

Studies on two new species of Microsporida hyperparasitic in adult *Allocreadium fasciatusi* (Trematoda, Allocreadiidae)

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SUMMARY

The vegetative and sporogonic stages of *Unikaryon allocreadii* sp.nov. and *Nosema gigantica* sp.nov., from the parenchyma of adult flukes, *Allocreadium fasciatusi* in the gut of the freshwater fish *Aplocheilus melastigma* from India, are described and figured. *U. allocreadii*, which is uninucleate throughout development and is disporoblastic, differs from *U. legeri* in that it occurs in adult flukes from a freshwater host. *N. gigantica* has diplokaryon nuclei, at least in the sporogonic stages, and is much larger than all other microsporidia in platyhelminths.

Hatching of the spores and pathogenicity are described and the mode of transmission to the flukes is discussed. Infected flukes, which are sluggish and opaque, are often displaced from the normal site of infection.

INTRODUCTION

While conducting studies in 1973 on the biology of *Allocreadium fasciatusi* Kakaji, 1969, one of us (R. M.) found adult flukes, which appeared sluggish and opaque, in the intestines of the small, freshwater fish *Aplocheilus melastigma* McClelland, 1839, collected at Waltair, India. The opaque flukes were infected with a microsporidium with small, broadly ovoid spores. We have assigned this species to the genus *Unikaryon* and we propose to name it a new species. *Unikaryon allocreadii* sp.nov. The infections were present in adult flukes at most times of the year. Among the flukes collected in February 1976 another microsporidium with much larger, ovoid spores was found additionally in double infections with *U. allocreadii*. Although all the stages of the life-cycle of the second species have not been observed, certain features suggest that it belongs to *Nosema* and we propose to call it *Nosema gigantica* sp.nov.

The fluke *A. fasciatusi* grows to the adult stage in the intestine of the fish and its larval stages develop in two intermediate hosts, the first being a snail, *Amnicola travancorica* Benson, 1960 in which two generations of rediae and the cercariae develop. Several species of copepods act as the second hosts, harbouring encysted

metacercariae (Madhavi, 1977). No microsporidia have been detected in the larval stages.

This paper gives an account of the prevalence of infection in *Allocreadium*, the developmental stages of the hyperparasites and the pathology of hyperinfection and also discusses features of special interest in the transmission of these Microsporidia.

MATERIALS AND METHODS

The fish hosts *Apolecheilus melastigma* were collected from a stream at Waltair, Andra Pradesh (India). Flukes collected from the intestines of these fish were examined in saline, and infected specimens were detected by their opacity under reflected light. Some infected flukes were used for smears and others were sectioned. For making smears, flukes were teased apart on a slide in saline or in water. The smears were dried in air, fixed in methanol and stained for 30–40 min with Giemsa's stain directly, or after hydrolysis for 10 min in 1 M HCl. Some wet smears were fixed in Carnoy's fluid, hydrolysed for 8 min and stained with Schiff's reagent in order to demonstrate nuclei by the Feulgen reaction. Fresh spores in saline were also examined. For sections, infected flukes were fixed in Bouin's or Carnoy's fluid, dehydrated in ethanol, embedded in wax and sectioned at a thickness of 5 µm. The sections were stained with iron haematoxylin or by the Feulgen or periodic acid-Schiff techniques. Normal flukes, similarly sectioned and stained, were also examined.

RESULTS

Prevalence of infection

The number of fishes examined, their trematode infections and the infections of *U. allocreadii* in the trematodes are given in Table 1. The microsporidian infections occurred throughout the period of observation except during September and December. The failure to find microsporidia during December 1975 was considered to be abnormal since they had been found in December of other years and their absence at this time was probably due to the low number of flukes examined and the fact that many of them (10 out of 18 examined) were immature.

Complete records for previous years were not kept, but it was noted that there was regular depletion or total absence of hyperinfections in the late summer months, with reappearance towards October. Immature and mature flukes could be found throughout the year, but peak numbers of immature forms occurred in September at a time when there were few adults. This, coupled with the observation that microsporidian infections in October were mainly light, with vegetative phases predominating over spores, suggested that the onset of microsporidian infection was coincident with the peak period of transmission of the trematodes.

Infected and uninfected trematodes were never found together, all trematodes in a host fish were either infected or non-infected.

