

LIGHT AND ELECTRON MICROSCOPE STUDY OF A *LANKESTERELLA PETITI* N. SP., (APICOMPLEXA : LANKESTERELLIDAE) INFECTING *BUFO MARINUS* (AMPHIBIA : ANURA) IN PARÁ, NORTH BRAZIL

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Summary :

Captive toads, *Bufo marinus*, were either inoculated with blood or fed with blood and liver from two other toads which were showing sporozoites of a *Lankesterella* sp., in their erythrocytes. Stages ranging from late zygotes to sporulated oocysts were encountered in various tissues of two of the experimental animals. Development of the parasite was shown to be in the endothelium of the blood vessels, which explains distribution of resulting sporozoites in a wide range of different tissues. Ultrastructure studies revealed wall-forming-like organelles in the late zygote which are seemingly involved in the deposition of the thick wall of the sporulating oocyst. This wall is perforated in several places, allowing extensions of the parasite, with contained mitochondria, to extend out to the limits of the parasitophorous vacuole. These features, together with the presence of one to several refractile bodies bounded by a coat of electron-dense droplets, appear to be unique to *Lankesterella*.

KEY WORDS : *lankesterella petiti* n. sp. Apicomplexa. *Bufo marinus*. toad. Brazil. ultrastructure. zygote. oocyst.

MOTS CLÉS : *lankesterella petiti* n. sp. Apicomplexa. *Bufo marinus*. crapaud. Brésil. ultrastructure. zygote. oocyste.

Résumé : ÉTUDE EN MICROSCOPIE OPTIQUE ET ÉLECTRONIQUE DE *LANKESTERELLA PETITI* N. SP. (APICOMPLEXA : LANKESTERELLIDAE) PARASITE DE *BUFO MARINUS* (AMPHIBIA : ANOURE) DE L'ÉTAT DE PARÁ, NORD BRÉSIL.

Des spécimens de *Bufo marinus* en captivité sont infectés soit par inoculation de sang, soit par ingestion de sang et de foie de deux autres crapauds présentant des sporozoïtes de *Lankesterella* dans leurs hématies. Les différents stades depuis le zygote âgé jusqu'à l'oocyste sporulé ont été obtenus dans différents tissus de deux des crapauds infectés. Il est démontré que le développement du parasite s'effectue dans l'endothélium des vaisseaux sanguins, ce qui explique que les sporozoïtes qui en sont issus se trouvent dans beaucoup de tissus différents. Les études ultrastructurales montrent que des organites, rappelant les granules constitutifs de la paroi du zygote âgé, semblent également intervenir dans la formation de l'épaisse paroi de l'oocyste en cours de sporulation. Cette paroi est perforée en différents endroits, ce qui permet l'issue d'expansions cytoplasmiques contenant des mitochondries hors des limites de la vacuole parasitophore. Ces faits, ainsi que l'existence d'un ou plusieurs corps réfringents entourés d'un manteau de gouttelettes denses, semblent particuliers aux *Lankesterella*.

INTRODUCTION

Sporozoites regarded as those of *Lankesterella* spp., have been recorded in the blood and haemopoietic tissues of anurans from all continents, including the neotropics (Pessôa & Cunha, 1967). The life-cycle of this parasite was first elucidated by Nöller (1912, 1920) who described the merogonic stages of *L. minima* (Chaussat, 1850) Labbé, 1899, in tissues of *Rana esculenta*, in Germany, and went on to demonstrate the role of leeches in the transmission of this parasite from frog to frog. Desser *et al.* (1990) showed that it was also possible to infect the tadpole stage of *Rana catesbeiana* by the bite of experimentally infected leeches.

The ultrastructure of sporozoites of *L. hylae*, of tree-frogs (Stehbens, 1966a), confirmed the apicomplexan nature of *Lankesterella*, while that of late oogonous

and sporogonous stages of *L. minima* indicated a considerable structural divergence of that parasite from the coccidia of reptiles and higher vertebrates (Desser *et al.*, 1990). Interestingly, this includes the genus *Schellackia*, which is till now regarded, by most, as a member of the family Lankesterellidae.

