LIFE HISTORY AND ULTRASTRUCTURE OF *EIMERIA BUFOMARINI* N.SP. (APICOMPLEXA : EIMERIIDAE) OF THE GIANT TOAD, *BUFO MARINUS* (AMPHIBIA : ANURA) FROM AMAZONIAN BRAZIL

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Summary :

Eimeria bufomarini n.sp. (Apicomplexa : Eimeriidae) is described from the toad *Bufo marinus* (Amphibia : Anura) in north Brazil, by both light and electron microscopy. The oocysts average 9.2 × 9.0 µm and the sporocysts 6.25 x 3.7 µm. Oocysts mature in the epithelial cells of the intestine and, due to their extreme fragility, usually rupture and release their sporocysts in the host. Among other peculiarities, ultrastructure studies failed to reveal typical Type I and Type II oocyst wall-forming bodies characteristically found in coccidia of terrestrial vertebrates. The presence was shown, instead, of electron-dense bodies similar to those found in the coccidia of fish. This feature, the common possession of a soft, non-resistant wall together with an endogenous sporulation among anuran and piscine coccidia suggests phylogenetic relationships.

KEY WORDS : *Eimeria bufomarini* n. sp. Apicomplexa. *Bufo marinus*. Amazon. Brazil. ultrastructure. life history.

MOTS CLES : Eimeria bufomarini *n. sp. Sporozoa.* Bufo marinus. *Amazonie. ultrastructure. cycle biologique.*

Résumé *: EIMERIA BUFOMARINI* N. SP. (SPOROZOA : EIMERIIDAE) DU CRAPAUD GÉANT (AMPHIBIA: ANOURA) EN AMAZONIE BRÉSILIENNE : ÉTUDE EN MICROSCOPIE OPTIQUE ET ÉLECTRONIQUE AVEC DES COMMENTAIRES SUR LA PHYLOGÉNIE DES COCCIDIES D'ANOURES.

Eimeria bufomarini n. sp. (Sporozoa : Eimeriidae) est décrite en microscopie optique et électronique, chez le crapaud Bufo marinus *(Amphibia : Anoura) du Nord du Brésil. Les oocystes mesurent 9,2 × 9,0 µm et les sporocystes 6,25 × 3,7 µm. Les oocystes murissent dans les cellules épithéliales de l'intestin et, en raison de leur extrême fragilité, ils se rompent et libèrent leurs sporocystes à l'intérieur de l'hôte. Ils ont pour principale particularité l'absence des vésicules de type I et II initiant la paroi, qui sont caractéristiques des vertébrés terrestres. Par contre des corps denses semblables à ceux qui sont connus chez les coccidies de poissons ont été observés. L'association des caractères – paroi de l'oocyste molle et fragile et sporulation endogène – indiquent des relations phylogéniques entre les coccidies d'Anoures et celles de poissons.*

INTRODUCTION

The coccidia of amphibians have received relatively little attention compared with those of other vertebrates : most available reports are on holarctic species, and are dated to the earlier part of this century (see Pellerdy, 1974). The oocysts of many anuran coccidia sporulate and desintegrate in the gut, and infection is not readily detected when faeces are examined. In addition, the usual abundance of lumen protozoans further diminishes the prospect of finding oocysts or sporocysts. In the present communication we give a description of a previously undescribed coccidian from *Bufo marinus* (Bufonidae) from Pará State, north Brazil.

MATERIALS AND METHODS

Toads were captured by hand on the outskirts of Belem, and in the vicinity of Salvaterra on the Island of Marajo, north of Belém. Infections were detected by the examination of squash preparations of gut tissue, Giemsa-stained smears and histological sections of the intestine. Faecal examination, following Zinc sulphate ($ZnSO_4$) floatation of material from the infected animals, failed to reveal oocysts or sporocysts.

Twenty mature oocysts of the parasite were measured in fresh squash preparations of the intestine, using an ocular micrometer, × 8 eyepieces and a × 100 neofluar objective. The drawing was based on a combination of direct observations made on the oocysts being measured, and photomicrographs. The latter were prepared using a Zeiss Photomicroscope III and Kodak TMX 402 film. All measurements in light microscope studies are in micrometers (µm) and are given as means, with the range in parentheses, followed by the shape-index (= ratio of length/width).

Other endogenous stages were studied in smears fixed in aqueous Bouin's fluid and stained by a modified Giemsa method (Lainson, 1958), and in histological sections of the intestines. For this purpose, segments of the gut were fixed in 10 % neutral, buffered formaldehyde and either embedded in glycol methacrylate medium (GMA of Agar Co., U.K) or paraffin wax. Sections of GMA-embedded material were cut at 3.0-4.0 µm with a glass knife on a Serval JB4 microtome. Some were stained with Meyer's haemalum and eosin: others were post-fixed in aqueous Bouin's fluid for 20 minutes, washed in 70 % ethyl alcohol until colourless, and stained in Giemsa in the same manner as were the smears. Paraffin-wax embedded intestine was cut at 4.0-5.0 µm and stained with haematoxylin and eosin or Giemsa.

