

Goussia hyperolisi n. sp.: a coccidian infection in reed frog *Hyperolis viridiflavus* tadpoles which expires towards metamorphosis

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ABSTRACT: *Goussia hyperolisi* n. sp. is described from the guts of *Hyperolis viridiflavus* tadpoles taken from a fish pond at Sagana, Kenya. Infection expired as metamorphosis approached. No infection could be found in post-metamorphosed and older frogs. Infected tissues were studied by light and electron microscopy. Endogenous stages, meronts and gamonts were in cells of the gut epithelium within a parasitophorous vacuole with folded rims. In the macrogamonts, small bodies with a dense core were the only structures reminiscent of wall-forming bodies. The wall membranes which formed following zygote formation detached and merged with the parasitophorous vacuole wall to form the oocyst wall. During the regression of the infection, the one or several sporulated oocysts in the infected host cell, an epithelial or intraepithelial leukocyte, became embedded in a multilaminated matrix, the yellow body, and were eventually destroyed.

KEY WORDS: *Goussia hyperolisi* n. sp. · Coccidia · *Hyperolis viridiflavus* Kenya Tadpoles Yellow bodies

INTRODUCTION

Many named species of *Eimeria* described from anurans have soft-walled oocysts which sporulate within their host's gut epithelial tissue (Paperna 1995a, Paperna & Lainson 1995). The sporocysts of these species open via longitudinal sutures rather than having an apical opening plugged with a stieda body. These features are shared with many piscine coccidia included in the genus *Goussia* Labbe, 1896 (Paperna 1995b). This led Molnar (1995) to place *E. neglecta* Nöller, 1920, which he redescribed, into the genus *Goussia*. The unique form of development of anuran coccidia which segregates them from the eimerians of the higher vertebrate taxa had already been noticed by Laveran & Mesnil in 1902, but their proposal for a separate subgenus *Paracoccidium* for the anuran species was ignored. To date most of the described species of anuran eimeriid coccidia have been reported from metamorphosed anurans (Upton & McAllister 1988). From tadpoles only *Goussia neglecta* has been described (Nöller 1920, Molnar 1995). In this communi-

cation, we present the results of a light and electron microscopic study of another eimeriid coccidium of the genus *Goussia* from the digestive tract of tadpoles of the reed frog *Hyperolis viridiflavus* (Dumeril & Bibron) from Sagana fish ponds in Central Kenya.

MATERIALS AND METHODS

After finding coccidian infection in reed frog *Hyperolis viridiflavus* tadpoles collected in a fish pond on the Sagana farm in Central Kenya, more tadpoles as well as adult reed frogs were collected from the same habitat and brought to the laboratory. There, within 1 to 14 d after collection the animals were sacrificed (by anaesthesia using chloroform) and examined for infection.

Prepared intestinal smears were air dried and Giemsa stained after fixation in absolute methanol. Samples of the same infected tissues were fixed in Neutral Buffered Formalin for histology. Fixed tissue, after dehydration in graded ethanols, was embedded in glycol-methacrylate medium (GMA of Agar Comp., UK). Sections, 3 to 4 µm thick, were cut with a glass knife on a Sorval JB4 microtome and stained with

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Table 1 Autopsy results of *Hyperolis viridiflavus* tadpoles (T), post-metamorphosis (Fm) and adult frogs (Fa). Neg.: negative for *Goussia hyperolisi* n. sp. infection

Date	Stage	Merog./Gamonts	Oocysts	Oo+spor	Yellow bodies	Neg.
1 Sep	T; 4 legs		++	++		
23 Sep	T; 2 legs		+	++++	++	
23 Sep	T; 0 legs					Neg.
23 Sep	T; 4 legs					Neg.
23 Sep	T; 4 legs					Neg.
23 Sep	T; 4 legs				+	
23 Sep	T; 4 legs		++	++++	+++	
23 Sep	T; 4 legs			+	++	
23 Sep	T; 2 legs	+	+	++		
23 Sep	T; 0 legs			++	+	
23 Sep	T; 0 legs					Neg.
10 Oct (Coll. 23 Sep)	2 legs (stunted)	+	+	++		
10 Oct (Coll. 23 Sep)	0 legs (stunted)			+	+	
23 Sep	Fm					Neg.
23 Sep	Fm				+	Neg.
23 Sep	Fm					Neg.
23 Sep	Fm					Neg.
10 Oct	Fa; 11 specimens (feces)					Neg.

Meyer's haemalum-eosin (MHE). For electron microscopy, tissues were fixed in 2.5% cacodylate (0.1 M, pH 7.4) buffered glutaraldehyde for 24 h at 4°C, repeatedly rinsed in cacodylate buffer, post-fixed in 1% osmium tetroxide in the same buffer for 1 h, rinsed in the buffer and in distilled water, stained en bloc with aqueous uranyl acetate for 3 h, rinsed in distilled water, dehydrated in ascending ethanol concentrations and embedded in Agar 100® (Agar Comp.). Thin sections were cut on a Reichert 'Ultracut' with a diamond knife, stained on grid with uranyl acetate and lead citrate, and examined in a Jeol 100CX transmission electron microscope (TEM). The means of measurements are given with (\pm) standard deviation and number measured (n).

