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Light and Electron Microscopic Studies on Various Developmental Stages of *Hoferellus cyprini* (Doflein, 1898)

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SUMMARY

The development of *Hoferellus cyprini* (Myxosporea) in common carp was studied by light and electron microscopy. It has been established that in the autumn the early developmental stages are located in a cell syncytium formed from the epithelium of the renal tubules. The authors share the opinion of Lom and Dyková [13] that each intracellular trophozoite corresponds to a primary parasitic cell which contains secondary, tertiary and quaternary cells having formed by internal cleavage. From December the trophozoites are released into the tubular lumen, and the secondary cells are transformed into coelozoic plasmodia. Spores are formed inside the plasmodia in the ureter or renal tubules in April. The sporoblast cells correspond to the quaternary cells of the trophozoites. On the surface of each of the two spore valves 10 striations run longitudinally and continue in bristles at the caudal end of the spores. Morphologically, the spores are consistent with the description of *Mitraspora cyprini* Fujita, 1912. Therefore, the authors regard *M. cyprini* as a synonym of *Hoferellus cyprini* (Doflein, 1898).

Introduction

Since its first description by Doflein [5], during a long period, *Hoferellus cyprini*, an enigmatic parasite, had been reported from common carp only by Mercier [15] and Plehn [19]. Subsequently, its occurrence was mentioned mainly in text-books [3, 20]. Only in 1981 was the parasite demonstrated again [12] from common carp living in Czechoslovakia. Subsequently, Körting and Herrmans [11] reported it from the Federal Republic of Germany, and Grupcheva et al. [9] from Bulgaria. In Hungary, Molnár [16] was the first to observe *H. cyprini* developmental stages; however, he identified them as a *Myxobolus* cyst. Plehn [19] is the only investigator who dealt with the development of *H. cyprini:* according to her in the winter months the vegetative stages develop in the tubular epithelium; then, in the early spring they enter into the tubular lumen and form spores.

In a previous paper Molnár et al. [17] reported that in the Hungarian pond farms *H. cyprini* was a frequent parasite of common carp. In the same publication they gave voice to their perception that *H. cyprini* was synonymous with the species *Mitraspora cyprini* Fujita, 1912 and its development followed a one-year cycle. In their opinion the early intracellular stage of the development of *H. cyprini* takes place in the epithelial cells of the renal tubules in the summer and autumn. In winter the intracellular forms are gradually released into the lumen of the renal tubules. Spore formation occurs during the spring, in the plasmodia situated in coelozoic position, in the tubular lumen, and in the ureter. In another paper Molnár and Kovács-Gayer [18] studied how the intracellular forms got into the lumen of renal tubules. The developmental cycle suggested by them essentially falls in line with the observations of Plehn [19] and is basically identical with the one reported by Ahmed [2] for *Mitraspora cyprini* parasitizing the goldfish.

Detailed morphological studies on *H. cyprini* were performed only by Lom et al. [14] and Lom and Dyková [13], who arrived at the conclusion that the intracellular stages

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of *H. cyprini* were nothing else but the developmental stages of some other myxosporean, probably *Sphaerospora renicola,* that had got to a dead-end of development. They contested the existence of the species *H. cyprini.*

In the present paper, on the basis of light and electron microscopic studies on the intracellular and coelozoic developmental stages of *H. cyprini,* we furnish evidence of its existence as well as its identity with *Mitraspora cyprini.*

Material and Methods

The test material consisted of two- and three-summer common carp derived from different pond farms of Hungary, primarily from a Transdanubian fish farm in which the infection rate was 70%. From this latter farm, common carp specimens were submitted for examination at two-week intervals in the winter.

The kidneys of the fish were examined in fresh squash preparations, similarly to the mucus squeezed out from a short portion of the ureter. From the infected organs impression smears were made and stained with Giemsa. For histological examination, the same organs were fixed in Bouin's solution or in 10% formalin buffered with NaH_2PO_4 and NaOH (pH 7.2). The frozen and paraffin-embedded sections were stained with haemotoxylin and eosin and according to the method of Farkas-Mallory [10].

Part of the samples taken for electron microscopy was fixed in 10% formalin buffered with NaH_2PO_4 and NaOH (pH 7.2) whereas the other part in 5% glutaraldehyde and 1% OsO_4. After drying in an ascending series of ethanol, the samples were embedded in Durcupan ACM resin. Sections were cut with a Reichert ultramicrotome and examined in a Philips 201 CS electron microscope.