Table 1. Infection of *Allocreadium fasciatusi* and of *Unikaryon allocreadii* in the fish *Aplocheilus melastigma*

Date	Number examined	Number of fishes			Number of flukes	
		Infected with <i>A. fasciatusi</i>	Containing infected flukes	% of fishes containing infected flukes	Collected	Infected with <i>U. allocreadii</i>
April 1975	32	9	4	12.5	14	5
May 1975	39	6	2	5.1	7	2
June 1975	30	8	2	6.6	11	3
July 1975	25	16	1	4.0	85	3
August 1975	30	6	1	3.3	12	1
September 1975	25	22	0	0	170	0
October 1975	23	12	2	8.6	47	7
November 1975	30	11	3	10.0	13	3
December 1975	20	9	0	0	18	0
January 1976	27	10	3	11.1	34	11
February 1976	23	6	3	13.0	21	12*
March 1976	30	7	1	3.3	9	1
April 1976	26	10	3	11.5	46	14
May 1976	25	10	4	16.0	21	8
June 1976	11	3	1	9.09	13	2
July 1976	20	3	2	10.0	3	2

* 8 flukes from a single host had double infection with *Nosema gigantica*.

Description of *Unikaryon allocreadii* sp.nov.

Schizonts were uninucleate or binucleate. All had clear hyaline cytoplasm forming a peripheral layer surrounding the nuclei. The majority had compact densely staining nuclei, which were either uniformly dark (Pl. 1A) or had one or more pale-staining areas within the chromatin mass (Pl. 1B, C). These nuclei were sharply defined from the hyaline cytoplasm by a ring of pale cytoplasm. A fine strand between the two nuclei of some binucleate schizonts (Pl. 1C) was suggestive of the final link between daughter nuclei separating from the uninucleate state. Nuclei of binucleate schizonts were never in diplokaryon form (Pl. 1C-F).

Certain uninucleate schizonts, which appeared more attenuated than others, possessed a larger and characteristically reticulate nucleus (Pl. 1G, H). Binucleate forms with this type of nucleus were not seen. They may have represented a pre-sporont stage.

Uninucleate schizonts were rounded and measured $4.7 \pm 1.5 \times 4.3 \pm 1.2 \mu\text{m}$ ($n = 20$). Binucleate schizonts were usually ovoid and measured $5.8 \pm 1.0 \times 3.4 \pm 0.5 \mu\text{m}$ ($n = 12$).

Sporonts were spindle-shaped cells (Pl. 1I-M) in which the cytoplasm was less uniformly stained than that of the schizonts. At first they possessed a single, central nucleus which was often irregular. Nuclear division (Pl. 1L) gave rise to binucleate sporonts (Pl. 1M). The daughter nuclei migrated to the poles of the sporont (Pl. 1N) which then became dumbbell-shaped (Pl. 1O, P) and separated into 2 sporoblasts. Sporoblasts were broadly ovoid cells with a gran-

ular nucleus either rounded and terminal (Pl. 1R) or in the form of a subterminal band (Pl. 1Q).

Sporonts measured $5.2 \pm 0.4 \times 2.3 \pm 0.2 \mu\text{m}$ ($n = 10$). Sporoblasts were very uniform in size, measuring $2.9 \pm 0.3 \times 2.1 \pm 0.2 \mu\text{m}$ ($n = 10$).

Spores when fresh (Pl. 1T) were broadly ovoid, slightly asymmetrical, with one side flatter than the other. There was a large vacuole usually antero-lateral in position. Fresh spores measured $3.5 \pm 0.3 \mu\text{m} \times 2.7 \pm 0.2 \mu\text{m}$ ($n = 50$).

In Giemsa-stained preparations the spores often appeared to have collapsed, with one side (probably that associated with the vacuole) distinctly concave. The single nucleus was large and round and lay towards the convex side. A conspicuous granule was often seen near, but not actually at, the anterior pole (Pl. 1S).

Description of Nosema gigantica sp.nov.

Stages of schizogony and sporogony were difficult to distinguish from one another. Large, rounded cells with 1, 2 or 4 nuclei could have belonged to either sequence (Pl. 2A-D). These cells possessed granular cytoplasm and nuclei in which the chromatin was distributed as a network of coarse granules. Division of the tetranucleate stages could have given rise to binucleates and these in turn to uninucleates. Smaller cells which were distinctly ovoid were identified as sporoblasts. These were uninucleate or binucleate (Pl. 2E-H), the latter giving rise directly to binucleate spores (Pl. 2I, J). Where 2 or 4 nuclei were present in any of these stages they were arranged as diplokarya (pairs of nuclei in apposition).