For electron microscopy, tissue segments were fixed in

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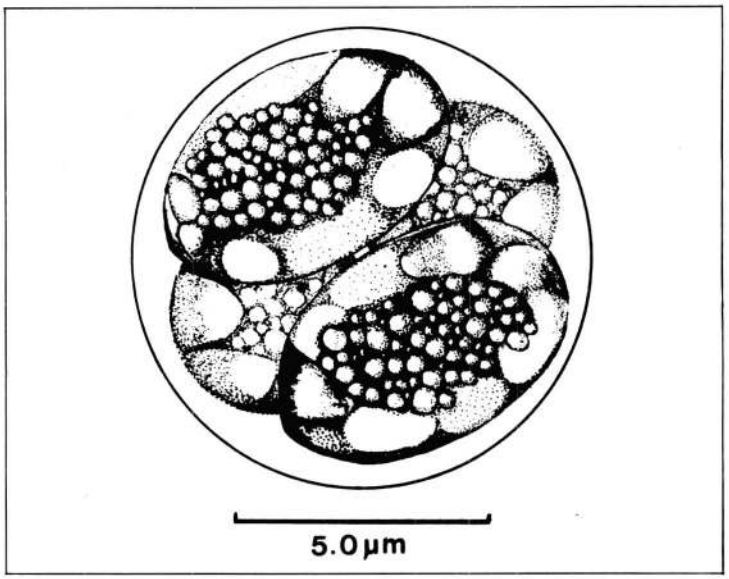
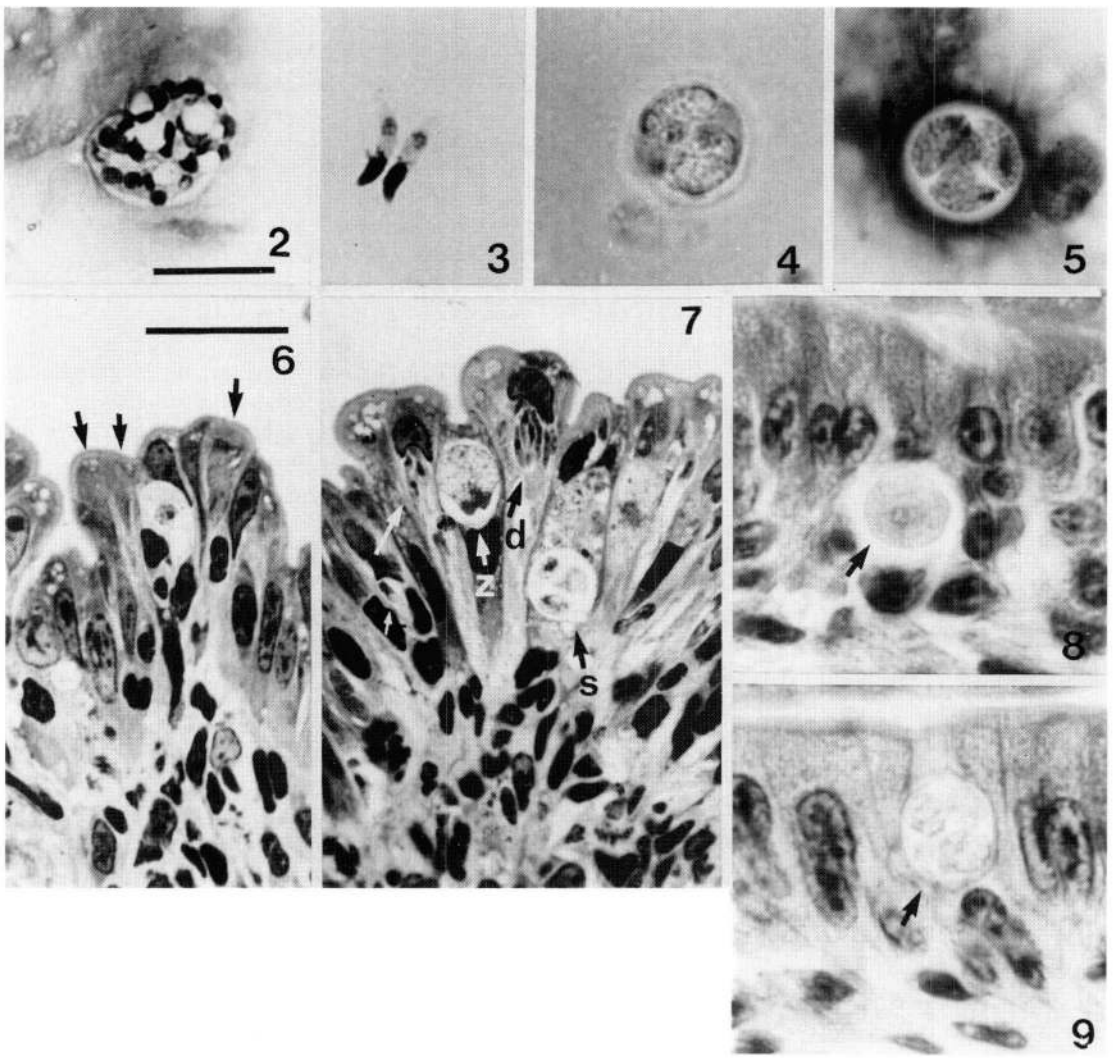


Fig. 1. – Line-drawing of a living, mature oocyst of *Eimeria bufomarini* n.sp., in a squash preparation of epithelium from the small intestine of *Bufo marinus*.