Results

Light microscopy

Regarding the intracellular *Hoferellus* stages, our results are fully consistent with the observations of Lom and Dyková [13]. In the autumn months, histological preparations stained with haematoxylin and eosin frequently contained circumscribed areas 100–180 × 170–200 μm in cross section, involving a given segment of the renal tubules:

In these areas the tubular epithelial cells were filled up with masses of *Hoferellus* trophozoites 6 to 8 μm in diameter (Fig. 1).

The trophozoites situated intracellularly expanded the cells enormously and pushed the cell nucleus towards the basement membrane. The borders of the cells were indistinct; however, the parasites were demarcated from the tubular lumen by a narrow layer of plasma on which the brush border was well recognizable (Fig. 1). Early infection, restricted to a few cells only, was not observed.

The intracellular parasites revealed certain segmentation also by histological methods, and in impression smears stained with Giemsa 7–8 new individuals were clearly demonstrable within the parasite released from the host cell. The intracellular developmental stages started to get out into the renal tubules in December and January. At that time both the trophozoites situated intracellularly in the tubular epithelial cells and the plasmodia that had just entered the tubular lumen were demonstrable in the kidney (Fig. 2).

In the winter months following December, plasmodia of 15–40 × 15–30 μm in diameter (Fig. 3) were observed in the ureter and, less frequently, also in the renal tubules. The plasmodia were found freely in the lumen of the urether or loosely attached to its wall. There were 3–10 sporoblasts within a plasmodium.

Sporogenesis commenced in the plasmodia situated in the ureter in April, and *Mitraspora*-type spores having striations were formed [17]. In intensive infection, spore formation took place already in the tubular lumen.

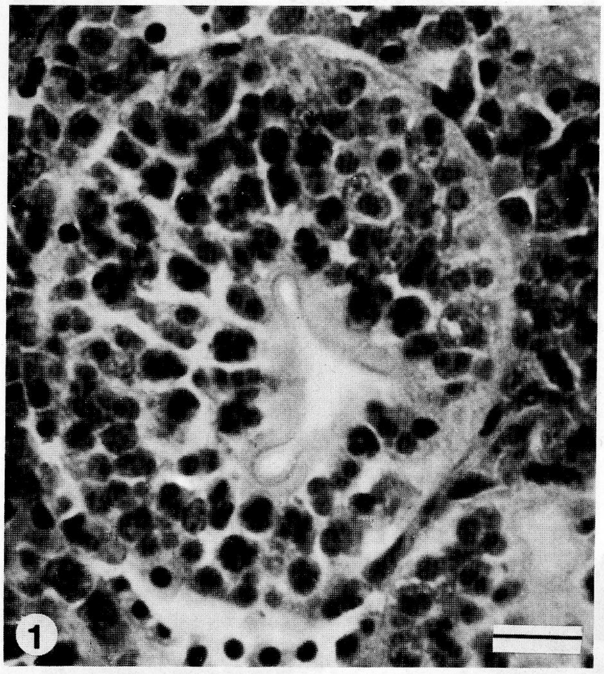
Electron microscopy

The intracellular developmental stages were studied on a material fixed in December.

The cell borders between the tubular epithelial cells infected by *H. cyprini* trophozoites were indistinct, the cytoplasms of the cells merged and the cells formed a large syncytium (Figs. 1 and 4). In the part of the cytoplasm neighbouring the brush border short reticula, irregular mitochondria and numerous electron-dense granules were seen. Scattered in the syncytial cellular material among the parasites were tubular mitochondria. The nuclei of the syncytium formed by the tubular epithelial cells exhibited vacuolar enlargement and became poor in chromatin. Little electron-dense material was attached to the more or less intact nuclear membrane.

The syncytium was filled with developmental stages of the parasite (Fig. 4): these stages filled the cytoplasm completely; however, towards the lumen of the renal tubule a relatively intact cytoplasm layer had remained on which intact microvilli were seen. The structure of the trophozoites embedded in the cytoplasm was identical with that reported by Lom and Dyková [13]. These parasites consisted of primary cells in which secondary, tertiary and quaternary specimens were distinguishable. Within the primary cell there was always only one secondary cell; in turn, the latter contained 3 to 5 tertiary cells (Fig. 5). The primary cells studied by us surrounded the secondary cells as a narrow margin, their cytoplasm contained small mitochondria, occasionally vesicula and short, convoluted endoplasmic reticula. The nucleus of the primary cell had usually lost its typical structure and was seen in the cytoplasm as an amorphous mass.