Only 4 tetranucleate stages were seen, and their size range was $9.8 \times 7.8 \mu\text{m}$ – $12.2 \times 7.8 \mu\text{m}$. Measurements of 20 uninucleate and binucleate stages together gave mean values of $8.5 \pm 1.0 \times 6.7 \pm 1.3 \mu\text{m}$. The size range was 6.8×5.3 – $10.8 \times 9.8 \mu\text{m}$.

Spores were not seen fresh. In Giemsa-stained smears they were ellipsoid in shape and slightly flattened anteriorly (Pl. 2I-L). Some detail of the internal structure could be determined, including the anterior polar cap (Pl. 2J, arrowed), some coils of the polar filament and 2 nuclei in diplokaryon form. Measurements of 50 spores gave mean values of $7.9 \pm 0.5 \times 4.9 \pm 0.3 \mu\text{m}$.

Hatching of spores

A remarkable feature of the two species was the ease with which spore hatching took place. During preparation of the Giemsa-stained smears many spores of both species everted their filaments and gave remarkably clear pictures of the pattern of sporoplasm emergence.

In *U. allocreadii* it was observed that the filament was almost completely, if not completely, everted before the sporoplasm began to emerge from the spore cavity (Pl. 3A). There was probably no further elongation of the filament during the emergence. The single nucleus passed through the tube, causing slight expansion of the diameter as it did so (Pl. 3B-F), and finally emerged with its cytoplasm as a very delicate, round or irregular sporoplasm with a highly irregular nucleus. Two chromatic dots of unknown nature were observed on opposite sides of the nucleus and the cytoplasm of the sporoplasm was barely visible after attenuation

in the smear (Pl. 3G, H). Extruded filaments measured up to 30 µm, and from the number of undulations (Pl. 3F) it was estimated that the filament was probably coiled about four or five times within the spore.

Spores of *N. gigantica* were not often seen completely hatched, probably because eversion of the very long filament in the smears was mechanically interrupted. Several views of the eversion were obtained: Pl. 3I shows a filament doubled back on itself; Pl. 4A shows a filament with the tip free, and part remaining coiled within the spore; Pl. 4B shows an almost complete extrusion, where the filament measures 190 µm and the two dense areas (arrowed) within the tube probably represent the 2 sporoplasm nuclei on their way to the exterior.

Pathogenicity

Infected flukes were easily distinguished by their opacity and sluggish nature. When placed with normal flukes in saline, the infected ones lost their activity much earlier than the normal ones. The microsporidian infection was confined to the parenchymatous tissue and appeared first in the peripheral parenchyma and subcuticular cells and then spread to the deeper parenchyma. Other organs were not infected. In heavy infections, the spores filled the parenchymatous cells and masked or destroyed their prominent nuclei (Pl. 4C, D). In flukes containing double infections, the spores of *N. gigantica* were restricted to the peripheral parenchyma while those of *U. allocreadii* were located also in the deeper parenchyma.

The heavily infected flukes were nearly always those which were mature or gravid. They were capable of producing viable eggs but there were fewer eggs in the uterus at any one time than in normal flukes, which may have been due to reduced fecundity or to impairment of the activity of the uterus.

The heavily infected flukes were often found in the posterior part of the intestine, distant from the normal sites of infection, the stomach and anterior intestine. This may have resulted from the inability of the sluggish flukes to maintain station against the peristaltic movements of the gut.

DISCUSSION

Fifteen species of Microsporida have been named from Platyhelminthes and two others, positively identified as Microsporida have been recorded but not named. Information relating to most of these has been summarized by Canning (1975) and the remaining two, *Nosema xiphidiocercariae* and *Nosema rhionicae* were described by Voronin (1974). Most of those species infecting trematodes were found in larval stages and only *Pleistophora danilewskyi* (Pfeiffer, 1895), *Microsporidium distomi* (Lutz and Splendore, 1908) and *Pleistophora ghigii* (Guyénot and Naville, 1924) were found in adults.