RESULTS

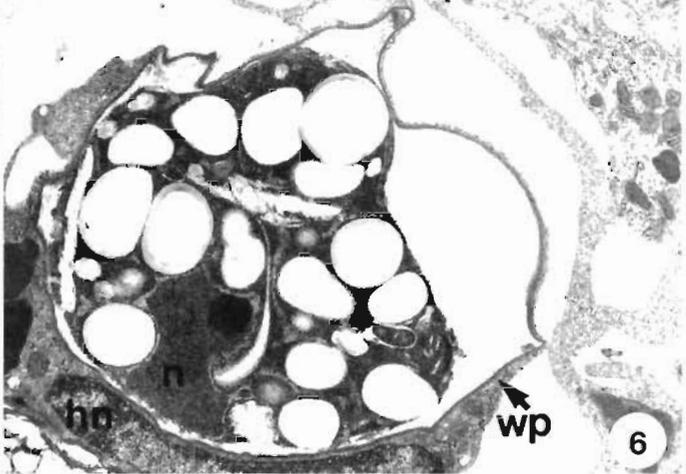
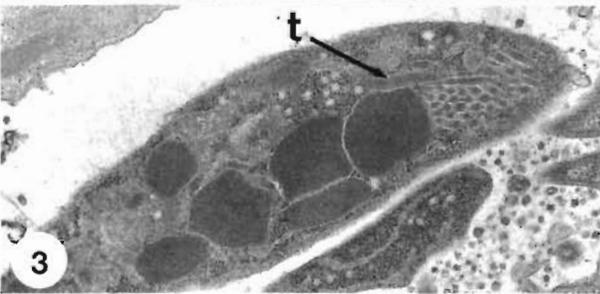
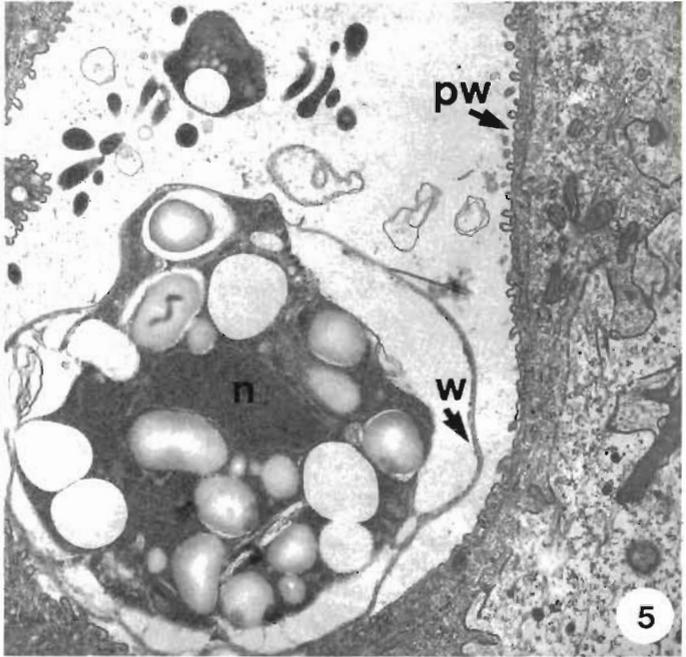
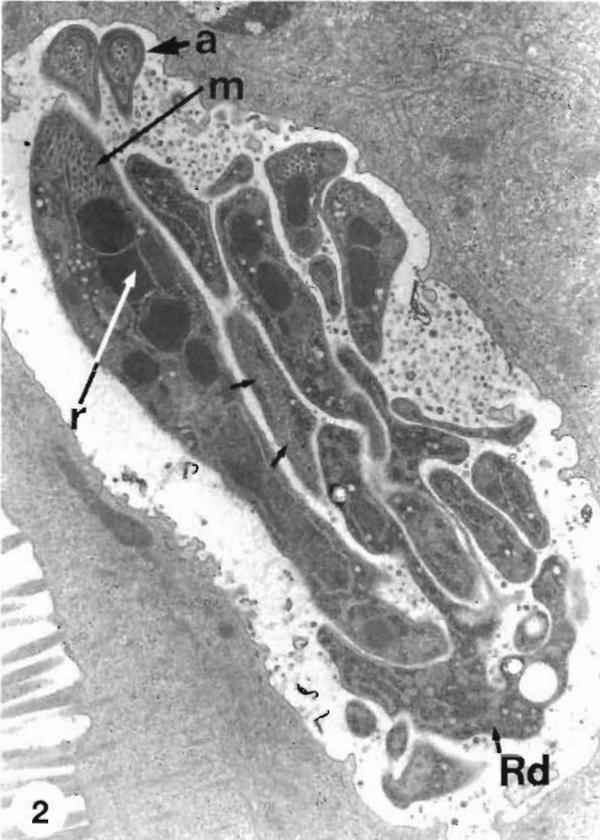
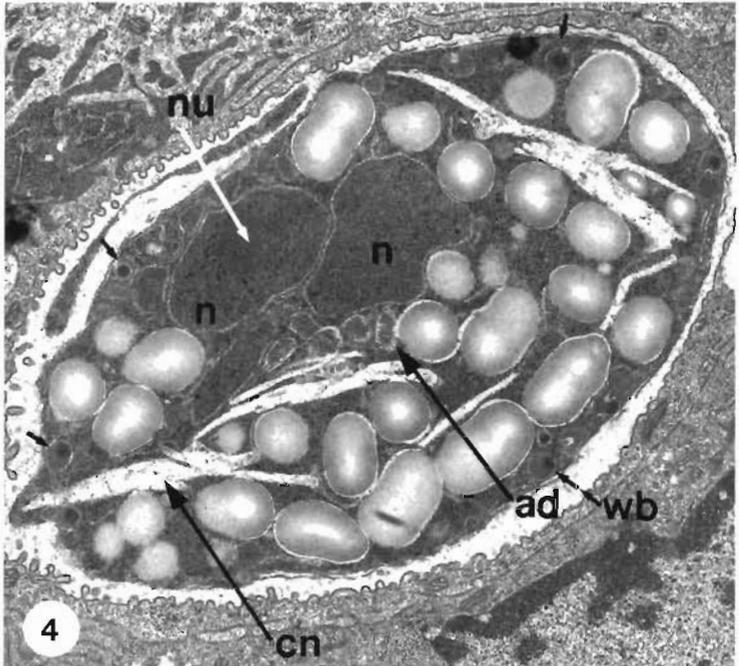
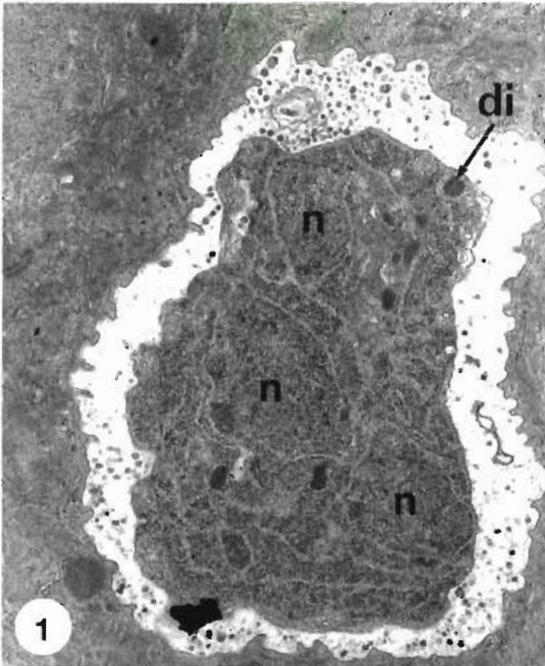
Reed frog tadpoles from Sagana ponds were first examined and found to be infected on September 1, 1994. Both tadpoles and adult frogs were subsequently collected on September 23 of the same year. In the first and second collections most tadpoles had 4 legs, and

only a few were still at the 2 or no-leg stage. Of the 10 examined tadpoles 6 revealed coccidian infections in various stages of regression, consisting mostly of sporulated oocysts, in part located inside yellow bodies. The gut epithelium also contained many yellow bodies without any remains of parasites. Two tadpoles, one with 2 legs and one without legs, left in the laboratory for a further 17 d in a 1-l dish in tap water at an ambient temperature of 26 to 28°C failed to develop to metamorphosis. When sacrificed, these tadpoles demonstrated a similarly regressed infection consisting predominantly of sporulating oocysts, accompanied by many yellow bodies. However, they also revealed a very small number of early meronts (or gamonts). Four post-metamorphosis frogs and feces collected from 11 additional adult specimens retained in captivity were negative (Table 1).