Figs. 2-9. – Endogenous stages of *E. bufomarini* n.sp., in the small intestine of *Bufo marinus*. 2. Almost mature meront. 3. Free merozoites. 4. Zygote. 5. Oocysts with four sporoblasts. All stages as seen in Giemsa-stained intestinal smears : scale bar = 10.0 µm. 6. Young, elongate gamonts at the tips of the gut epithelial cells. 7. Scattered merozoites (arrows), segmented meront (d), zygote (z) and sporulated oocyst (s). 8. Macrogamont. 9. Mature oocyst with sporozoites. All stages as seen in histological sections of the small intestine stained by Meyer's haemalum eosin or Giemsa. Scale bar = 10.0/µm.

2.5 % glutaraldehyde in cacodylate buffer (0.1m, pH 7.4) for 24 hours at 4.0 °C, rinsed repeatedly in the same buffer, post-fixed in 1.0 % osmium tetroxide in the same buffer for one hour and, after rinsing in the same buffer, dehydrated in graded alcohols and embedded in Epon. Thin sections, cut on a Reichert Ultracut ultratome with a diamond knife, were stained on grids with uranil acetate and lead citrate and examined with a Joel 100CX TEM.

RESULTS

EIMERIA S.L. BUFOMARINI N.SP. (Figs. 1-26)

Diagnosis : oocysts predominantly spherical, to subspherical, 9.2 × 9.0 (8.7-10.0 × 8.7-10.0), shape-index 1.0 (1.0-1.1). The oocyst wall is colourless and extremely delicate; seemingly of a single layer. There is no micropyle, oocyst residuum or polar bodies. Sporocysts 6.25 × 3.7 (6.0-6.5 × 3.7-4.0), shape-index 1.7 (1.6-1.7), with an almost imperceptible Stieda body-like knob. Two refractile bodies are present in each sporozoite, as detected by transmission electron microscopy (TEM), but these are seen with difficulty by light-microscopy due to the bulky sporocyst residuum which obscures most of the sporozoites.

Type host : *Bufo marinus* (Amphibia : Anura : Bufonidae)

Location in host : epithelial cells of the intestine

Sporulation : endogenous. The oocysts sporulate within the epithelial cells and, usually, freed mature sporocysts are discharged into the gut lumen. Whole oocysts gain entrance into the lumen only if the host cell collapses prior to completion of sporulation.

Type material : Sections of infected intestine of toad BmXl/3495-2 are deposited in the Museum National d'Histoire Naturelle, Paris (N° 303LN). Further sections and also intestinal smears from same toad are held in the Department of Parasitology, Instituto Evandro Chagas, Belém, Pará, Brazil, and in the Department of Animal Sciences, Faculty of Agriculture, Rehovot, Israel.

Type locality : Salvaterra, Island of Marajo, Para State, north Brazil. Material also obtained from the outskirts of Belém, Pará.

Prevalence : infections were detected in five of seventeen toads captured in a disused well in Salvaterra, Marajo, and in one of thirteen from a suburban site in Belém.

Pathogenicity : no evidence of pathogenicity was found in any of the infected toads.

Etymology : the specific name of *E. bufomarini* is derived from the generic and specific name of the host, *Bufo marinus*.

Merogony and gametogony : development is within the epithelial cells of the intestine.

In Giemsa-stained smears it was not possible to differentiate between developing meronts and microgamonts, and undivided parasites sometimes reached up to 7.0 × 5.0. However, two distinctly different dividing stages were seen. One of these, with pale blue cytoplasm, produced up to 11 nuclei when measuring only 4.2 in diameter, 26 nuclei at about 8.4 (Fig. 2) and 36 in forms reaching 10.5 × 10.0. Other parasites possessing a denser, strongly stained cytoplasm, reached from 8.0-15.0 × 7.0 and at the most contained only 6-8 nuclei. It seems likely that some of the former division stages represented developing microgamonts.