The two species recovered from *A. fasciatusi* were found only in adults, although larval stages in the two intermediate hosts were regularly examined. No microsporidian infections have been detected in the intestinal cells or other tissues of the fish. No other helminths capable of transmitting the spores were present in the gut and metacercarial stages of other flukes present in the muscles and gills

were free of infection. It is thus probable that the adult flukes are infected directly by spores present in the gut of the fish, though infections as metacercariae in the second intermediate hosts cannot be eliminated. *A. melastigma* is a small fish reaching a maximum size of 32 mm and is present throughout the year in large numbers in the stream. It feeds on algae (like *Spirogyra*) and copepods and other arthropods. The all or nothing infection rate of flukes in the fish suggests that the microsporidia are parasitic in some small invertebrate, serving as food for the fish, rather than that the spores are free in the water after dispersal from dead flukes. The autumnal rise in infection of the flukes could be related to seasonal infection of reservoir hosts or perhaps to a change in feeding habits of the fish.

Kramer (1960) produced the first convincing evidence that the microsporidian sporoplasm passes through, and is ejected from the tip of, a tubular polar filament. Using three binucleate species, he showed the passage of the two nuclei along the filament and their extrusion in a cytoplasmic globule at the tip. Since this time, the mechanism for the extrusion has been discussed by several workers (Lom & Vavra, 1963; Sprague & Vernick, 1968; Ishihara, 1968; Lom, 1972; Weidner, 1972) and an evaluation of the various theories has been given by Sinden & Canning (1974). Most of these authors agree that the filament is everted during extrusion. The osmophilic core of the filament observed in electron microscopy may be discharged and form part of the glycoprotein coat which has been demonstrated around the extruded filament by Weidner (1972).

The present demonstration of the passage of the sporoplasm through the filament shows that the whole filament is extruded before the sporoplasm begins to emerge. It thus appears that an initial force is required to evert the filament and that a second pressure is required to move the sporoplasm from the spore cavity to the tip of the filament. The ease with which the hatching process takes place and the large size of the *N. gigantica* spores indicate that these species would serve as excellent models for the study of the extrusion process.

Taxonomic position

The smaller of the two microsporidia from *A. fasciatusi* was uninucleate throughout its development and was disporoblastic, producing 2 sporoblasts and ultimately 2 spores from a single fusiform sporont. The spores lay free in the host tissue, not surrounded by a pansporoblast membrane. This falls within the definition of the genus *Unikaryon* (Canning, Lai & Lie, 1974) which has been placed in the family Unikaryonidae and sub-order Apansporoblastina by Sprague (1977). Two other species of *Unikaryon*, *U. legeri* named by Dollfus (1912) as *Nosema legeri* and transferred to *Unikaryon* by Canning & Nicholas (1974) and *U. piriformis* Canning, Lai & Lie (1974) are also parasites of Platyhelminthes, occurring in the metacercariae of *Meigymnophallus minutus* and rediae of echinostomes respectively. The size of the spores of the three species is similar. The piriform shape of the spores and the long polar filament (150 µm) of *U. piriformis* are distinctive, but the slight differences in general appearance and spore shape of the present species are hardly sufficient in themselves to justify its separation from *U. legeri*. However, infection of the adult fluke rather than the larvae in freshwater hosts as opposed

to marine hosts indicate that it is likely that they are distinct. For this reason we have considered it as a distinct species.

The larger microsporidium described in this study can be distinguished from all others in Platyhelminthes by the large size of its stages. It is currently considered characteristic of the genus *Nosema* that the sporont divides by binary fission to give 2 free sporoblasts each with nuclei in diplokaryon arrangement. Although in this species there was no means of distinguishing between schizonts and sporonts and the details of sporogony were not determined, sporoblasts not enclosed by a pansporoblast membrane and possessing diplokaryon nuclei were recognized. The ovoid shape of the spores is in accord with the generic diagnosis. These features suggest that the parasite should be assigned to the genus *Nosema*.

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EXPLANATION OF PLATES

PLATE 1

Stages of development of *Unikaryon allocreadii* (Giemsa-stained, except T).

A, B. Uninucleate schizonts.

C-F. Binucleate schizonts; note attachment between nuclei in C and D.

G, H. Uninucleate stages with reticular nuclei, possibly pre-sporonts.

I-M. Sporonts.