The cytoplasm of the secondary cell was rich in structures: besides the tertiary cells present in it, it contained numerous vesicula, ribosomes and mitochondria of varied form. Mainly at the periphery of the cytoplasm, there were formations of complex structure 1.5 to 2 μm in diameter, which contained a finely granular electron-opaque substance centrally, and a ring of vesicula and mitochondria at the periphery (Fig. 5). The outlines of the tertiary cells were clearly discernible within the secondary cells; however, neither the finer details of the strongly electrondense structures nor outlines of the quaternary cells were recognizable.



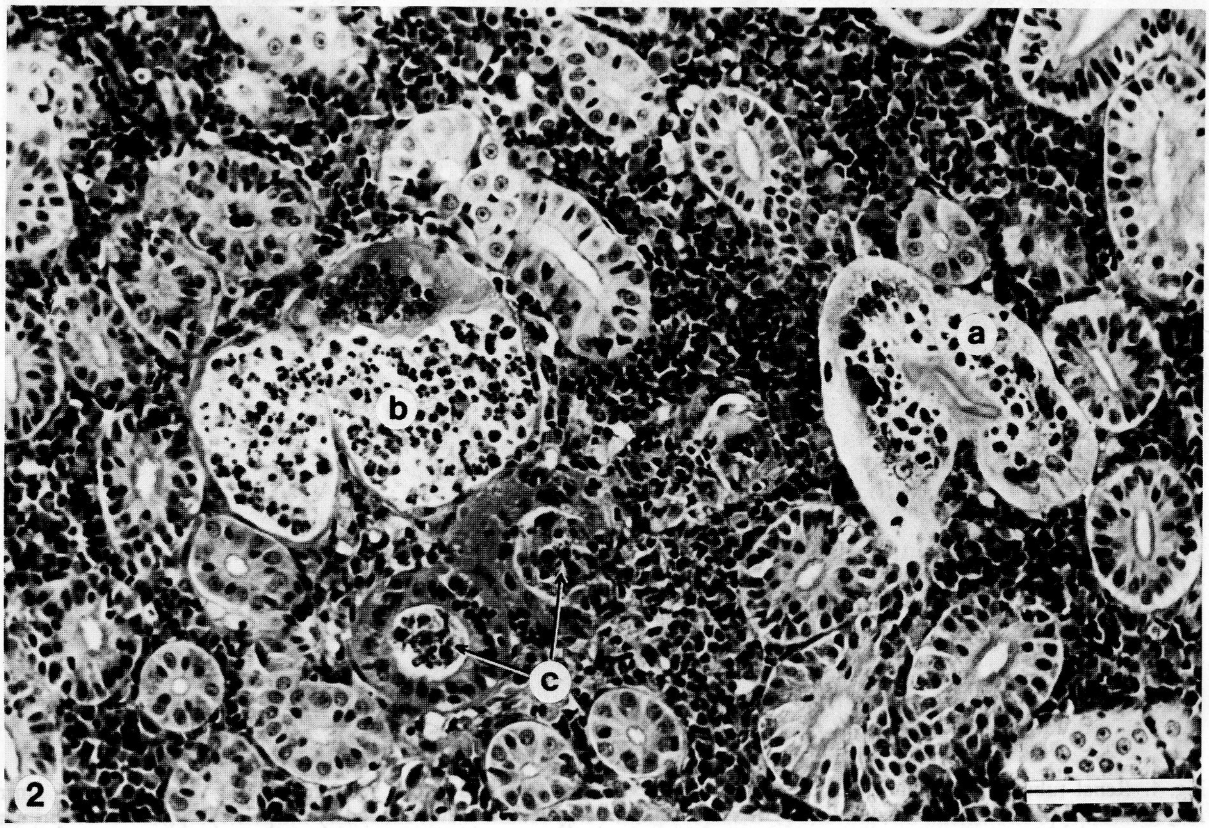
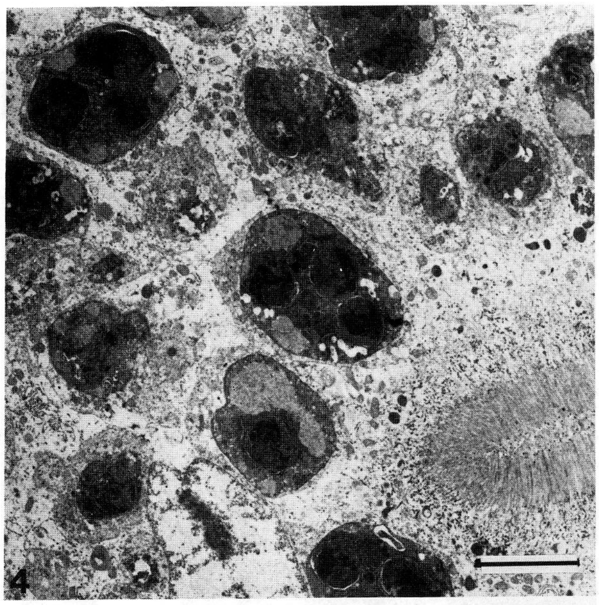
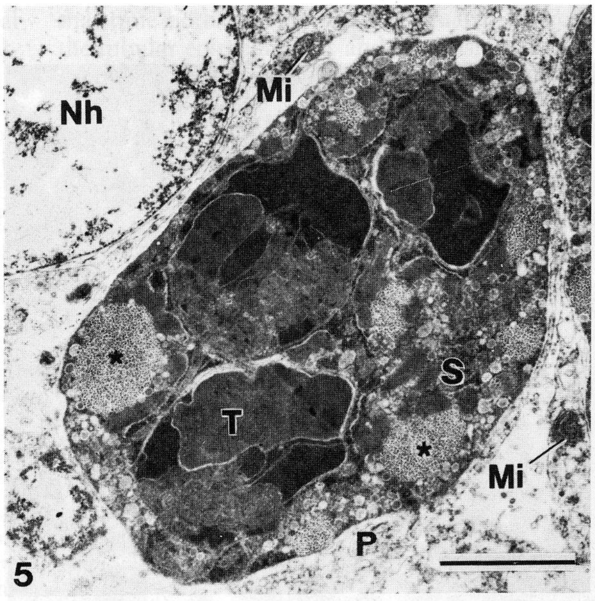
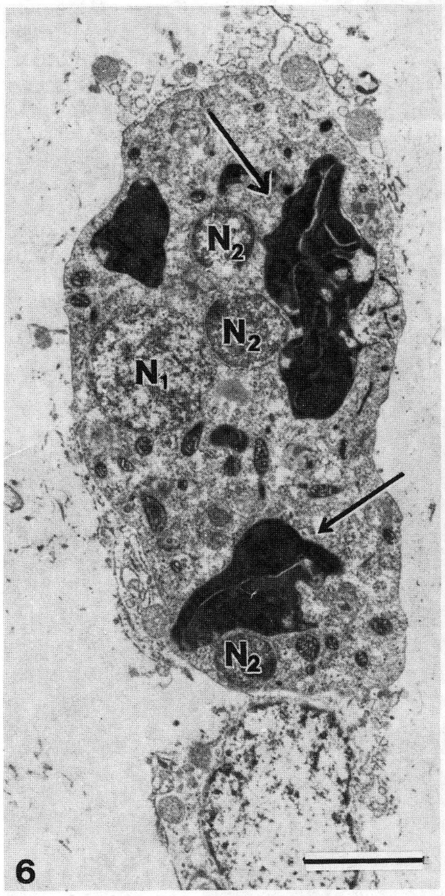