Electron microscopic observations

Non-differentiated, $4.5\text{--}5.2 \times 2.3\text{--}3.6 \mu\text{m}$ meronts (Fig. 1) and a formation of merozoites $9.5 \times 3.9 \mu\text{m}$ in

Figs. 1 to 6. TEM images of *Goussia hyperolisi* n. sp. from *Hyperolis viridiflavus* tadpoles. Fig. 1. Meront ($\times 12600$). n: parasite nucleus; di: dense inclusions. Fig. 2. Formation of merozoites extending from the meront residuum (Rd); arrows: mitochondrion ($\times 11500$). a: apical complex; m: micronemes; r: rhoptries. Fig. 3. Enlargement of the merozoite's anterior portion ($\times 16200$). t: duct-like formation. Fig. 4. Macrogamont ($\times 12900$). nu: nucleus; cn: canaliculi; ad: adnuclear inclusions; wb (and arrows): wall-forming-body-like organelles. Fig. 5. Zygote or early oocyst with detached wall, still enclosed parasitophorous vacuole by (PV) with intravacuolar folds (PV wall: pw) ($\times 11300$). Fig. 6. Young oocyst with its envelope adhering to the PV wall ($\times 7800$). hn: host-cell nucleus; wp: merged oocyst wall



size (Fig. 2) were found located in cells of the gut epithelium between the nucleus and the brush border within a parasitophorous vacuole (PV) with wavy to folded rims, fringed with many intravacuolar folds and filled with globular debris. Meronts were bound by a single membrane. Their cytoplasm contained extensive endoplasmic reticulum (ER), mitochondria and some electron-dense inclusions (Fig. 1). The very elongated 7–8 μm long merozoites were still connected to a meront residue and were bound by a pellicle. The conoid and the apical ring were detectable in the tips of some merozoites (Fig. 2). Micronemes filled the merozoite's anterior end and several exceptionally large rhoptries filled the pronuclear zone; the latter are connected through duct-like formations to the apical region (Fig. 3). The cytoplasm was filled with very small amylopectin granules. In some, an elongate mitochondrion was traced. The meront's residuum contained amylopectin granules, of the same size as in the merozoites, and a few lipid vacuoles (Fig. 2).

