Microsporidian hyperparasites and bacteria associated with *Pseudodiplorchis americanus* (Monogenea: Polystomatidae)

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This is the first report of a microsporidian hyperparasite in a monogenean. Every stage in the life cycle of the polystomatid *Pseudodiplorchis americanus* was infected (prevalence 11%). Ultrastructural examination revealed microsporidians within most tissues, but the heaviest aggregations occurred in the external surface layer of the larvae, especially the tegumental ciliated cells where they may interfere with exciliation. Adult worms also harboured a bacterium (prevalence 55%) that was largely restricted to the gut lumen and therefore probably ingested. Transmission of the microsporidian may occur via ingestion of host tissue, but infection within the germ cells and encapsulated embryos *in utero* demonstrates transovarian and transuterine transfer. The oncomiracidia can be already infected at the time of transmission, carrying the microsporidians to newly invaded vertebrate hosts. Heavy microsporidian infections are associated with cellular damage in developing and adult monogeneans and may have a direct effect on the population biology of *P. americanus*.

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Nous signalons ici, pour la première fois, la présence d'une microsporidie hyperparasite chez un Monogène. Tous les stades du polystomatidé hôte, *Pseudodiplorchis americanus*, portaient des parasites (prévalence de 11%). L'examen ultramicroscopique a révélé la présence de microsporidies dans presque tous les tissus, mais les points les plus infectés se trouvaient dans la couche externe des téguments des larves, particulièrement dans les cellules tégumentaires ciliées, là où ils peuvent entraver l'exciliation. Les vers adultes étaient affectés aussi par une bactérie (prévalence de 5%) confinée surtout à la lumière de l'intestin et donc probablement ingérée. La transmission des microsporidies se fait peut-être par ingestion des tissus de l'hôte, mais les infections dans les cellules germinales et les embryons encapsulés *in utero* indiquent l'existence d'une transmission transovarienne et transutérine. Les oncomiracidies sont peut-être déjà infectées au moment de la transmission et transmettent alors les microsporidies à des vertébrés hôtes fraîchement infectés. Les infections microsporidiennes graves s'accompagnent de lésions cellulaires chez les monogènes adultes et les monogènes en développement et peuvent avoir un effet direct sur la dynamique des populations de *P. americanus*.

[Traduit par la rédaction]

Introduction

There are relatively few reports on hyperparasites of helminths. Viral infections have been observed in 7 species of monogeneans, aspidogastreans, and digeneans (Ip and Desser 1984; Fournier 1980 and references therein), and a similar number of helminths have been reported with infections of bacteria. One of the earliest accounts concerning the association of bacteria with polyopisthocotylean monogeneans suggested a symbiotic relationship (Llewellyn 1957). However, Morris and Halton (1975) showed that bacteria in the gut of *Diclidophora merlangi* occur only if the parasite is maintained *in vitro* and therefore are likely to be contaminants. Extracellular bacteria have also been described in *Diplodiscus subclavatus* and *Megalodiscus temperatus* (see Halton 1967; Morris 1973). Intracellular bacteria have been observed in *Euzetrema knoepffleri*, *Polystoma integerrimum*, *Zeuxapta seriolae*, and *Cichlidogyrus hallitypicus* (see Bekkouche and Dupouy 1974; Fournier *et al.* 1975; Rohde 1986; El-Naggar and Kearn 1989). The digenean *Crassicutis archosargi* is parasitized by a myxosporidian and a flagellate, *Hexamita* species (Overstreet 1976). Microsporidian hyperparasites are more common in larval and adult digeneans (Azevedo 1987; Azevedo and Canning 1987; Azevedo and Corral 1987; Canning and Madhavi 1977; Canning *et al.* 1974, 1979) but also occur in acanthocephalans (Loubes *et al.* 1988). We report here the occurrence of microsporidians and bacteria in the polystomatid monogenean *Pseudodiplorchis americanus*.