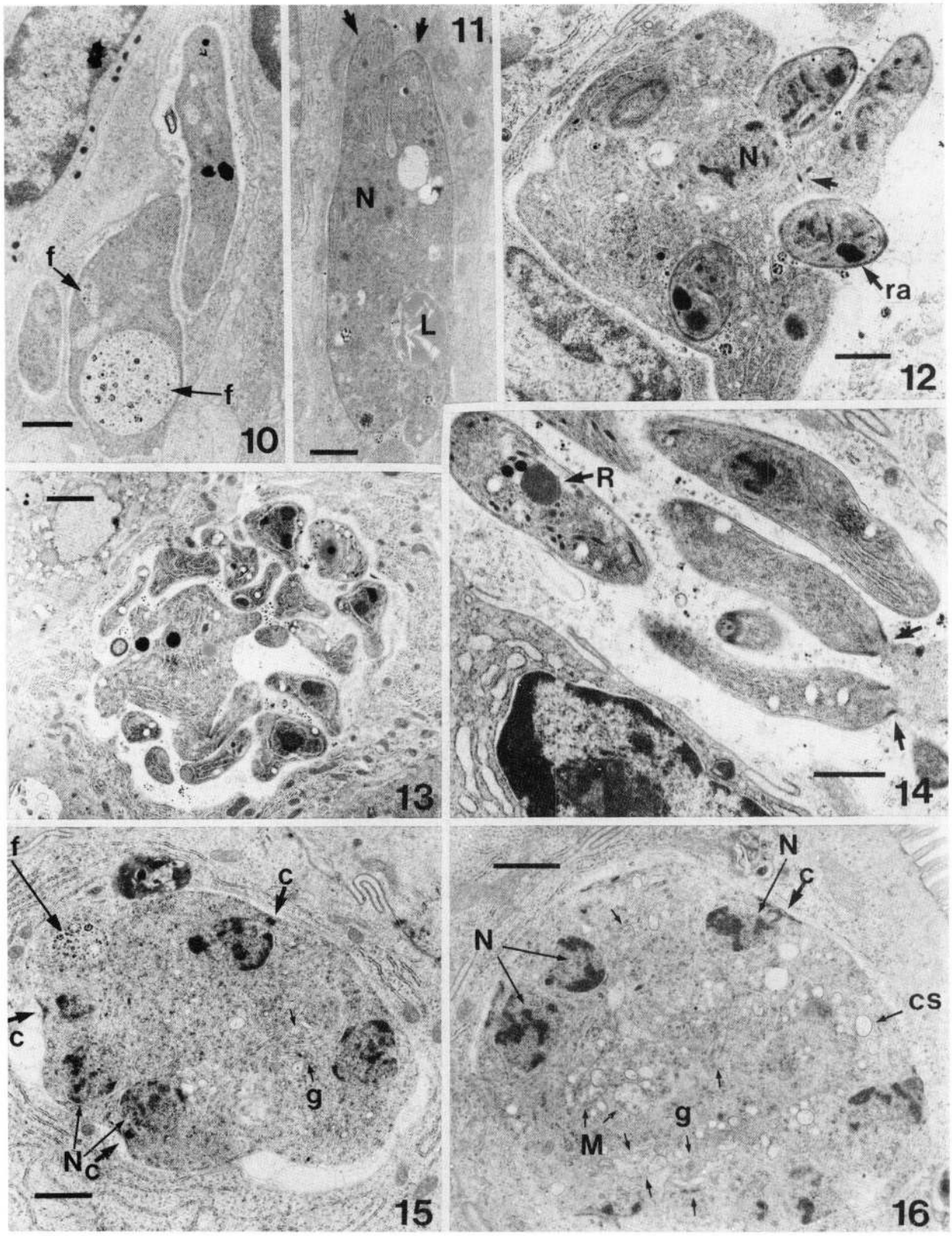
Two types of mature meronts were noted. One of these produced up to 15 longer and thinner merozoites, measuring 5.6-8.4 × 1.0-1.4 (Figs. 3,7) : the other gave rise only up to eight merozoites, which were much stouter and averaged 7.0 × 2.1. Our impression is that it was from the latter that gamonts subsequently developed. Histological sections showed there to be a residuum following separation of the merozoites, which seem to remain for some time in clusters at the division site, and where some undergo a further division, apparently by endodyogeny (see TEM studies, below).

Histological sections (and TEM) showed that the gamonts maintained an elongate form throughout their growth (Figs. 6, 18-21), but rounded up at maturity. Like the meronts, they were located at the tips of the host cells, just below the brush border (Fig. 6). There was, however, a marked tendency for all developmental stages to round up when dislodged from their host cells, in smears.