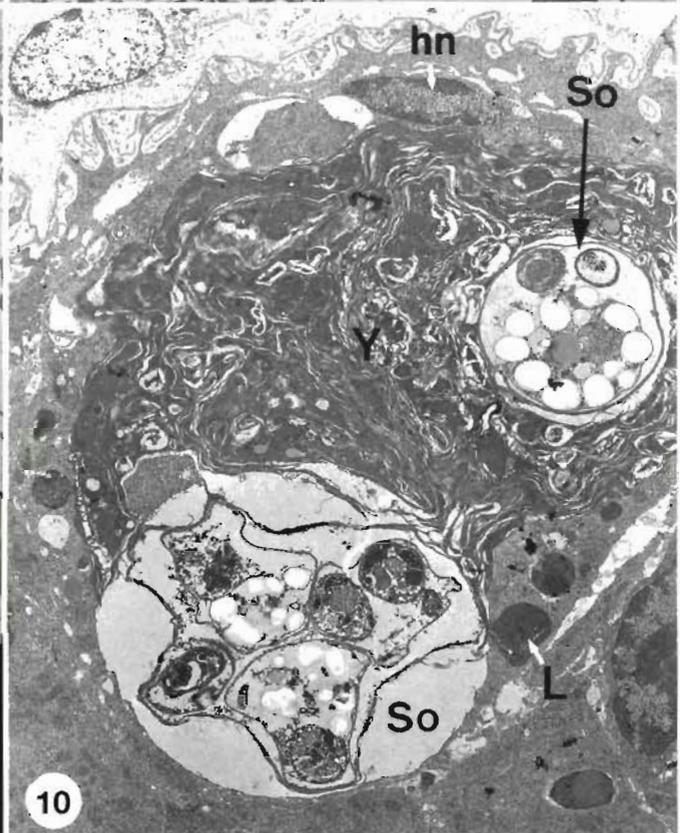
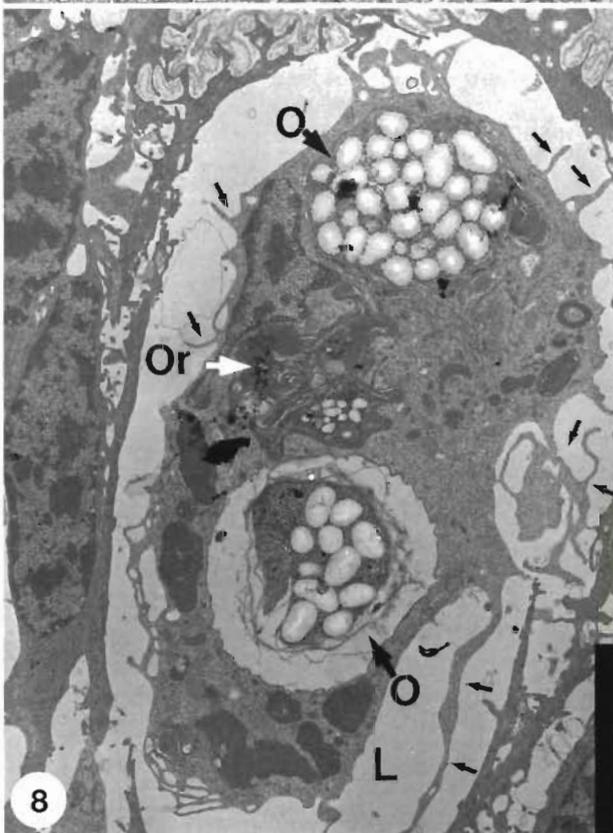
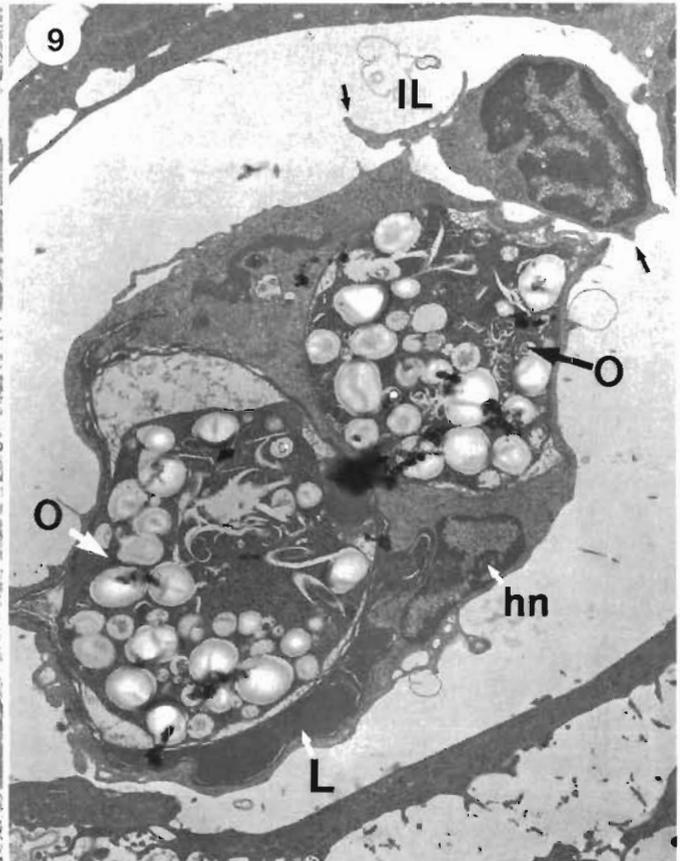
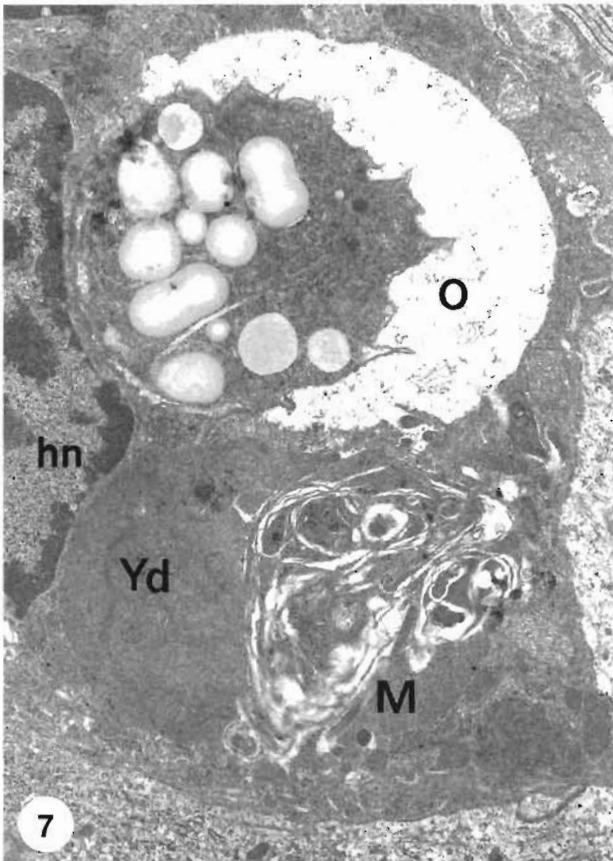
The 7–9 \times 4–5 μm macrogamonts (Fig. 4) were lodged in a PV with a wavy wall and intravacuolar folds. Superimposed on this wall was a seemingly peeling and fractured (due to processing?) secondary membrane. Their condensed cytoplasm contained 3 bodies of nucleolemma-bound nucleoplasm, which seemed to be segments of a lobed nucleus. One segment contained a conspicuous nucleolus. The cytoplasm also contained several conspicuously large canaliculi, a network of ER, variable numbers of amylopectin granules, inclusions with contents similar in density to that of the cytoplasm, adnuclear organelles and a few small wall-forming-body-like organelles with high-density cores.