Materials and methods

During the course of ultrastructural studies on *Pseudodiplorchis americanus*, samples of natural populations of the host, the spadefoot toad, *Scaphiopus couchii*, were collected in the San Simon valley, southeast Arizona, U.S.A., during July and August 1986. The toads were dissected soon after capture, or were transported to England and maintained in the laboratory at Queen Mary and Westfield College in sterile potting compost (John Innes No. 2). Hosts were anaesthetised by immersion in 2% MS 222 (Sandoz), and worms representative of all stages in the life cycle (16 encapsulated larvae, 20 hatched free-living oncomiracidia, 43 juveniles recovered from the respiratory tract and 10 from the gut, 10 subadults from the bladder, 30 gravid adults, and 1 dead adult) were recovered and processed for transmission electron microscopy. Specimens were fixed in 2.5% buffered glutaraldehyde for 0.5–3 h, washed overnight in 0.1 M sodium cacodylate buffer, postfixed for 1 h in 1% osmium tetroxide, dehydrated, and embedded in Taab resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate and viewed in a Jeol 100S or 100C electron microscope operated at 80 kV.

Other polystomatids examined for hyperparasite infections included an adult specimen and 3 juveniles of *Neodiplorchis scaphiopodis* (from the respiratory and urinary tracts of *Scaphiopus bombifrons*,

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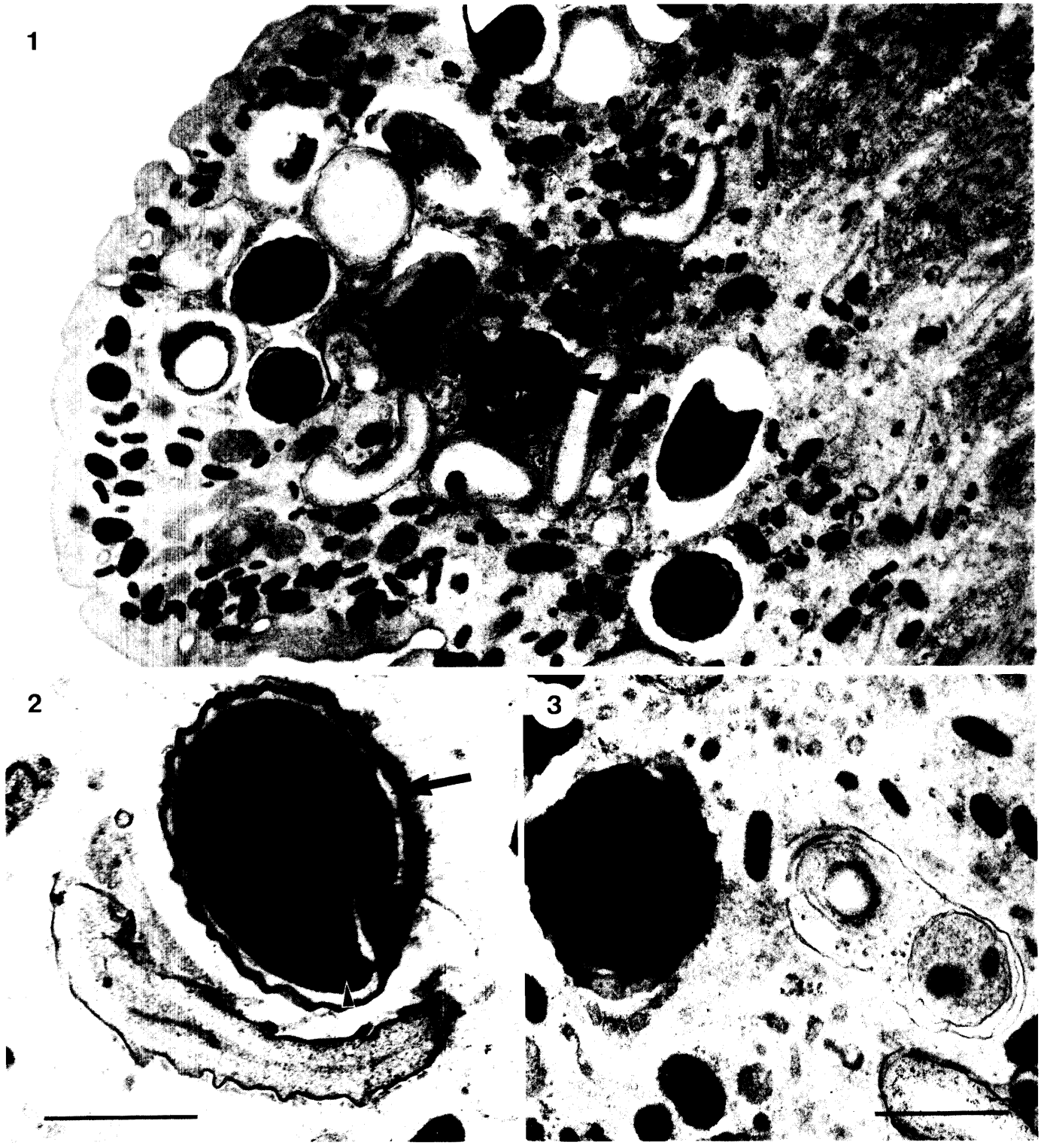