N-P. Division of sporonts into sporoblasts.

Q, R. Sporoblasts.

S. Stained spores; note the large nucleus and small polar granule.

T. Fresh spores; note the antero-lateral vacuole.

PLATE 2

Stages of development of *Nosema gigantica* (Giemsa-stained).

A-D. Large stages, with 1-4 nuclei, which are probably schizonts.

E-H. Sporoblasts; G shows one uninucleate and one binucleate sporoblast.

I-L. Spores; note the diplokaryon nuclei, the flattened anterior end and the polar cap (arrowed in J).

PLATE 3

A-H. Polar filament eversion and sporoplasm emergence in *Unikaryon allocreadii*. The compact sporoplasm nucleus passes through the everted filament. The cytoplasm of the sporoplasm is barely visible after emergence (G, H) and the nucleus is now irregular and granular; two chromatic dots (arrowed) lie on the opposite sides of the nucleus in G.

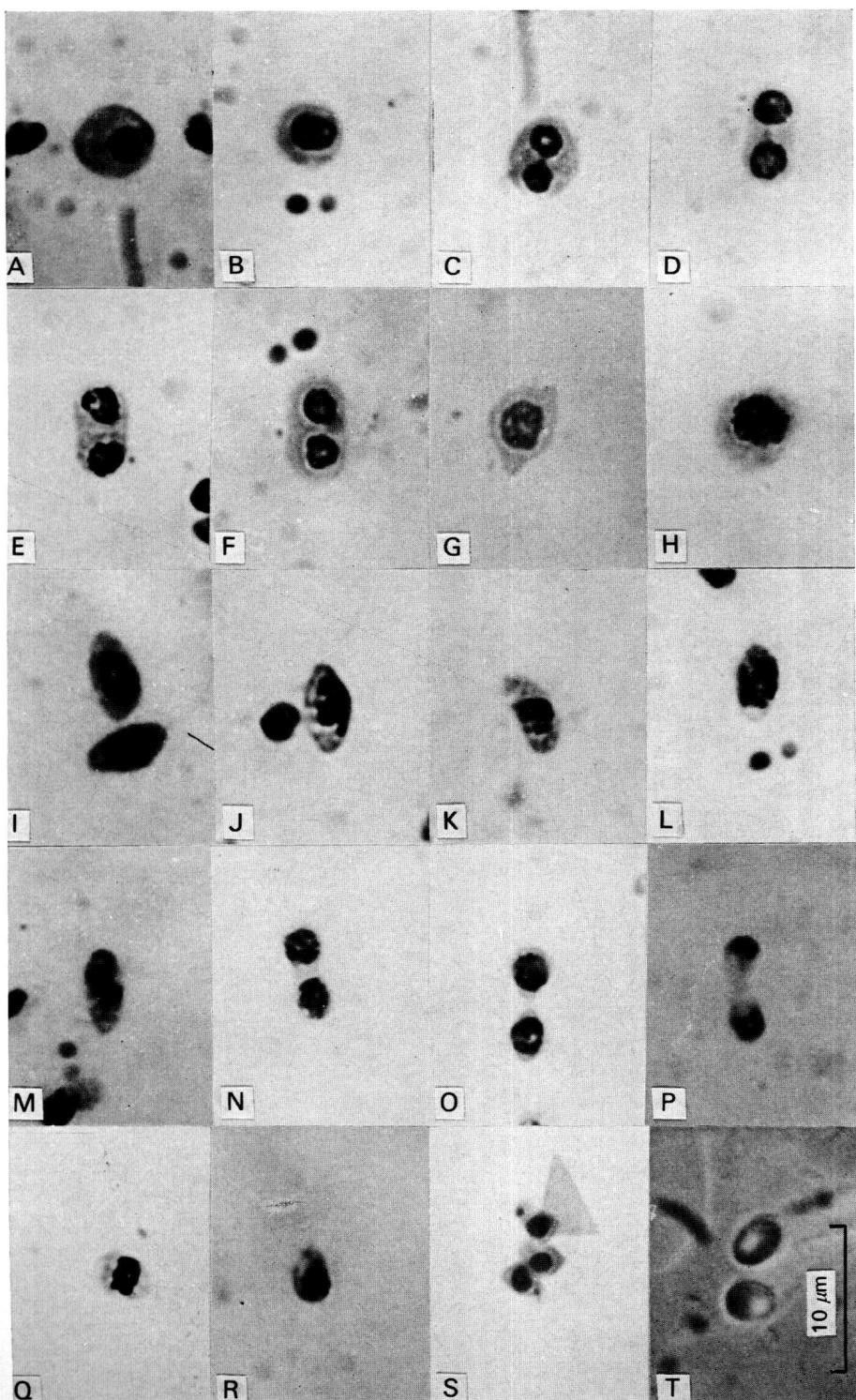
I. Filament of *Nosema gigantica* coiled back on itself during emergence.