Fig. 1. Cross-section of a portion of a renal tubule infected by *Hoferellus* trophozoites. The cytoplasm of the tubular epithelial cell has changed into a syncytium and is filled with trophozoites. The host cell's nuclei are pushed near the basement membrane. On the luminal surface the brush border is relatively intact. H-E, bar = 20 µm. – Fig. 2. Renal tubules infected by *Hoferellus cyprini;* (a) intracellular forms; (b) tropozoites freshly released into the lumen of the renal tubule; (c) intraluminal trophozoites. H-E, bar = 100 µm. – Fig. 3. Plasmodia in the lumen of the ureter (arrow). H-E, bar = 100 µm.







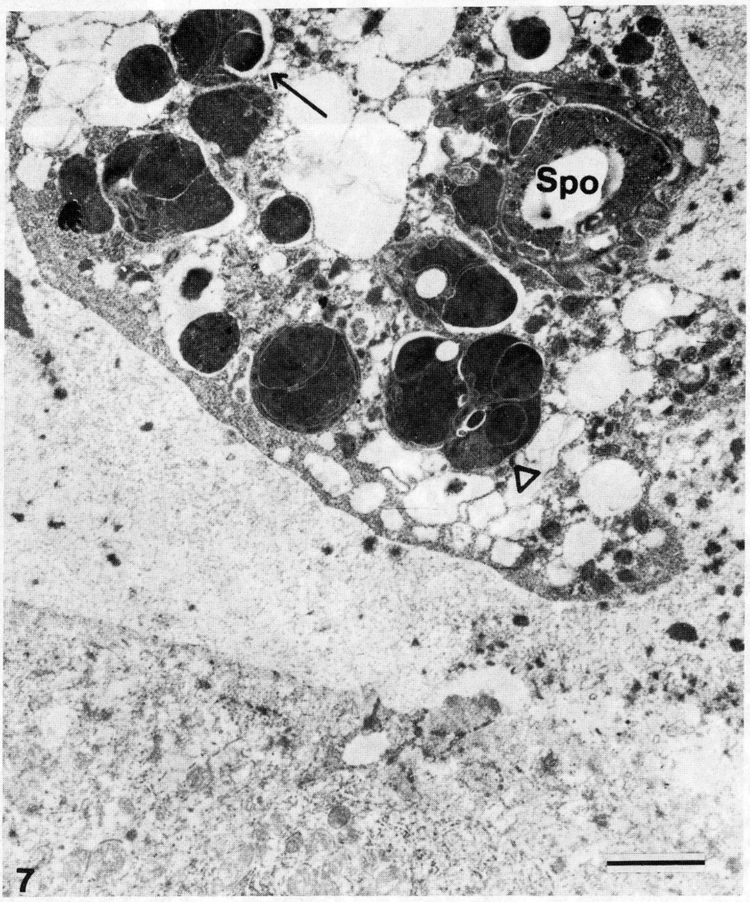
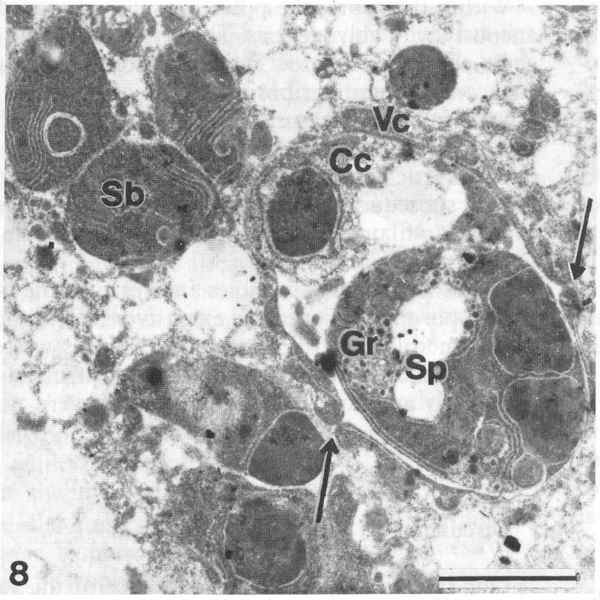
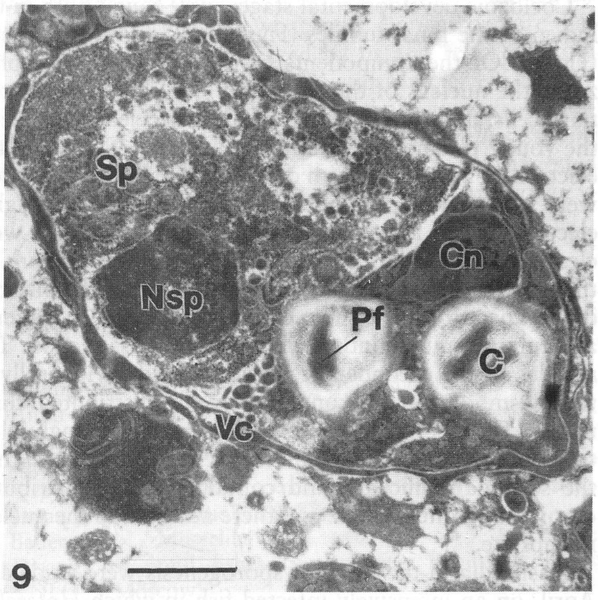


Fig. 4. Renal tubular epithelium infected by trophozoites and transformed into a syncytium. Bar = 5 µm. – Fig. 5. Intracellular *Hoferellus* trophozoite near the nucleus (Ns) of a host cell. The trophozoite is surrounded by the pale cytoplasm of the primary cell (P) containing mitochondria (Mi). In the cytoplasm of the secondary cell (S) three tertiary cells (T), and electrone opaque substance are seen. Bar = 2 µm. – Fig. 6. Young plasmodium in the lumen of the ureter. In the cytoplasm of the plasmodium developed from the secondary cell the nucleus of the plasmodium (N_1), the nuclei of the tertiary cells (N_2) and the quaternary cells (arrow) are seen (*). Bar = 2 µm. – Fig. 7. Plasmodium in the stage of sporogenesis in the lumen of the renal tubule. The foamy cytoplasm of the plasmodium contains distinct quaternary cells (arrow), quaternary cells arranged into a sporoblast (Δ) and formed spores (Spo). Bar = 2 µm.