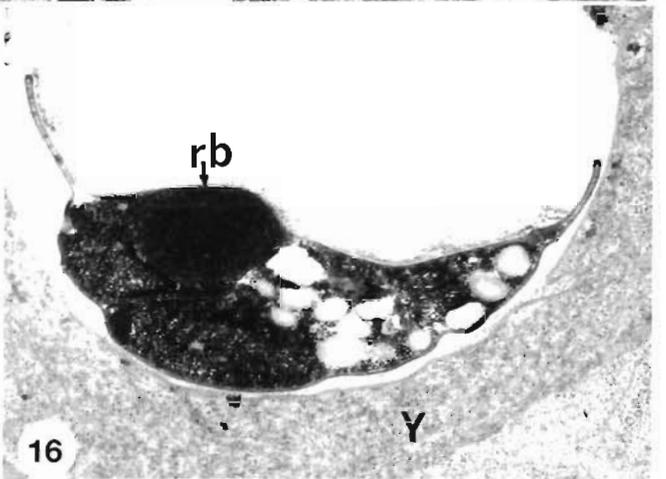
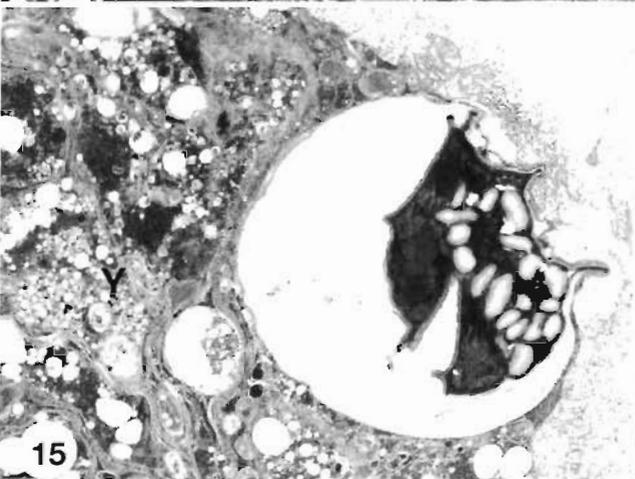
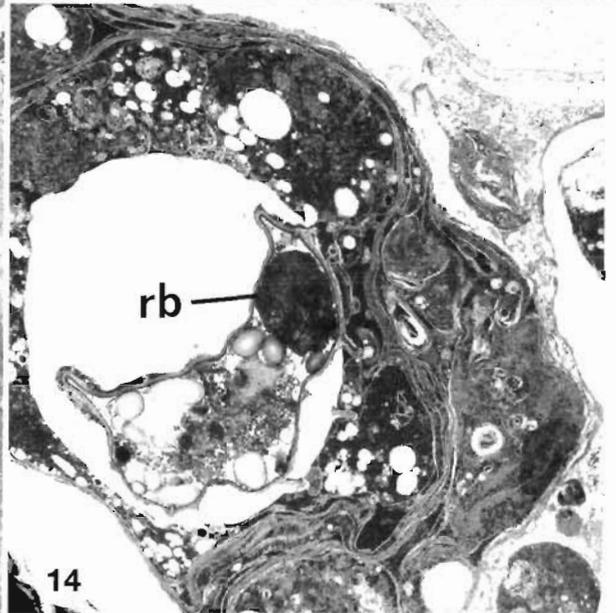
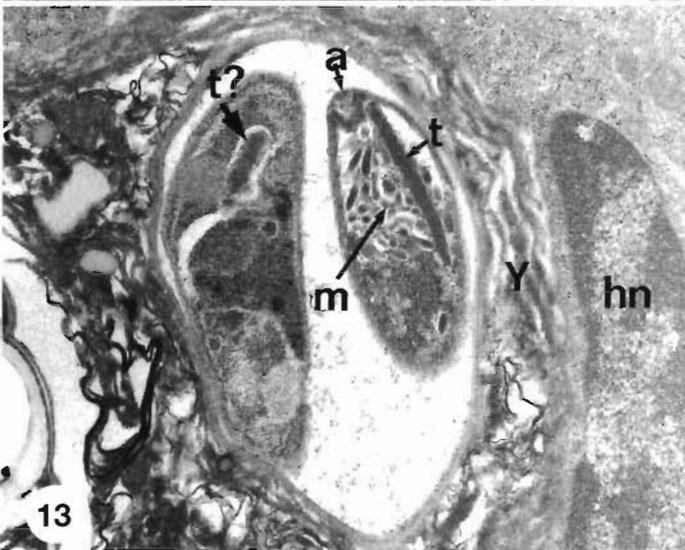
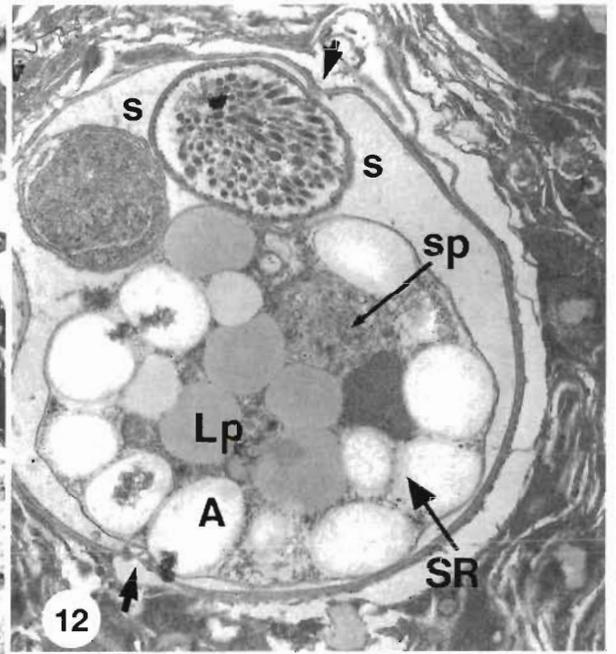
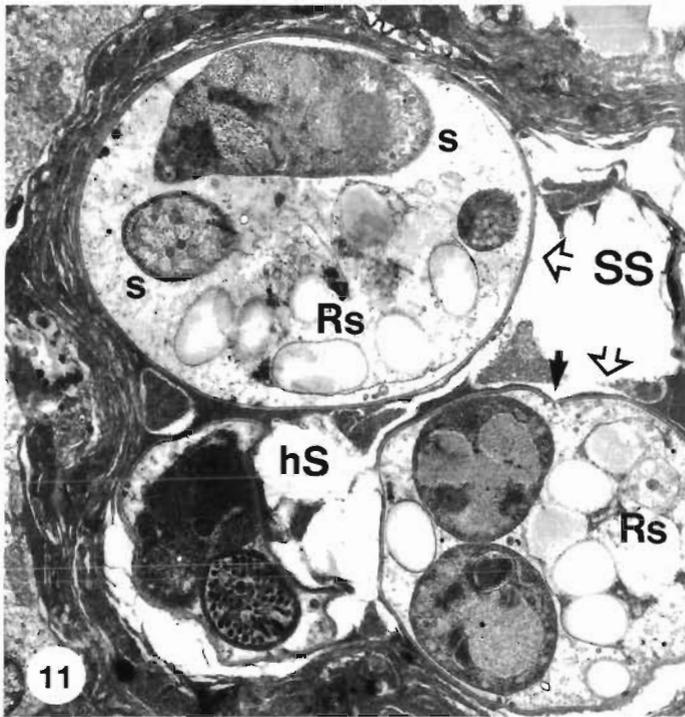
Later stage macrogamonts (zygotes) and oocysts demonstrated variable degrees of processing damage, which appeared to result from decreased permeability to fixatives or embedding media. The late-stage macrogamonts or early-stage oocysts became enclosed in an additional, thickened 44–64 nm thick membrane (Fig. 5) which subsequently adhered to the rims of the PV to become the oocyst wall (Fig. 6). Host cells of 7–9 \times 6–8 μm oocysts demonstrated progressive cytoplasmic degradation, either becoming homogeneously dense or breaking into a multilaminated matrix (Fig. 7). Host cells with one to several variable-stage oocysts or their residues became rounded and separated from the sur-

rounding tissue. The rims of such cells had pointed processes reminiscent of those seen on the surface of leukocytes. The cytoplasm was evidently progressively degenerating, becoming filled with electron-dense inclusions (lysosomes), and the start of multilamination could be seen at the rims of the oocyst-containing PVs (Figs. 8 & 9). Sporocysts, in the early stage and already with sporozoites, were contained within their host cell inside a large body of degenerated cytoplasm (Fig. 10). Sporocysts were enclosed within a double-layer 77–85 nm thick wall with distinct longitudinal sutures. The sporocyst cytoplasm was filled with amylopectin granules and lipid vacuoles at a ratio of 2:1. The sporocyst residuum gradually disintegrated and disappeared once sporozoite differentiation was completed (Figs. 11 & 12). Early-stage sporozoites revealed an apical complex accompanied by numerous micronemes as well as a fibrillar, tubular structure (which might be connected to the rhoptries complex?), a nucleus and dense aggregates of ribosomes. Neither refractile nor crystalloid bodies could be identified in the newly formed sporozoites (Figs. 11 to 13). The walls of the sporocysts hardened toward the completion of sporulation, which resulted in their further distortion due to their resistance to fixation and processing. Aged sporozoites within the hard-walled sporocysts contained a fair number of amylopectin granules. Other organelles could only be traced with difficulty; a large electron-dense body was tentatively identified as a refractile body (Figs. 14 to 16).