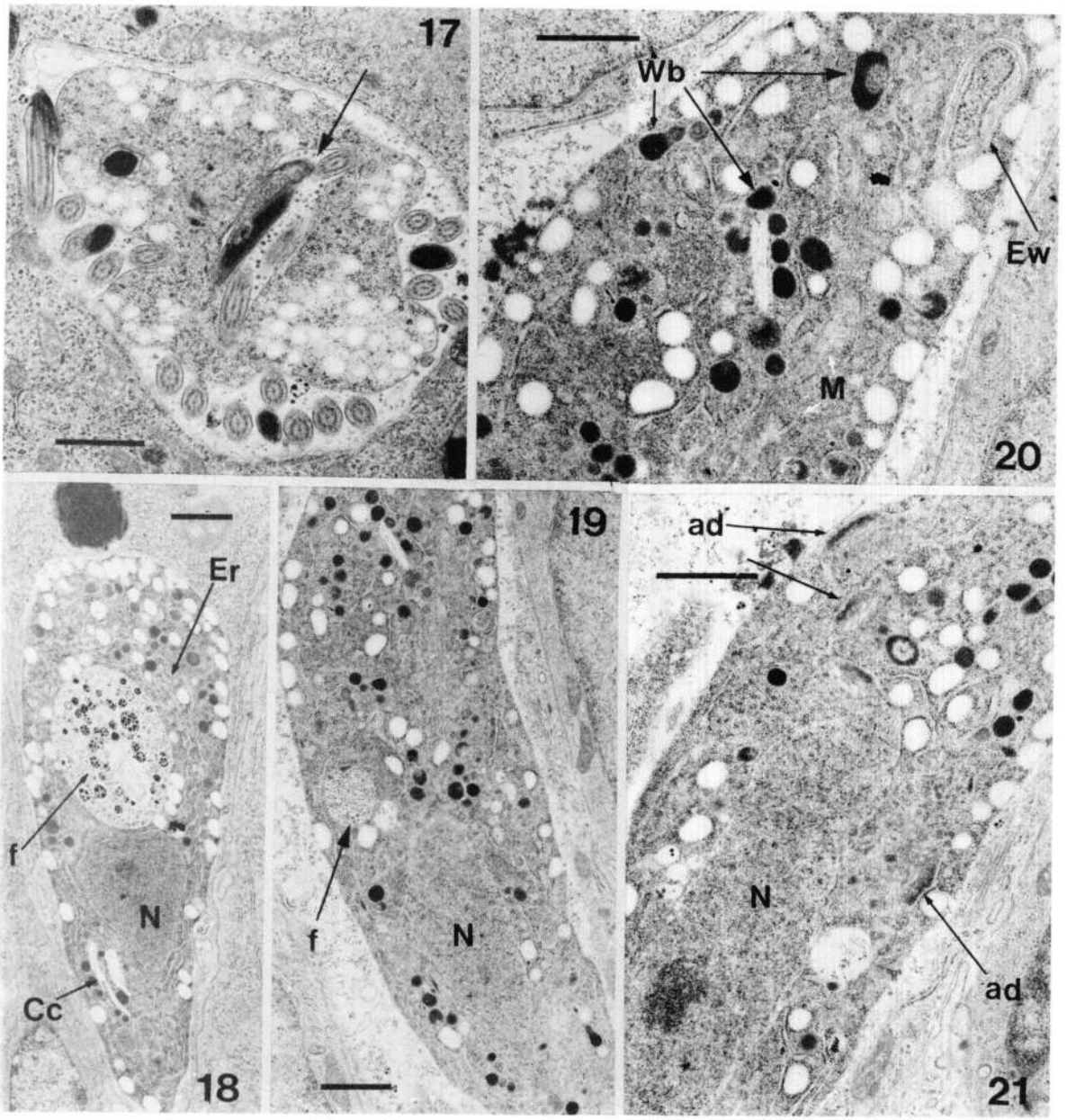
The youngest forms considered to be developing macrogamonts possessed a large, pale nucleus with a distinct nucleolus, and measured 4.0-5.0 × 3.0, as seen in smears. Developing microgamonts were difficult to identify in such material and measurable specimens were not located. In sections, however, mature forms with up to ten detectable microgamonts measured an average of 7.0 × 8.0.

Zygotes, seen in sections, were subspherical and measured about 7.5 × 5.0 (Fig. 8): at this stage some host cells showed signs of degeneration. Unsporulated oocysts seen in smears measured 10.5 × 8.0 (Fig. 4) but rounded up to 10.0 × 10.0 when sporulated (Fig. 5). Measurements for the mature, living oocysts (Fig. 1) have already been given, under the heading Diagnosis. The slightly increased size in Giemsa-stained smears is doubtless due to a flattening of the oocysts. As to be expected, all parasites viewed in histological sections appear smaller than those seen in smears, due to shrinkage.