FIG. 1. Microsporidians in the tegument of *P. americanus*. Electron-dense spores and electron-lucent bodies within the surface layer of a parasite retrieved from the host's gut. The spherical body (arrow) enclosing six dense globules and amorphous material may be an intermediate stage in the protozoan's development. Scale bar = 1 μm. FIGS. 2 and 3. Electron-dense spores within the surface layer of an immature *P. americanus*. The spore in Fig. 2 is surrounded by a thick irregular wall (arrow), the only internal structures visible are the electron-lucent spheres (arrowhead), which may represent cross sections of the polar tube. Scale bars = 0.5 μm.

also from Arizona), 3 adult worms and 5 encapsulated embryos of *Protopolystoma xenopodis* (a urinary bladder parasite of *Xenopus laevis*, from South Africa), and an adult specimen of *Polystomoidella oblonga* (from the bladder of *Sternotherus odoratus*, from eastern U.S.A.).

Results

Distribution of microorganisms

Two types of microorganisms were observed in *Pseudodiplorchis americanus*: microsporidians and bacteria. Micro-

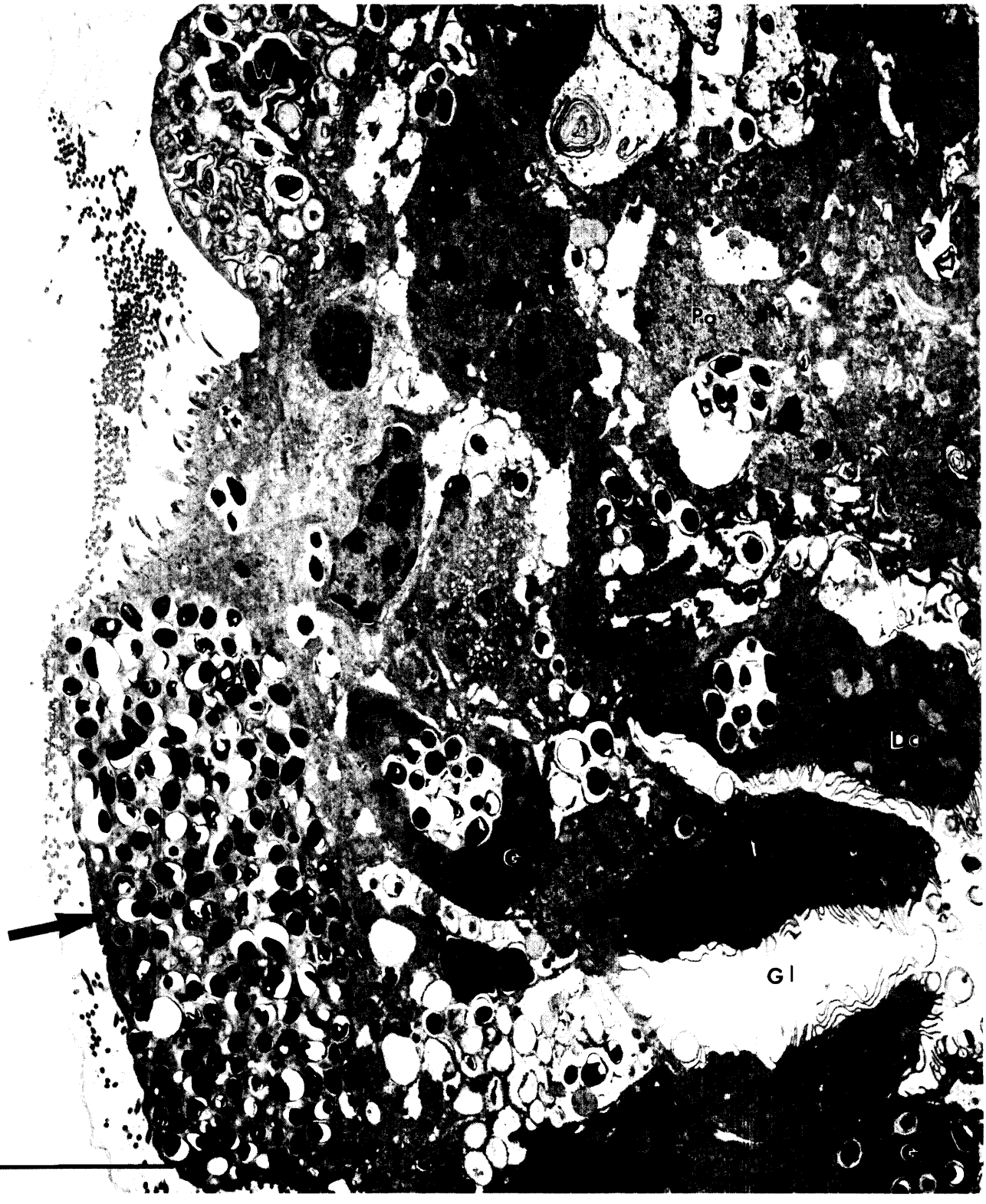

FIG. 4. Heavily infected encapsulated oncomiracidium of *P. americanus*. The ciliated cell (arrow) is packed with spores, another accumulation of microsporidians occurs in the adjacent tegument and has caused distention of the surface layer. Electron-dense whorls (W) lie next to some of the spores. Microsporidians are also present in the parenchyma (Pa), glands (G), digestive cells (Dc), and gut lumen (Gl). Scale bar = 10 μm.

sporidians, identified in 14 specimens, were present at all stages of the helminth's life cycle (Table 1). By contrast, bacteria were restricted to 7 adult worms, occurring at a prevalence of 5.4%.