PLATE 4

A, B. Filament emergence in *Nosema gigantica*. Part of the filament is still coiled within the spore in A. The two dark objects (arrowed) within the filament in B are probably the sporoplasm nuclei. Note that the magnification of B is about half that of the previous illustrations.

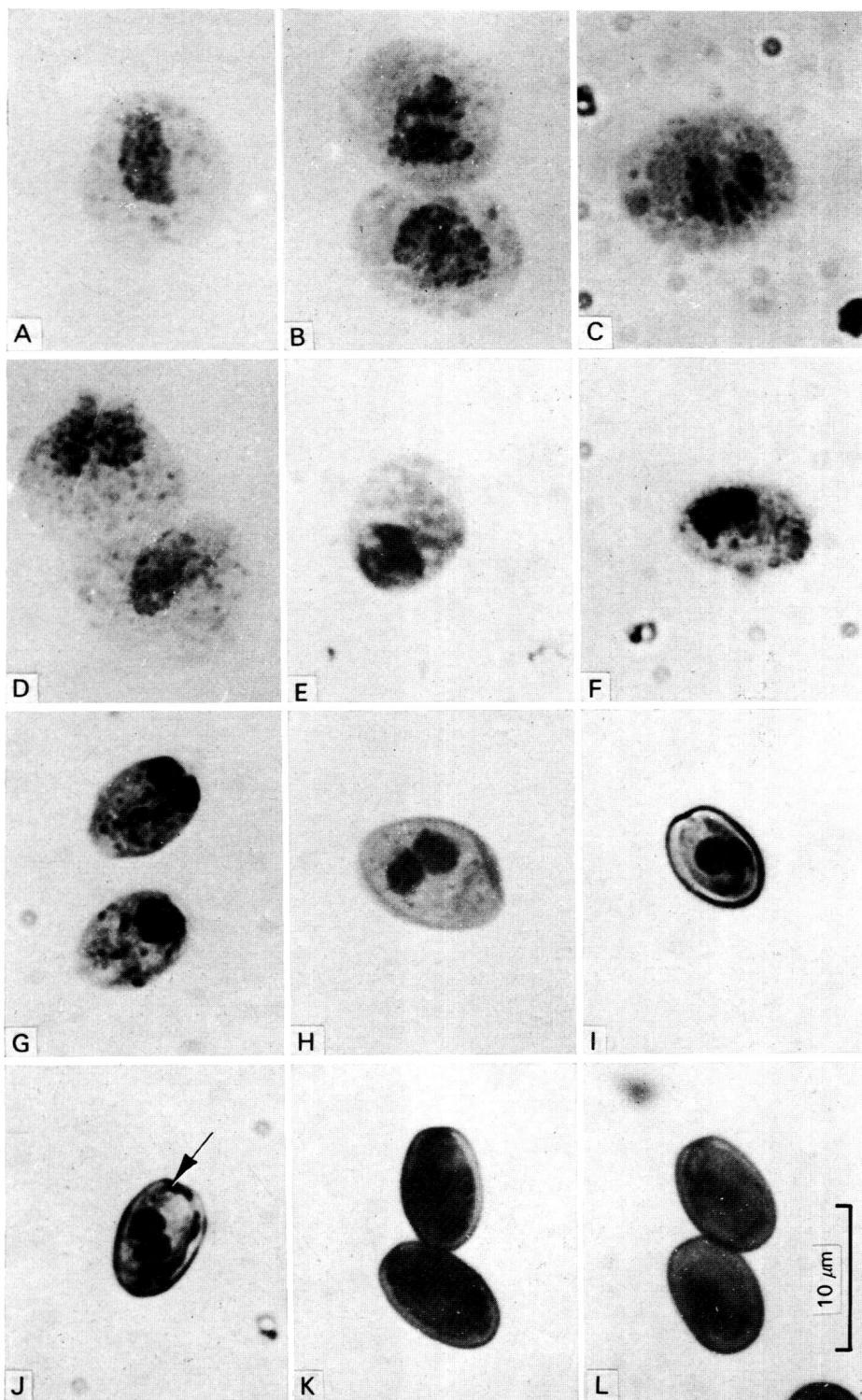
C, D. Sections of the gut of *Aplocheilus melastigma* containing flukes *Allocreadium fasciatum* infected with *Unikaryon allocreadii*. The peripheral parenchyma is packed with spores. In D, patches of spores (arrowed) appear in the deeper parenchyma among other organs which are not infected.