Ultrastructure studies showed that although the mero-



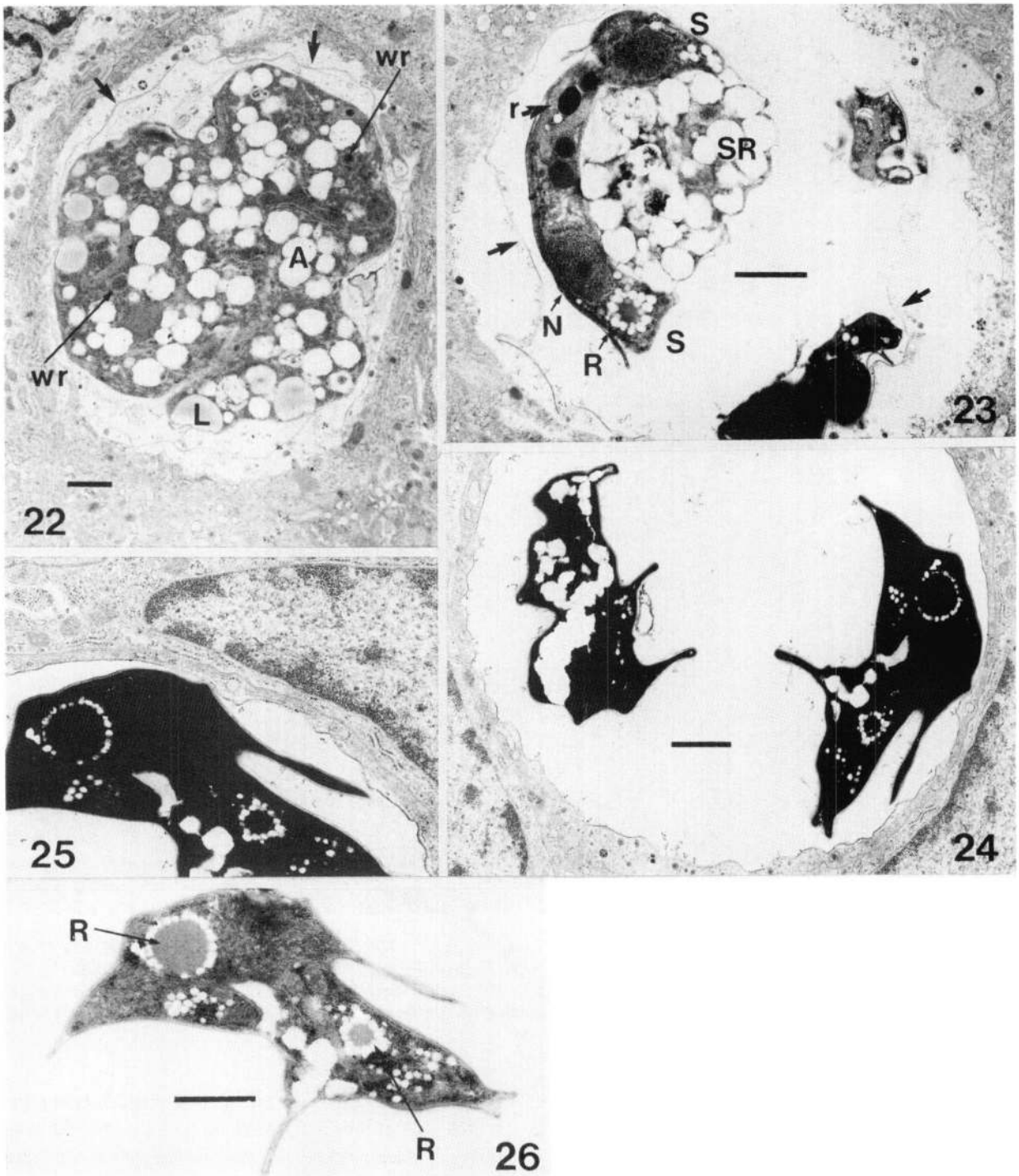
Figs. 10-16. – Ultrastructure of endogenous stages of *E. bufomarini* n.sp., in the small intestine of *Bufo marinus* (scale bars = 1 µm). 10. Trophozoites and young gamont with large food vacuole (f), each in different parasitophorous vacuoles, in the same host cell. 11. Merozoite, seemingly dividing by endodyogeny : arrow indicates two differentiating apical complexes; L, lipid vacuole; N, merozoite's nucleus. 12. Dividing meront; N, nucleus; ra, rhoptry anlagen. 13. Merozoites differentiating from a meront leaving a meront residuum. 14. Formation of merozoites emerging from the meront's residuum; arrow: thickened pellicular junction; R, refractile body. 15,16. Young microgamonts with several nuclei (N), mitochondria (M and small arrows), Golgi complexes (g, between small arrows) and many cisternae (cs).



Figs. 17-21. – Ultrastructure of endogenous stages of *Eimeria bufomarini* n.sp., in the small intestine of *Bufo marinus*; scale bars = 1 µm. 17. Mature microgamont with microgametes (arrowed). 18. Young macrogamont with canaliculi (Cc), distinct endoplasmic reticulum (Er) and large food vacuole (f). 19. Mature macrogamont. 20,21. Enlarged view showing details of electron-dense bodies simulating wall forming bodies (Wb), mitochondria (M), endoplasmic reticulum whorls (Ew) and Golgi-like aggregates with electron-dense adjunct (ad).

zoites of a given meront at first lay in a common parasitophorous vacuole (PV) (Fig. 10), they later became separated in individual, adjoining vacuoles. Some were seen to be undergoing division prior to the formation of further meronts, seemingly by endodyogeny (Fig. 11). As a result, stages which were still with the characteristic merozoite pellicle, rhoptries and micronemes were found together in the same PV with other stages which had lost their rhoptries and had differentiated into juvenile meronts bounded by a single limiting membrane. Juvenile and dividing meronts (and gamonts) contained food vacuoles filled with medium and high electron-dense globules, and which sometimes occupied almost the whole width of the parasite (Figs. 10, 18). The globular substance was also seen in the PV.

Ultrathin sections showed mature meronts to contain a few amylopectin granules (probably represented by large vacuoles, seen in the parasites examined in Giemsa stained smears – Fig. 2), many large mitochondria, and dense rough and smooth endoplasmic reticulum (ER) : the cytoplasm sometimes showed remnants of micronemes and food-vacuole globules (Fig. 12). Differentiation of the merozoites is by exogenesis – at



Figs. 22-26. – Ultrastructure of *E. bufomarini* oocysts in the intestinal epithelium of *Bufo marinus*; scale bars = 1 µm. 22. Young oocyst with remains of the electron-dense bodies (wr); arrows indicate oocyst superimposed (second) wall membranes. 23. Oocysts, showing cross section of a sporocyst through sporozoite (S) and a residuum (SR) : N, sporozoite's nucleus; R, refractile body surrounded with amylopectin granules; r, rhoptries. 24. Cross section through oocyst with hard-walled sporocysts. Oocyst wall juxtaposed to the PV wall. 25. Enlarged view of 24, revealing details of the interphase between the host-cell and the oocyst. 26. Same, less contrusted print allowing detection of some details of the sporocysts, including sporozoite's refractile body.

the surface of the meront (Figs. 12-14) – and the pellicle of the emerging merozoite forms a thickened juncture with the meront wall (Fig. 14). Developing merozoites have one or two rhoptry anlagen (Fig. 12) and some free merozoites were seen to have a refractile body (Fig. 14). The presence of a conspicuous meront residuum was confirmed (Fig. 13).