The majority of specimens infected with microorganisms were retrieved from toads that had been maintained in the laboratory for at least 4 months. However, a gravid adult dissected from the bladder of a toad within 24 h of capture was

TABLE 1. Occurrence of microsporidians in specimens of *Pseudodiplorchis americanus* examined with transmission electron microscopy

	n	Frequency (%)
Encapsulated embryos (*in utero*)	16	6.3
Recently hatched oncomiracidia	20	20.0
Juveniles from the respiratory tract	43	4.7
Juveniles from the gut	10	10.0
Subadults from the bladder	10	0.0
Gravid adults	30	20.0
All stages in the life cycle	129	10.9

also infected with microsporidians and bacteria. Microorganisms were not detected in the small samples of the other polystomatids examined.

Microsporidians

Two distinct microsporidian stages were identified: electron-dense spores and electron-lucent bodies. Both structures often occurred in close proximity, but some specimens of *P. americanus* contained only spores. The spores were oval or rod-shaped (2 × 1 μm) and were surrounded by a thick trilaminate wall (Figs. 1–3). The only internal structure visible was the polar tube. Scoring of the resin around the spores suggested they are composed of a very hard substance. The electron-lucent heteromorphic bodies that were similar in shape and dimensions to the spores may represent early stages of spore formation (Fig. 1). Others, which were C-shaped and only 0.1 μm in diameter, appeared degenerative (Figs. 1 and 2). The outer electron-dense wall had a convoluted appearance and was probably composed of at least two layers. The cytoplasmic contents were granular, but no cell organelles were recognised.