The present paper records further evidence for the unique ultrastructure of *Lankesterella* of anurans, following a study of the zygotes and early oocyst formation of *Lankesterella petiti* n. sp. a hitherto undescribed species of this parasite in Brazilian toads, *Bufo marinus*.

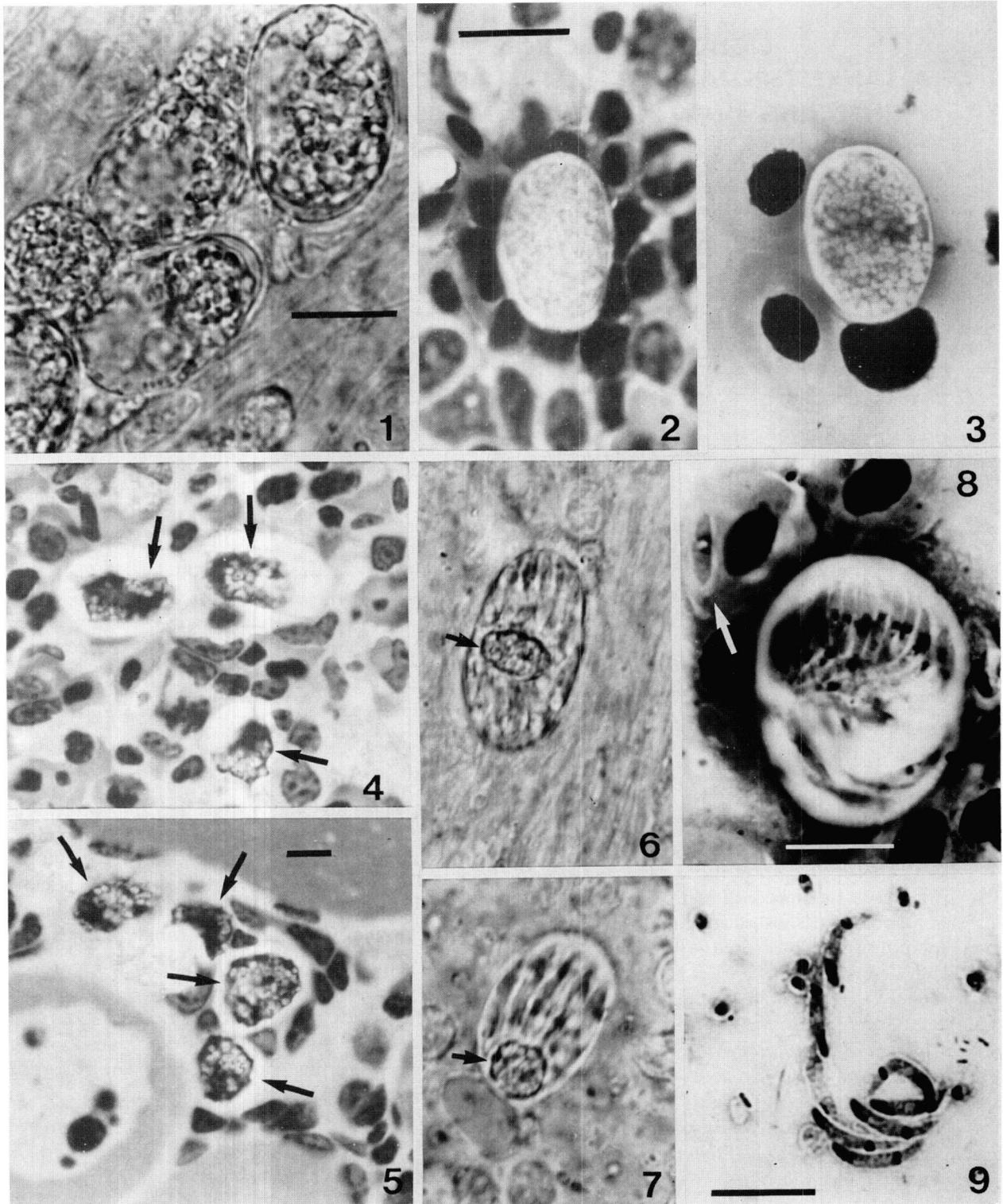
MATERIALS AND METHODS

Two *Bufo marinus*, collected from the outskirts of Belém, showed numerous *Lankesterella*-like sporozoites in thin blood films of their peripheral blood. Six other toads, of the same species, but collected in the vicinity of Salvaterra, Island of Marajó, north of Belém, presented no signs of similar blood parasites.