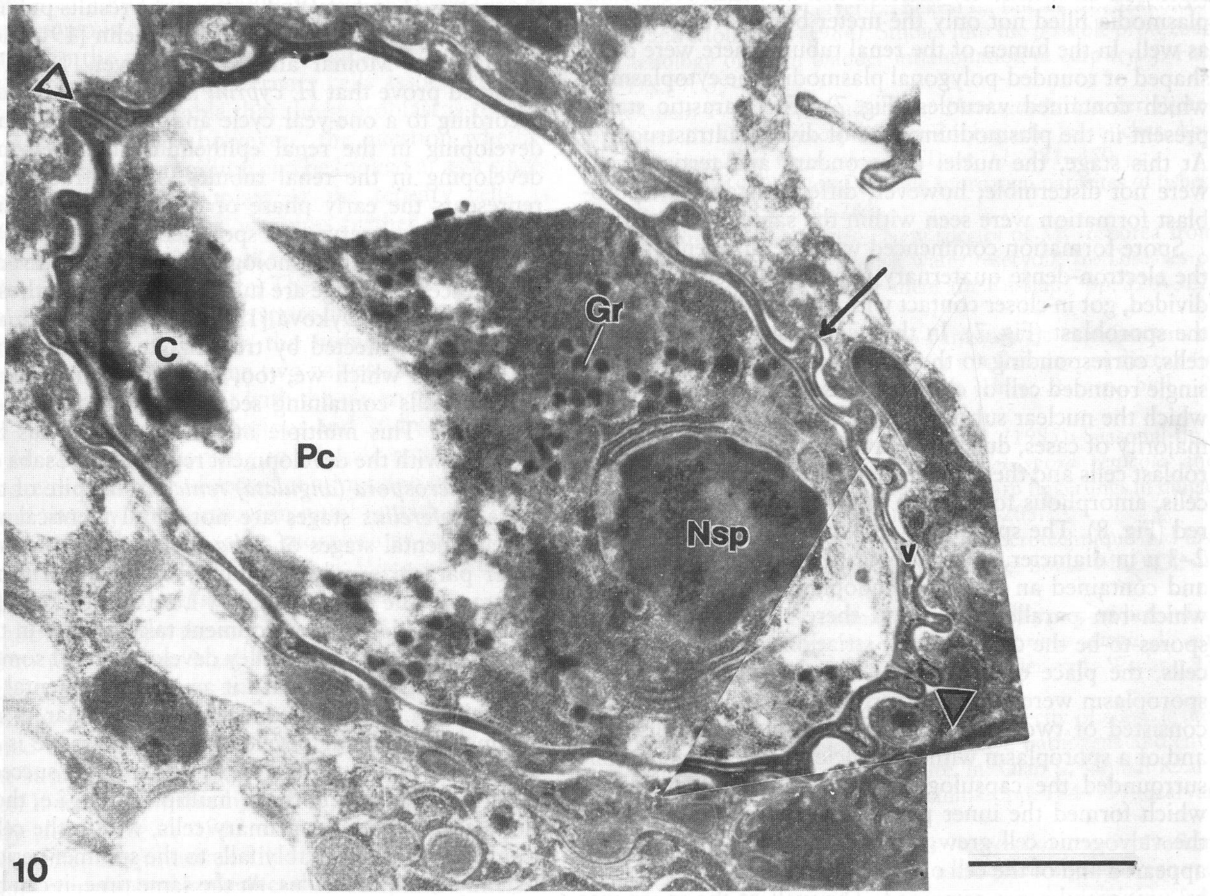


Fig. 8. Young spore in the plasmodium. Most of the spore is made up by the sporoplasm (Sp) having two nuclei. The cytoplasm of the sporoplasm contains fingerprint-like endoplasmic reticules and electron-dense (probably glycogen) granules (Gr). Of the capsulogenic cells (Cc), the nucleus of only one is seen. The spore wall (Vc) exhibits well-visible striations and sutures (arrow). Beside the spore sporoblast cells (Sb) of rich structure and associated with one another are seen. Bar = 2 µm. – Fig. 9. Young spore. Spore capsules (C), polare filament (Pf), nucleus of capsulogenic cell (Cn), sporoplasm (Sp), one of the nuclei of the sporoplasm (Nsp), spore wall (Vc). Bar = 1 µm. – Fig. 10. Longitudinal section of a mature spore: capsule (C), place of capsules lost for a technical reason (Pc), nucleus of the sporoplasm (Nsp) electron-dense granules (Gr), striations on the surface of the spore (arrow), sutures (Δ). Bar = 1 µm.