The hyperparasites were most frequently observed in the surface layer of the tegument, occurring in small clusters (up to 8) or scattered amongst the tegumental vesicles and mitochondria. In larval specimens, spores were found in large numbers within tegumental ciliated cells; the intensity of infection was often so high that cell organelles were completely obscured (Fig. 4). However, only small numbers of microsporidians were present in the surrounding tissues. The ciliated cells of *P. americanus* oncomiracidia are usually shed 1–2 h post-invasion (Cable 1989). Four worms recovered from the host 75–90 min post-invasion were in the process of exciliation, a 5th specimen had intact ciliated cells that were packed with microsporidians. In many of the infected ciliated cells, the number of cilia appeared to be reduced, and in 1 cell, the outer plasma membrane was discontinuous. Two other parasites (105–120 min post-invasion) had completely lost their locomotory cells, but a 3rd infected specimen still retained its ciliated cells.

Microsporidians were not observed in the male reproductive tissue, but spores and electron-lucent bodies were visible in the ovary, vitelline follicles and ducts, uterus wall, and developing egg capsules (Figs. 5 and 6). The majority of the spores within egg capsules were located within the cytoplasmic lining of the capsule; others were observed in the lumen and within the cytoplasmic connections by which nutrients are transferred from parent parasite to developing embryos (Cable and Tinsley 1991). The heaviest infections were observed in some fully developed encapsulated oncomiracidia that contained microsporidians in all of their tissues.

Other aggregations of microsporidians were observed within the parenchyma and less frequently in the gland cells, the sensory bulbs of uniciliate receptors (Fig. 7), protonephridial canals, and the supportive cup of photoreceptors. Spores were found in the gut of only 1 specimen, an oncomiracidium that was released from its parent's genital pore soon after the adult was recovered from the bladder of the toad. The larva appeared unusual in having a black gut which was very conspicuous at low stereomicroscope magnification (×25): ultrastructural examination revealed that the gut lumen was packed with microsporidians. A few electron-dense spores were also present in the surface layer of this specimen.

Bacteria

The rod-shaped bacteria (approximately 2.6 × 0.45 μm) were most frequently observed within the gut lumen of adult worms (Fig. 8). Smaller numbers were present within the digestive cells, parenchyma, gland cells, vitelline ducts, and uterus wall. An isolated bacterium was observed within the cytophore of a spermatid rosette, but there was no evidence of tissue degeneration. The highest levels of infection were observed in the disintegrating tissue of a dead worm that was removed from the host's bladder surrounded by a hard capsule.

Discussion

Two microorganisms are associated with the monogenean *Pseudodiplorchis americanus*: a bacterium and a microsporidian. The large number of bacteria within the gut of adult worms suggests these microorganisms are taken up from the amphibian host while the helminth is feeding. They probably enter when superficial epithelial cells are ingested with blood, but it is not known which (if any) of the tissues of *Scaphiopus couchii* are infected. A few bacteria were observed in association with the uterine wall but were never found in embryos or other immature specimens of *P. americanus*. Transmission of a bacterium via the gametes does occur in *Euzetrema knoepffleri*, but this organism has a pathogenic effect on the male reproductive cells (Fournier *et al.* 1975). In contrast, there was no evidence of tissue damage in the testis of *P. americanus*. The heaviest bacterial infection in *P. americanus* was observed in a dead adult worm, but this is likely to have been a post-mortem event.

The distribution of the microsporidian suggests that there is a frequent association between this organism and the monogenean. The microsporidians colonize a variety of tissues and were associated with visible damage within the tegument. The surface layer packed with spores was distended and devoid of other cell contents. Heavy burdens of microsporidians observed in the tegumental ciliated cells of oncomiracidia could impair locomotor function and interfere with transmission. However, in laboratory infections, post-invasion parasites recovered from the respiratory tract of toads showed ciliated cells still packed with microsporidians, so host invasion is not precluded. There was also an indication that exciliation may be prevented or delayed by heavy infections of microsporidians.