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Figs 1-9. – *Lankesterella petiti* n. sp., infecting the toad *Bufo marinus* in Amazonian Brazil. Figs 1-5. Experimental infections: light microscope views of undivided oocysts (zygotes). Fig. 1. Living parasites in a squash preparation of liver. Figs 2,3. In Giemsa-stained smears of spleen and blood after fixation in Bouin's fluid. Figs 4,5. As seen in histological sections of spleen and ovary: zygotes are arrowed. Figs 6-8. Mature oocysts. Figs 6,7. Living oocysts in squash preparations of lung and spleen from an experimentally infected toad showing sporozoites and conspicuous residual body (arrowed). Fig. 8. Mature oocyst in a Bouin-fixed, Giemsa-stained smear of kidney from a naturally infected, adult toad : an intra-erythrocytic sporozoite is arrowed. Fig. 9. Freed sporozoites, probably those from a ruptured oocyst of *Lankesterella*, in a Bouin-fixed, Giemsa-stained smear of intestinal tissue from a naturally infected, juvenile toad. Scale bars = 10 μ m; magnifications are the same for Figs. 2 and 3, for 4 and 5, and for 6-9.

Three of the latter animals were force-fed with a mixture of blood and liver fragments from the infected toads, and three were inoculated with the infected blood, by the intraperitoneal route.

At necropsy, a variable number of days later, only two of the experimental toads showed developmental stages and mature oocysts considered to be those of *Lankesterella*. One (BX6) had been fed with the blood/liver mixture, and was examined 16 days post-infection. The other (BX4) had received only the injection of infected blood, and was necropsied 25 days p.i. In only one of these toads (BX6) were the parasites sufficiently abundant to be detected by the electron microscope. Parasites were not found in the peripheral blood of either animal.

For light microscopy, air-dried dab-smears of tissues were fixed in absolute methyl alcohol for three minutes and stained in Giemsa (30 drops of stain to 15.0 ml of distilled water buffered to pH 7.4) for one hour. Companion smears were fixed for 20 m in aqueous Bouin's fixative and washed in 70.0 % ethyl alcohol until entirely colourless. These smears were then stained for 1 h 30 m in Giemsa, dehydrated and differentiated in a series of acetone/xylol mixtures 95:5, 70:30, 30:70, cleared in pure xylol and mounted in "Permount". Whilst these slides were in preparation, small fragments of the various tissues were crushed between slide and coverslip for direct light-microscope examination.

Fragments of tissues for histology were fixed in 10.0% neutral, buffered formalin and embedded in glycol methacrylate (GMA of Agar Company, UK). Sections of 3.0-4.0 μm were cut with a glass knife on a Sorval JB4 microtome, and stained with Meyer's haemalum, using eosin or eosin/phloxin as counter-stains.

Tissue for transmission electron microscopy (TEM) was fixed in 2.5 % cacodylate (0.1M, pH 7.4)-buffered glutaraldehyde for 24 h at 4.0°C, repeatedly rinsed, and post-fixed in 1.0 % osmium tetroxide in the same buffer for 1 h. After further rinsing in the buffer, tissues were dehydrated in ascending ethyl alcohol concentrations and embedded in Agar 812 medium (Agar Company, UK). Thin sections were cut on a Reichert "Ultracut" ultratome with a diamond knife, stained on grids with uranyl acetate and lead citrate, and examined with a Joel 100CX TEM.

