type of tissue response was observed in all fish hosts at any time p.i., and also in a smaller intrahepatic islet of pancreas, we could assume that amoebae reached pancreatic tissue via the portal vein and its branches. As revealed by isolations, they also got into other organs in small, hardly detectable numbers which did not provoke alterations.

Pathogenic potential of *H. vermiformis* observed in our preliminary experiments should be proven in a state of immunodepression. The latter was emphasized as a prerequisite for the entry of amoebae and their survival in cultured European catfish, *Silurus glanis,* by Nash et al. (1988).

REFERENCES

BONDAD-REANTASO M.G., ARTHUR J.R. 1989: Trichodinids (Protozoa: Ciliophora: Peritrichida) of Nile tilapia (*Oreochromis niloticus*) in the Philippines. Asian Fish. Sci. 3: 27–44.

CHEN S.C., TUNG M.C., CHEN S.P., TSAI J.F., WANG P.C., CHEN R.S., LIN S.C., ADAMS A. 1994: Systematic granulomas caused by a rickettsia-like organism in Nile tilapia, *Oreochromis niloticus* (L.), from southern Taiwan. J. Fish Dis. 17: 591–599.

ČERVA L. 1969: Amoebic meningoencephalitis. Axenic culture of *Naegleria*. Science 163: 576.

DE JONCKHEERE J.F. 1980: Growth characteristics, cytopathic effect in cell culture, and virulence in mice of 36 type strains belonging to 19 different *Acanthamoeba* spp. Appl. Environ. Microbiol. 39: 681–685.

DYKOVÁ I., LOM J., MACHÁČKOVÁ B., SAWYER T.K. 1996: Amoebic infections in goldfishes and granulomatous lesions. Folia Parasitol. 43: 81–90.

KALININA L.V., PAGE F.C. 1992: Culture and preservation of naked amoebae. Acta Protozool. 31: 115–126.

NASH G., NASH M., SCHLOTFELDT H.J. 1988: Systemic amoebiasis in cultured European catfish, *Silurus glanis* L. J. Fish Dis. 11: 57–71.

NATIVIDAD J.M., BONDAD-REANTASO M.G., ARTHUR J.R. 1986: Parasites of Nile tilapia (*Oreochromis niloticus*) in the Philippines. In: J.L. Maclean, L.B. Dizon and L.V. Hosillos (Eds.), The First Asian Fisheries Forum. Asian Fisheries Society, Manila, Philippines, pp. 255–259.

NERAD T.A., SAWYER T.K., LEWIS E.J., MCLAUGHLIN S.M. 1995: *Acanthamoeba pearcei* n. sp. (Protozoa: Amoebida) from sewage contaminated sediments. J. Euk. Microbiol. 42: 702–705.

PAGE F.C. 1967: Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. J. Protozool. 14: 499–521.

PAGE F.C. 1985: The limax amoebae: comparative fine structure of the Hartmannellidae (Lobosea) and further comparisons with the Vahlkampfiidae (Heterolobosea). Protistologica 21: 361–383.

PAGE F.C. 1988: A New Key to Freshwater and Soil Gymnamoebae. Freshwater Biological Association, The Ferry House, Ambleside, Cumbria LA22 OLP, 122 pp.

PAGE F.C., SIEMENSMA F.J. 1991: Nackte Rhizopoda und Heliozoea. Gustav Fischer Verlag, Stuttgart-New York, 297 pp.

ROGERS W.A., GAINES J.L. Jr. 1975: Lesions of protozoan diseases in fish. In: W.E. Ribelin and G. Migaki (Eds.), The Pathology of Fishes. University of Wisconsin Press, Madison, pp. 117–141.

TAYLOR P.W. 1977: Isolation and experimental infection of free-living amebae in freshwater fishes. J. Parasitol. 63: 232–237.