The static micrographs provide no direct visualization of infection mechanisms; nevertheless, there is evidence that both the reproductive and digestive tracts may provide routes for transmission. Microsporidians occurred in the ovary of reproducing *P. americanus*, demonstrating infection of ova before fertilization; in the vitelline follicles and ducts, provid-

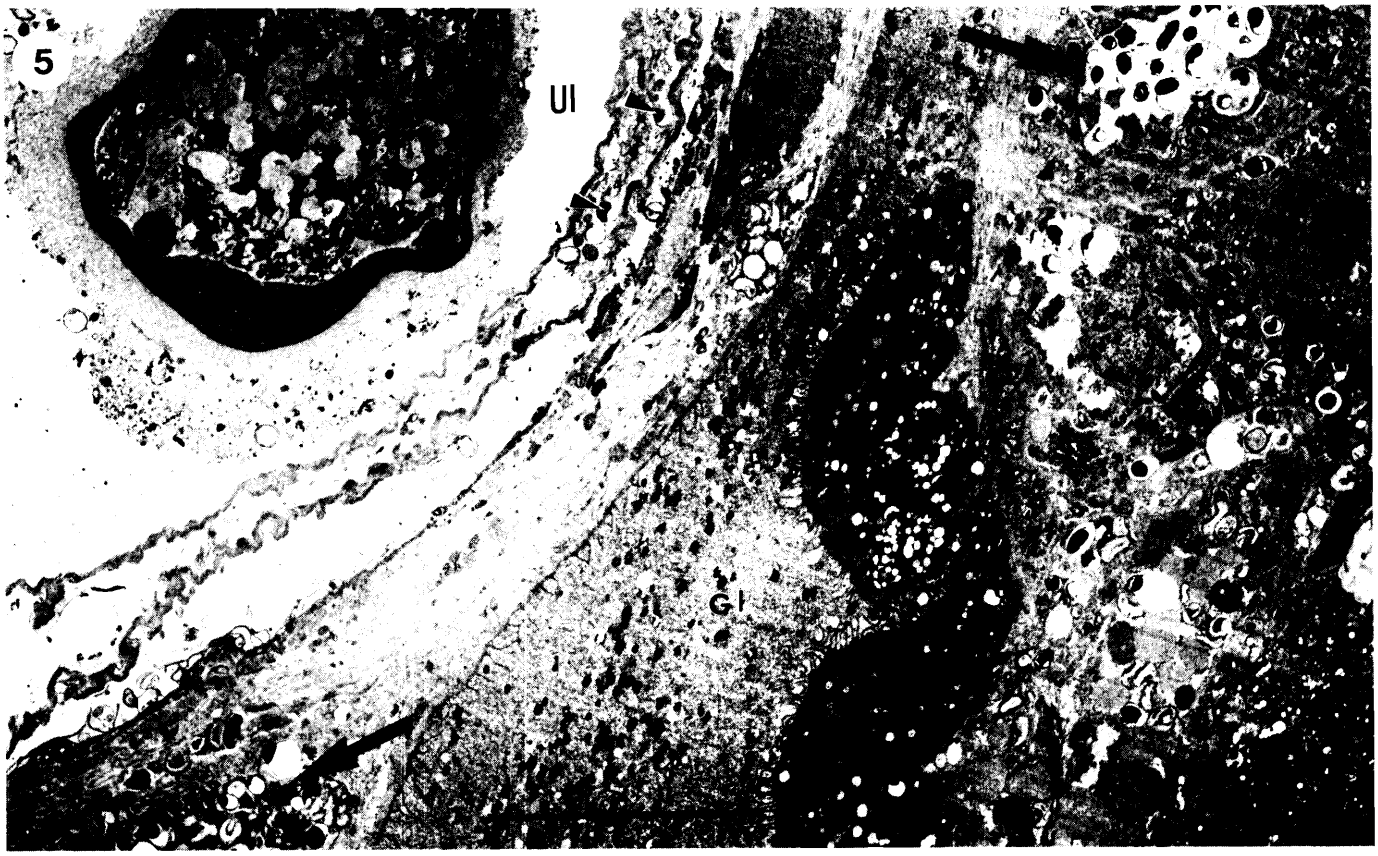

FIG. 5. Microsporidians associated with the uterus of an adult *P. americanus*. Aggregations of spores (arrows) are visible either side of the gut lumen (Gl) and lie in close proximity to the uterus lumen (Ul). Two spores (arrowheads) are enclosed within the membranous egg capsule of a developing larva. Scale bar = 10 μm. FIG. 6. Longitudinal section of a vitelline duct of an adult *P. americanus*. Microsporidians (arrowheads) lie within the duct wall. Scale bar = 2 μm. FIG. 7. Electron-dense spores (arrowheads) within the tegument of an adult *P. americanus* recovered from the host's bladder. One spore lies within the sensory bulb of a uniciliate receptor just below the basal body (*bb*). Scale bar = 2 μm.

FIG. 8. Bacteria within the gut lumen of adult *P. americanus*. Much of the gut lumen (Gl) is filled with rod-shaped bacteria (arrowheads). Scale bar = 3 μm.

ing an avenue for incorporation of microsporidians within the egg capsules; and in the uterus wall and egg capsules, indicating that encapsulated embryos may be infected during their development *in utero*. This represents an immediate source of contamination for the larvae and increases the prevalence of infestation in the next generation. The potential of these transfer routes is confirmed by the records of oncomiracidia heavily infected with microsporidians, both whilst still within the parental uterus and immediately after hatching in the external medium. Apart from this capacity for vertical transmission, the monogeneans may acquire infection by ingesting host tissues. Oncomiracidia do not begin to feed on blood until 48 h after host invasion (Tinsley and Earle 1983). Host blood is drawn into the gut lumen, and periodically the waste products of digestion are expelled through the mouth. Although microsporidians were not observed in the gut lumen of adult worms, an oncomiracidium was found, prior to its first blood meal, with a gut infestation of microsporidians. This suggests that regurgitation of gut contents by newly established parasites could liberate microsporidian spores into the host's body.