Coelozoic developmental stages were found both in the lumen of the renal tubules (Figs. 7–10) and in the ureter (Fig. 6). Of the components of the intracellular trophozoite, the nucleus of the secondary cell, nuclei of the tertiary cells, and the quaternary cells were clearly visible in the young plasmodium studied in January (Fig. 6). In the electron-opaque cytoplasm of the plasmodium, no borders were seen between the nuclei; however, with their highly electron-dense appearance the quaternary cells were clearly distinct from the cytoplasm of the plasmodium. In the finely granular matrix of the cytoplasm of the plasmodium, apart from the short endoplasmic reticula, vesicula and tubular mitochondria were seen in cross section. The nucleus of the secondary cell was located peripherally in the plasmodium, and contained evenly distributed chromatin. Tertiary nuclei were seen near the strongly electron-dense quaternary cells: they were smaller in size than the secondary nucleus, and contained finely distributed chromatin granules, here and there attached to the nuclear membrane.

Plasmodia in the stage of sporogenesis were studied in April, on an intensively infected fish in which *Hoferellus* plasmodia filled not only the ureter but the renal tubules as well. In the lumen of the renal tubules there were oval-shaped or rounded-polygonal plasmodia, the cytoplasm of which contained vacuoles (Fig. 7). The parasitic stages present in the plasmodium were of diverse ultrastructure. At this stage, the nuclei of secondary and tertiary cells were not discernible; however, different stages of sporoblast formation were seen within the same plasmodium.

Spore formation commenced with the differentiation of the electron-dense quaternary parasite stages. These cells divided, got in closer contact with one another and formed the sporoblast (Fig. 7). In their simplest form quaternary cells, corresponding to the sporoblast cells, consisted of a single rounded cell of electron-dense cytoplasm (Fig. 7) in which the nuclear substance was hard to recognize. In the majority of cases, due to the division occurring in the sporoblast cells and the arrangement in groups of the dividing cells, amorphous formations of complex structure appeared (Fig. 8). The sporoblast cells arranged in groups were 2–3 μ in diameter, their cytoplasm was rich in structures and contained an extensive endoplasmic reticular system which ran parallel. Here and there the outlines of the spores-to-be the characteristic striations of the valvogenic cells, the place of the suture, and the formation of the sporoplasm were clearly discernible (Fig. 7, 8). The spore consisted of two valvogenic and capsulogenic cells each and of a sporoplasm with two nuclei. The valvogenic cells surrounded the capsulogenic cells and the sporoplasm, which formed the inner part of the spore. Subsequently, the valvogenic cell grew thinner, characteristic striations appeared and of the cell only a firm, striated wall bordered by unit membranes remained. The wall of the spore was strengthened by longitudinal striations, the number of which was ten per spore valve. On the spore end opposite to the polar capsule, the striations continued in thin bristles 4.5 to 6 μm in length. The suture of the spore valves, i.e. the meeting-line of the cells forming the spore wall, was perpendicular to the plane of the polar capsules (Fig. 10).

Within the spore, the polar capsules developed simultaneously with valvogenesis. The cytoplasm of the capsulogenic cell contained few reticula, mitochondria and vesicula, and numerous ribosomes. The nucleus was situated eccentrically and was rich in chromatin. Already at the beginning of sporogenesis, a round, membrane-bound body of reticular structure (matrix) appeared in the cytoplasm: subsequently, first a ring-shaped and wide, then a thin polar filament running in a corkscrew-like manner was formed (Fig. 9).

In addition to large vacuoles and vesicula, the cytoplasm of the sporoplasm contained extensive RER, mitochondria and numerous free ribosomes. The nucleus was electron-dense, round or slightly oval. The nuclear membrane ran irregularly and large chromatin granules were attached to it (Fig. 10).

Discussion

Our studies furnish further evidence of the validity of the species *H. cyprini* and support the results published on the development of this species by Plehn [19], Molnár et al. [17] and Molnár and Kovács-Gayer [18]. The data obtained prove that *H. cyprini* is a parasite that develops according to a one-year cycle and has intracellular stages developing in the renal epithelium and coelozoic ones developing in the renal tubules. The intracellular stage represents the early phase of development, whereas the coelozoic stage serves for sporogenesis.