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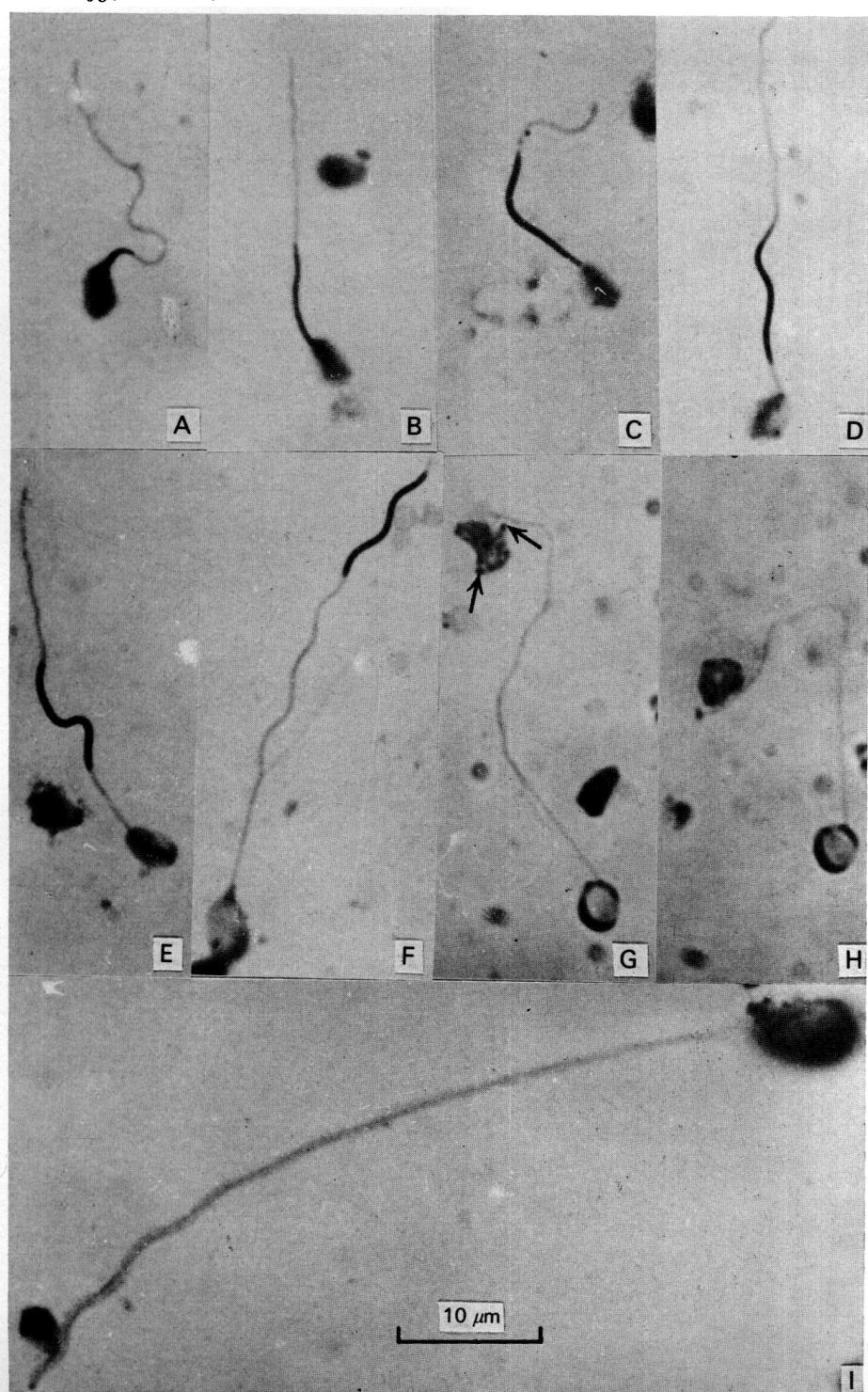