Previous reports have suggested that microsporidian infections are pathogenic to amphibian and helminth hosts. Infection of the European common toad, *Bufo bufo*, with the microsporidian *Plistophora myotrophica* results in atrophy of the striated muscles. The toads become emaciated and suffer heavy mortality: 75% of animals collected from one site died after infection (Canning *et al.* 1964). In our experience, very few *S. couchii* die during laboratory maintenance under simulated desert conditions (Tinsley and Earle 1983). However, a small number of toads did fail to feed, lost condition, and died, and it is possible that their deaths were caused by microsporidian infection. Laboratory studies suggest that helminth infections are not a significant cause of mortality in this toad (Tinsley 1990). The capacity for vertical transmission and for passage of microsporidians during feeding on host tissues prompts speculation that the monogenean *P. americanus* could be a vector of microsporidians from one vertebrate to another.

Electron micrographs showed little of the internal fine structure of the microsporidians, probably because of the very dense spore wall, and there was no direct evidence of developmental stages. However, the possibility that the spores are chance contaminants of the helminth, not part of a host–parasite association, must be excluded. Microsporidians were found in almost every tissue, and were associated in the tegument with significant structural damage; the presence of spores in the tissue of encapsulated larvae developing *in utero* must result from an intracellular proliferation and dissemination within the monogenean.

These pathological effects may influence the population biology of *P. americanus*. It is known that infection of digeneans with microsporidians may lead to reduced helminth fecundity, and eventually the host tissue is replaced by spores and completely destroyed (Canning *et al.* 1979). Transmission of *P. americanus* is very successful, resulting in 100% prevalence of infection amongst toads aggregated in breeding pools and a mean intensity of 100 larvae/host (Tinsley and Jackson 1988). However, few of these worms reach maturity: 1 year post-infection, at the next opportunity for transmission, only 50% of *S. couchii* carry a mean of 5 mature worms per infected toad (Tinsley 1989). The decline in the helminth population seems to occur during toad hibernation, but there are no obvious factors to account for this fall. Whilst it would be premature to deduce from ultrastructural observations alone that the microsporidian infections are a cause of mortality, part of the reduction in helminth parasite numbers may be caused by the microsporidian hyperparasite infection.

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