Changes in the host-cell cytoplasm first occurred around the PV, as evidenced by the formation of an increasingly thick layer of multilaminated material (Figs. 11 to 13). Sporocysts with their sporozoites ultimately disintegrated. The large body of seemingly necrotic substance remaining within the hypertrophied host cell contained very variable consistencies, incorporating remains of defunct sporocysts as well. The fine structural image of this material indicated a blend of vacuoles and electron-dense bodies, or multilaminated electron-dense matrix or both (Figs. 17 & 18). All stages of the sporulation process before the formation of yellow bodies, which were identified from light microscopically examined material (Figs. 19 to 22), were identified from TEM images, except for the final accumulation of the lipofuscin (ceroid)-type material in the yellow body (Figs. 22 & 23).

Figs. 7 to 10. TEM images of *Goussia hyperolisi* n. sp. from *Hyperolis viridiflavus* tadpoles. Fig. 7. Host cells infected with a zygote or early oocyst (O) demonstrating extensive degenerative changes: homogeneously dense (Yd) and multilaminated cytoplasm (M) ($\times 6800$). hn: host-cell nucleus. Figs. 8 & 9. Young oocysts (O) and their residues (Or) inside a seemingly leukocytic, detached cell with lysosomes (L) and with surface processes, seen also in an adjacent noninfected cell (IL) ($\times 4000$ & $\times 5100$). Fig. 10. Host cell almost completely occupied by multilaminated matrix yellow body (Y) containing sporulated oocysts (So); remaining intact cytoplasm contains lysosomes (L) ($\times 4300$)





Figs. 11 to 16. TEM images of *Goussia hyperolisi* n. sp. from *Hyperolis viridiflavus* tadpoles. Fig. 11. Oocysts inside a yellow body demonstrating sporocysts at various stages of sporulation: soft-walled sporocysts (SS) with already formed sporozoites (s), disintegrating sporocyst residuum (Rs) and hard-walled sporocyst (hS). Arrow points to suture ($\times 9200$). Fig. 12. Higher magnification of differentiating sporocyst with sporozoites (s) emerging from a residuum (SR) of cytoplasm (sp) rich in lipid vacuoles (Lp) as well as amylopectin granules (A). Arrows point on the sutures ($\times 12900$). Fig. 13. Two sporozoites inside a sporocyst revealing apical complex (a), a tube (connected to rhoptries) (t) and micronemes (m); hn: host cell nucleus; Y: yellow body ($\times 15900$). Figs. 14 to 16. Images of yellow body (Y) including hard-walled sporocysts containing sporozoites; rb: a presumed refractile body ($\times 8000$, $\times 8400$ & $\times 13800$)

Taxonomy

Goussia hyperolisi n. sp.

Type host: *Hyperolis viridiflavus* (Dumeril & Bibron), tadpoles.

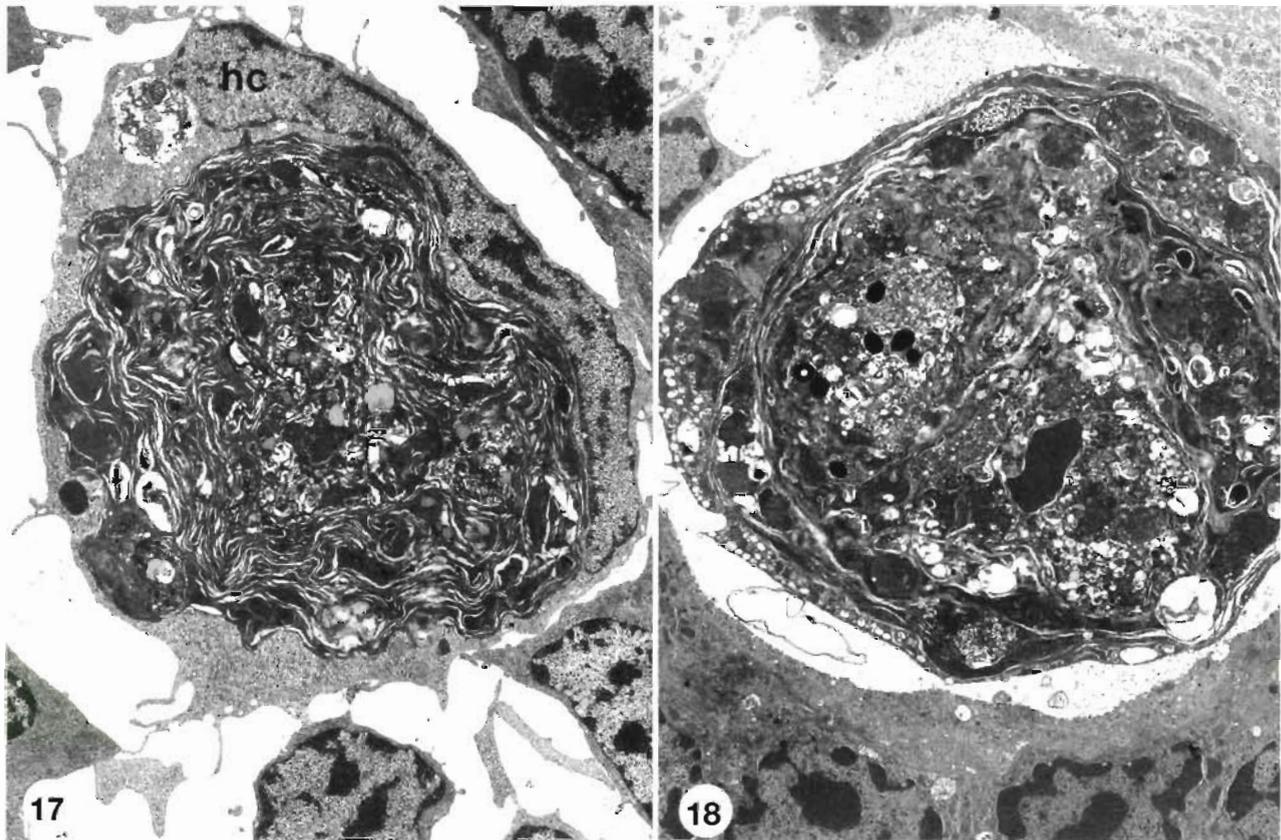
Type locality: Sagana fish ponds, Kenya.

Type material: Syntypes, Giemsa-stained slides in the Israel National Collection of Natural History (c/o I. Paperna).