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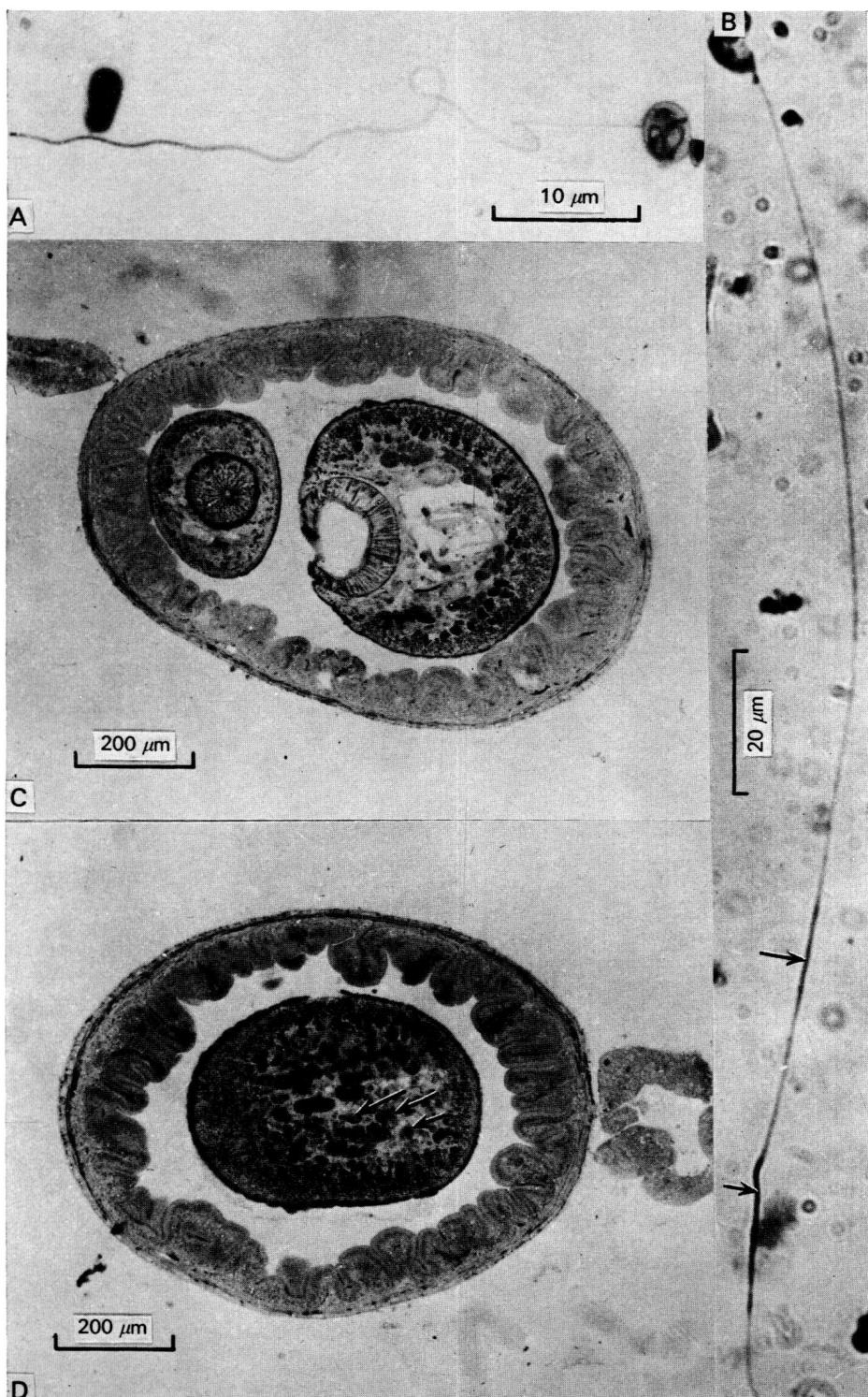
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