Viewed by TEM, developing microgamonts (Figs. 15, 16) are recognized by their characteristic peripherally arranged nuclei, adjoined with centrioles and a mitochondrion. Their cytoplasm contains a Golgi complex and an ER network, with a variable number of cisternae, large centrally located mitochondria and amylopectin granules: some contained small food vacuoles (Fig. 15). Mature parasites with differentiated microgametes were loaded with amylopectin granules (Fig. 17).

Macrogamonts are elongated bodies and, from an early stage in their development, they gradually become filled with amylopectin granules : some contained large food vacuoles, packed with globular aggregates (Figs. 18, 19). The cytoplasm of the macrogamont contains aggregated and scuttered ribosomes, a network of rough ER, canaliculae, mitochondria (Figs. 18, 20), Golgi-like aggregates with electron-dense adjunct, and electron-dense objects reminiscent of Type I wall-forming bodies (Fig. 20). The contents of some of these bodies was disaggregated into lucent granular material (Fig. 20). Zygotes, or young oocysts, were filled with large amylopectin granules, a few lipid vacuoles and the remains of the electron-dense bodies seen in the macrogamonts (Fig. 22). No sign could be seen of a rigid oocyst wall, either in the young oocyst or in the mature oocyst containing sporocysts with developed sporozoites (Figs. 23-26). In addition to the fine, soft limiting membrane of the oocyst, there developed a second delicate membrane (Fig. 22) which appeared after sporulation to become juxtaposed to the PV wall (Figs. 24, 25). The tough wall of the sporocysts clearly interfered with fixation and subsequent processing. Nevertheless, it was possible to detect amylopectin granules scattered in the cytoplasm of the sporozoites and concentrated around each of the two refractile bodies (Figs. 24-26). Large electron-dense bodies were thought to be rhoptries, and micronemes were sometimes apparent. The bulky sporocyst residuum contained many amylopectin granules (Fig. 23).

DISCUSSION

There are similarities in the oocyst size of *E. bufomarini* and that of *E. Iaminata* Ray, 1935 and *E. himalayani* Ray & Misra, 1942, both of which were described from *Bufo* spp., in India. Conspecificity is considered unlikely, however, due to geographic as well as host species differences. All other anuran coccidia described to date have significantly different morphometric characters.

Sporulation is known to be endogenous in most anuran coccidia (Pellerdy, 1974; Upton & McAllister, 1988; Chen & Desser, 1988), and where species have been examined in detail authors have noted the finding of sporulated oocysts and freed sporocysts in the gut lumen. Examples include *E. himalayani* (Ray & Misra, 1942), *E. Iaminata* (Ray, 1935), *E. neglecta* (Noller, 1920) and *E. prevoti* (Laveran & Mesnil, 1902; Boulard, 1975). Upton & McAllister (1988) have reviewed literature on the coccidia of anurans and commented on the delicate wall of the oocysts, which results in their rapid disruption in a similar way to those of piscine coccidia (Paperna & Cross, 1985; Lom *et al.*, 1991). *E. bufomarini* n.sp. forms no exception to this mode of development, and we have also noted endogenous sporogony of another as yet undescribed coccidium of the Brazilian frog, *Leptodactylus fuscus* (unpublished observations).

Laveran & Mesnil (1902) erected a new subgenus, *Paracoccidium*, to emphasise the phylogenetic significance of the type of oogenesis and sporulation described above, and it may be that a critical review and further ultrastructural information will justify their views.

Other peculiarities of E. *bufomarini* are the delayed departure of the merozoites from their parent's parasitophorous vacuole, and the elongate shape of the macrogamonts. Similarly vermiform macrogamonts have been described for *E. raillieti* of the anguid lizard *Anguis fragilis* (Lainson, 1959). Division of newly formed merozoites, usually by endodyogeny, has been recorded for *Schellackia landauae* of the lizard *Polychrus marmoratus* by Lainson *et al.*, (1976) and is also known among the coccidia of fish (Paperna, 1991). Additional peculiarities revealed in the ultrastructural study of *E. bufomarini* are the large food vacuoles with their globular contents, particularly conspicuous in the macrogamonts, and the exaggerated number of mitochondria. The Golgi-like structure, with electron-dense adjunct are characteristic of the mature macrogamonts, and similar bodies have previously been recorded in the meronts of some reptilian coccidia (Paperna & Landsberg, 1989) : their function is unknown.

Perhaps the most important finding in this description of *E. bufomarini* is the undoubted absence of the two types of wall-forming bodies which are so characteristic of reptilian, avian and mammalian eimeriid coccidia and the presence, instead, of electron-dense bodies resembling those found in the coccidia of fish (Steinhagen, 1991). Although data are at present avai-

lable from only the one species, *E. bufomarini*, this feature may be a general character of anuran coccidia. The fragile oocyst wall is another character which, in general, separates the coccidia of anurans from those of the terrestrial vertebrates; although in this respect it may be noted that the oocysts of the intranuclear *E. ranarum* (Labbe 1894) develop within the host, but are recorded as having a rigid, resistent oocyst wall.