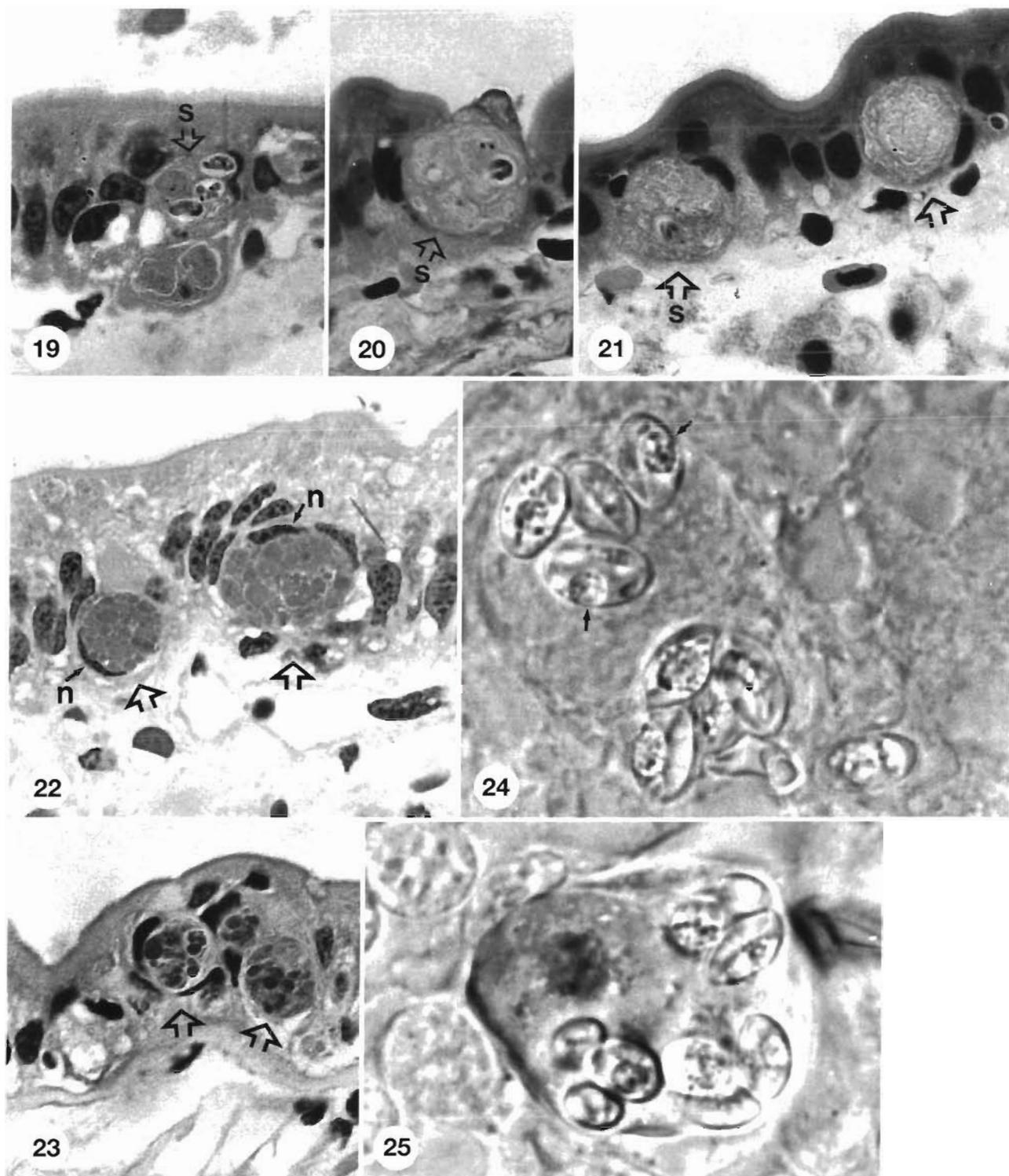
Description

Early oocysts in fresh impressions from the gut were 7.7 ± 1.0 (7.0 – 9.8) μm ($n = 11$) and sporoblasts $9.45 \pm$

0.91 (8.4 – 10.6) μm ($n = 6$) in diameter. Mature $7.22 \pm 0.25 \times 4.92 \pm 1.07$ (5.6 – 7.7×4.2 – 5.6) μm ($n = 8$) sporocysts were colorless, and in addition to the 7×1.4 – $2 \mu\text{m}$ sporozoites contained a 4.2 – $5.6 \times 2.8 \mu\text{m}$ residual body (Figs. 24, 25 & 26). In Giemsa-stained smears from the gut, round, amylopectin-loaded oocysts were $7.74 \pm 0.56 \times 7.04 \pm 0.44$ (7 – 8.4×6.3 – 7.7) μm ($n = 6$). Sporoblasts with 2 or 4 nuclei were 8.4 – 13.3×7.7 – $11.2 \mu\text{m}$ in size and sporulated oocysts reached $13.3 \times 11.2 \mu\text{m}$. Sporocysts, $6.58 \pm 0.75 \times 4.84 \pm 0.58$ (4.9 – 7.7×4.2 – 5.6) μm ($n = 11$) in size, were still enclosed within oocysts or freely scattered, and contained either 2 deep-red-staining nuclei or already-developed sporozoites. In histological sections, only oocysts and sporocysts were



Figs. 17 & 18. Yellow bodies within a detached cell of the gut epithelium of *Hyperolis viridiflavus* tadpoles. hc: intact host cell cytoplasm and an elongate nucleus ($\times 4700$ & $\times 5300$)



Figs. 19 to 23. MHE-stained histological sections of *Goussia hyperolisi* n. sp. infected *Hyperolis viridiflavus* tadpole gut ($\times 1420$). Fig. 19. Sporulated oocyst (s) in gut tissue. Figs. 20 & 21. Early yellow bodies (arrows), some still containing remains of sporocysts (s). Fig. 22. Yellow bodies filling up with the lipofuscin-like substance; the yellow-body cell nucleus is still noticeable (n). Fig. 23. Late (?) yellow body with dense granulated yellow substance.