In essence, the morphological features observed during the intracellular stage are fully consistent with those reported by Lom and Dyková [13] on *Hoferellus cyprini.* From the host cell infected by trophozoites, a giant syncytium develops, in which we, too, observed numerous primary parasite cells containing secondary, tertiary and quaternary cells. This multiple internal cleavage was basically identical with the development reported by Csaba et al. [4] for *Sphaerospora (angulata) renicola.* In spite of this fact, these *Hoferellus* stages are not at all identical with the developmental stages of *Sphaerospora renicola,* since the latter parasitize only the lumen of the renal tubules. In contrast to the opinion held by Lom and Dyková [13], we do not consider the development taking place in the renal epithelium to be a blind alley development of some myxosporean; rather, we regard it as a developmental stage of the valid *H. cyprini,* redescribed by Molnár et al. [17]. During intracellular development the parasite is actually multiplied. Unfortunately, we have not succeeded in observing the basic form of multiplication, i.e. the formation of the numerous primary cells, within the cell syncytium. This stage probably falls to the summer period insufficiently examined by us. At the same time, it can be stated that certain multiplication occurs also in the later intracellular stage, since 6 to 8 tertiary and/or quaternary cells are demonstrable in the secondary cell.

In our opinion, at the time of getting into the lumen, the intracellular *Hoferellus* stage containing multiple endogenous phases is transformed into a plasmodium which already contains the sporogonic developmental stages.

From the results of Molnár et al. [17] it is known that intracellular stages are not released into the renal tubules all at once but gradually from December to April. In all probability, from the trophozoites that get into the renal tubules early, less developed plasmodia will be formed, whereas from the ones that remain in the epithelial syncytium and are released from it only in the spring, plasmodia containing more developed sporoblasts will emerge. Thus, we presume that the tertiary and quaternary parasite cells of the intracellular phase already represent the sporogonic stage, and in the primary parasite cell situated in the host cell a similar process is enacted as within the plasmodium during sporogony. Obviously, the above fact, i.e. the prolonged release of *Hoferellus* trophozoits can be made responsible for the observation that from early-released, plasmodia spores are formed in the ureter or the urinary bladder, while from those released late, they are formed already in the lumen of the renal tubules.

Our results suggest that the primary cell surrounding intracellular trophozoites from the outside is destroyed soon after the trophozoites get into the lumen, and that the coelozoic plasmodia correspond to the secondary cells. The homogeneous cytoplasm of the plasmodium contains, in addition to the nucleus of the secondary cell, nuclei of the tertiary cells and the quaternary cells associated with them. It seems undoubtable that the sporoblast is formed by the quaternary cells; howevere, the question whether the nucleus of the plasmodium can be looked at as a single polysporoblastic pansporoblast or the tertiary cells as monosporoblastic pericytes, warrants further study.

The spores formed within the plasmodium are of typically *Mitraspora*-like structure. The sutures run longitudinally in the plane between the spore capsules, and there is a welldiscernible striation on the surface of the spores. There are 20 striations on the two valves constituting the spore wall. This is consistent with the observation of Achmerov [1] who found 20 striations and 20 bristles on the species *H. carassii*. According to Molnár et al. [17], the bristles are the frontally directed continuation of the striations, and so they are identical in number with the latter. Observations concerning spore morphology support the opinion of Molnár et al. [17], i.e. that *Mitraspora cyprini* is a synonym of *Hoferellus cyprini*.

By light microscopy the suture of the *H. cyprini* spores is hard to recognize in a reliable manner. Therefore, we share the opinion of Lom and Dyková [13] that the first descriptions of *Hoferellus* species [1, 5, 15] had probably illustrated the running of the sutures erroneously. Actually, the sutures run in a plane perpendicular to the polar capsules. If we assume this hypothesis, *Mitraspora cyprini* correctly described in 1912 corresponds to *Hoferellus cyprini* erroneously illustrated in 1898. What makes things even more complicated is the fact that Fujita [6] had reported *Mitraspora cyprini* from two hosts, the common carp *(Cyprinus carpio)* and the goldfish *(Carassius auratus)* at the same time. Since we do not know the host specificity of the parasite, it cannot be ruled out that two different parasite species live in the above two fish species. This possibility is suggested first of all by the fact that the *Hoferellus* species described from *Carassius* spp. [1, 7, 8] and *Mitraspora cyprini* described from the goldfish and studied by Ahmed [2] possess a somewhat more elongated spore that the *Hoferellus* spores observed by us and reported from the common carp by Doflein [5] and Mercier [15].

Apart from the possibility outlined above, we can establish that the developmental cycle of *Hoferellus cyprini* observed by us is fully consistent with the model suggested by Ahmed [2] based upon his studies on *Mitraspora cyprini.*

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Key words: Myxosporea – Developmental cycle – Morphology – *Hoferellus cyprini* – *Mitraspora cyprini*

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