While the above-mentioned features suggest a close affiliation of the anuran coccidia with those of fish, they may simply be primitive characters parallel to those seen not only in the piscine coccidia but also among the coccidia of invertebrate hosts and in some tissue-cyst forming coccidia such as *Sarcocystis* and *Frenkelia*.

Le Bail & Landau (1974) found *Bufo marinus* from French Guyana to commonly be infected with the haemogregarine *Schellackia balli* (Lankesterellidae), and we have noted what is probably the same parasite in this toad from north Brazil. Although the oocysts of *Schellackia balli* also develop in the epithelial cells of the small intestine, they can readily be distinguished from those of *E. bufomarini* by their possession of eight naked sporozoites, which develop directly from the zygote.

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REFERENCES

LE BAIL O. & LANDAU I. Description et cycle biologique experimental de *Schellackia balli* n.sp. (Lankesterellidae) parasite de crapauds de Guyane. *Annales de Parasitologie Humaine et Comparée*, 1974, *49*, 663-668.

BOULARD Y. Redescription d'*Eimeria prevoti* (Laveran et Mesnil, 1902) (Eimeriidae) parasite de la grenouille verte en Normandie. *Protistologica*, 1975, *11*, 245-249.

CHEN G.J. & DESSER S.S. The coccidia (Apicomplexa : Eimeriidae) of frogs from Algonquin Park, with descriptions of two new species. *Canadian Journal of Zoology*, 1989, *67*, 1686-1689.

LABBÉ A. Recherches zoologiques et biologiques sur les parasites endoglobulaires du sang des vertébrés. *Archives de Zoologie expérimentale et générale*, 1894, *2* (Ser 3), 55-258.

LAINSON R. Observations on the development and nature of pseudocysts and cysts of *Toxoplasma gondii*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 1958, *52*, 396-407.

LAINSON R. Observations on *Eimeria raillieti* in the "slow-worm", *Anguis fragilis* (Reptilia, Anguidae). *Journal of Protozoology*, 1959, *6*, 355-359.

LAINSON R., SHAW J.J. & WARD R.D. *Schellackia landauae* sp. nov. (Eimeriorina : Lankesterellidae) in the Brazilian lizard *Polychrus marmoratus* (Iguanidae) : experimental transmission by *Culex pipiens fatigans*. *Parasitology*, 1976, *72*, 225-243.

LAVERAN M.A. & MESNIL F. Sur deux coccidies intestinales de la *Rana esculenta*. *Comptes Rendus des Séances de la Société de Biologie et de ses Filiales et Associées*, 1902, *54*, 857-860.

LOM J., STEINHAGEN D., KORTING W. & DYKOVA I. Oocyst formation in the coccidian parasite *Goussia carpelli*. *Diseases of Aquatic Organisms*, 1991, *10*, 203-209.

NOLLER W. Zur Kenntnis der Coccidien des Wasserfrosches (*Eimeria neglecta* nov. spec.) (Befruchtung und Sporogonie von Lankesterella). *Archives für Protistenkunde*, 1920, *41*, 176-180.

PAPERNA I. Fine structure of *Eimeria vanasi* merogony stages in the intestinal mucosa of cichlid fishes. *Diseases of Aquatic Organisms*, 1991, *10*, 195-201.

PAPERNA I. & CROSS R.H.M. Scanning electron microscopy of gamogony and sporogony stages of *Goussia cichlidarum* Landsberg and Papema, 1985, a coccidian parasite in the swimbladder of cichlid fishes. *Protistologica*, 1985, *21*, 473-479.

PAPERNA I. & LANDSBERG J.H. Fine structure of endogenous stages of *Eimeria turcicus* developing in gall bladder epithelium of the gecko *Hemidactylus turcicus*. *South African Journal of Zoology*, 1989, *24*, 251-259.

PELLERDY L.P. Coccidia and coccidiosis, 2nd Edition. Verlag Paul Parey, 1974, Berlin & Hamburg.

RAY H.N. On a new coccidian, *Eimeria laminata* n. sp., from the intestine of an Indian toad, *Bufo melanostictus* Schneid. *Parasitology*, 1935, *27*, 369-373.

RAY H.N. & MISRA, P.L. Observations on a new coccidium *Eimeria himalayanum* n.sp., from the intestine of a Himalayan toad, *Bufo* sp. *Proceedings of the Indian Science Congress*, 1942, *28*, 169 (Abstract 3).

STEINHAGEN D. Ultrastructural observations on merogonic and gamogonic stages of *Goussia carpelli* (Apicomplexa, Coccidia) in experimentally infected common carp *Cyprinus carpio*. *European Journal of Protistology*, 1991, *27*, 71-78.

UPTON S.J. & MCALLISTER C.T. The Coccidia (Apicomplexa : Eimeriidae) of Anura, with descriptions of four new species. *Canadian Journal of Zoology*, 1988, *66*, 1822-1830.

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