Figs. 24 & 25. Sporulated oocysts of *G. hyperolisi* n. sp. in fresh squash preparation from *H. viridiflavus* intestine ($\times 2460$)

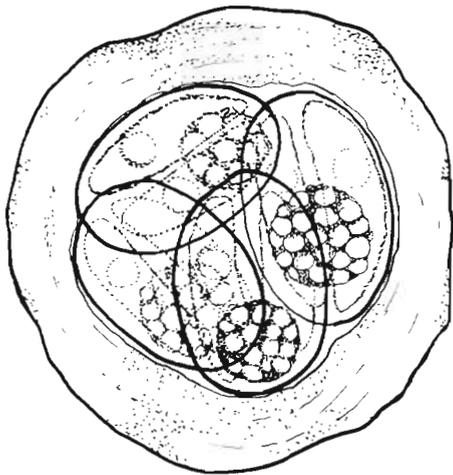


Fig. 26. Line drawing of sporulated oocyst enclosed within a yellow body ($\times 4700$)

found in the gut tissue, predominantly in the epithelial layer. Usually several sporulated oocysts (up to 8) or freed sporocysts were seen aggregating in an inclusion of degenerate substance inside hypertrophied host cells (Figs. 19 to 21, 24 & 25). A few sporulated oocysts, however, were seen lodged inside the underlying mucosal connective tissue (lamina propria). Yellow bodies remained after the infection had terminated (Figs. 20 & 21). In Giemsa-stained smears these bodies reached a diameter of $15\ \mu\text{m}$, had a coagulated consistency and stained violet gray.

Remarks

This is the first described eimeriid species from African-continent anurans (see Upton & McAllister 1988). Sporocysts of the presently described species from African reed frog tadpoles open via longitudinal sutures, which is a characteristic of members of the genus *Goussia*. The coccidium is very similar to *G. neglecta* (Nöller 1920) redescribed by Molnar (1995) from *Rana rhidibunda* and *R. esculenta* tadpoles in Hungary. Sizes of the oocysts and sporocyst of the presently reported species diverge somewhat from those of *G. neglecta*, with a sporocyst shape index of 1.1–1.3 versus 1.8 in the latter. Furthermore, conspecificity on account of geographical and host (at the generic level) differences seems to be unlikely.

DISCUSSION

Infections by *Goussia* spp. seem to be widespread among tadpoles of diverse genera. Lee Berger of the CSIRO Australian Animal Health Laboratory, Geelong,

Victoria, Australia, presented the senior author with a histological slide prepared from the intestine of a *Lymnodynastes tasmanicus* tadpole heavily infected with sporulating *Goussia*-like coccidian oocysts, similar to the one presently described. Molnar (1995) pointed out the close relationship between *G. neglecta* and congeneric species infecting fish, and associated them with the aquatic nature of their hosts, as compared to the terrestrial habitat of frogs and toads. A close affinity to piscine species is not restricted, however, to tadpole coccidia: Coccidia occurring in metamorphosed frogs and toads also have a thin, fragile oocyst wall, sporocysts lacking a stieda body (though some do open through an apical device, rather than a longitudinal suture; Chen & Desser 1989) and show endogenous sporulation, which takes place inside the gut epithelium (Paperna 1995a, Paperna & Lainson 1995). Similar to the previously described *Eimeria bufomarinii*, from *Bufo marinus* (Paperna & Lainson 1995), and to piscine coccidia (Paperna 1995b), wall-forming bodies, as they occur among reptilian, avian and mammalian coccidia (Scholtzseck 1979, Ostrovska & Paperna 1987), are lacking. In this context it is also worth noting the observed merging of the oocyst envelope with the PV wall, which recalls the process of oocyst wall formation in several piscine coccidia (Desser & Li 1984, Paperna 1995b).

Of the few other fine structural characteristics observed which are absent from the previously described anuran Coccidia, adnuclear inclusions have been reported in some reptilian coccidia (Ostrovska & Paperna 1987); the tube-like structure apparently connecting rhoptries to the apical complex in merozoites and sporozoites has also been observed in hemogregarine merozoites (Paperna & Sabnai 1982) and *Sarcocystis bradyzoites* (Paperna unpubl. data). Peculiar to the presently described coccidium were the intense folding of the PV wall and the large size of the rhoptries.

While describing *Goussia neglecta*, Nöller (1920) noted the disappearance of infection from the tadpoles as they neared their metamorphosis to frogs. In the reed frog, infection also terminated in tadpoles which failed to develop to the metamorphosis stage. This suggests that infection is time-restricted and expires independently of the metamorphosis process. It is worth noting that, unlike in pond conditions, in laboratory-kept tadpoles reinfection is less likely to occur. It remains to be seen, however, whether the parasite can become established and develop in gut cells of frogs. The host cells of oocyst stages in the tadpoles resemble intraepithelial leukocytes rather than ordinary mucosal epithelial cells. This is suggested from their elongate nuclei and the processes extending from their surface, which is often disengaged from the neighboring cells. Intraepithelial lymphocytes (IELs) have been

shown to be the host cells of invading sporozoites and possibly other stages as well (Lawn & Rose 1982, Fernando et al. 1987, Vilenkin & Paperna 1997). The infected host cells in the tadpole gut seemed to become surrounded by additional intraepithelial leukocytes. The spontaneous termination of the infection suggests the involvement of defense processes. It is highly reminiscent of the spontaneous recovery phenomena involving a formation of 'yellow bodies' reported in infections by the piscine coccidia *G. carpelli* (Kent & Hedrick 1985, Jendrysek et al. 1994) and *G. sinensis* (Baska & Molnar 1989). Although yellow bodies occur in guts of fish not infected with Coccidia, in tadpoles, as in fish, their appearance seems to be linked with the final stage of the coccidial infection in the host cells (Molnar 1984, Lom et al. 1991). The presence of several oocysts in such bodies is the consequence of multiple infection rather than of the merging of several host cells as suggested previously (Lom et al. 1991). In *G. carpelli* infections, yellow bodies interfere with the release process of ripe sporocysts into the gut lumen, and hence their evacuation from their host (M. Vilenkin & Paperna unpubl.). In tadpoles, as in fish, sporulated oocysts locked within the formed yellow body gradually disintegrate.

Kent & Hedrick (1985) demonstrated ceroid and lipofuscin in yellow bodies of *Goussia carpelli*, while electron microscopic images of yellow bodies revealed electron-dense, degenerating membranes, connected with secondary lysosomes. Multilaminated structures and lysosomal electron-dense inclusions also occurred in the tadpole yellow body. The globular lipofuscin seen in the tadpole gut yellow bodies under light microscope seem to have been extracted during processing for electron microscopy. Migration or displacement of coccidial oocysts or other stages below the gut mucosal basal lamina is a common phenomenon among coccidia of fish (Molnar 1984) as well as of other vertebrates (Pasternak & Fernando 1984).

Acknowledgements. The authors thank Mrs G. Rotish of the Museum of Kenya for the identification of the frogs and Prof. E. S. Mitema, head of the Department of Public Health, Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Nairobi, for providing research facilities to the first author. This work was partially supported by a research grant to the first author from the Israeli Ministry of Science.

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