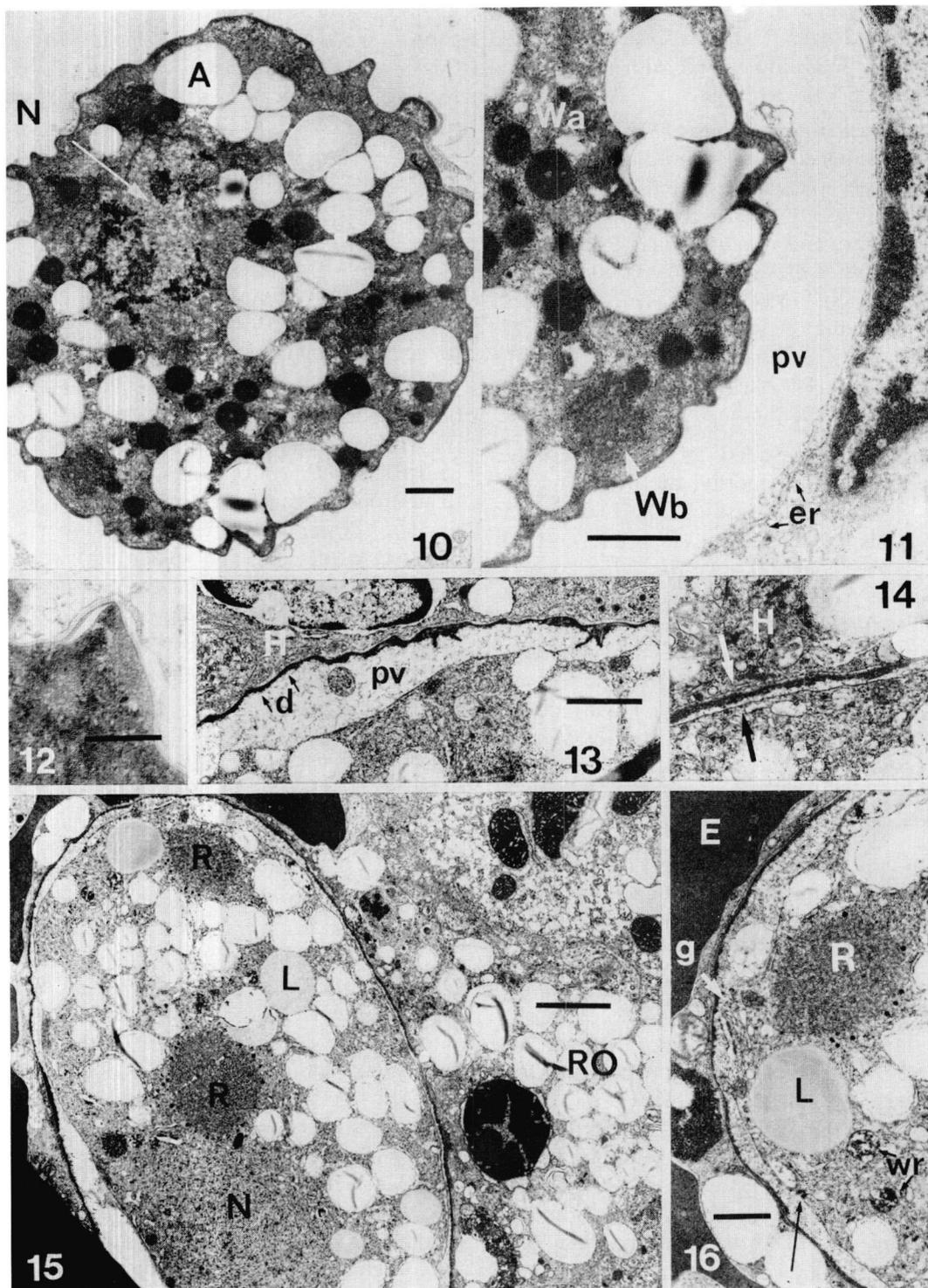
RESULTS

Toad BX6, experimentally infected following the ingestion of sporozoites in liver and blood, was sacrificed 16 days p.i., and large

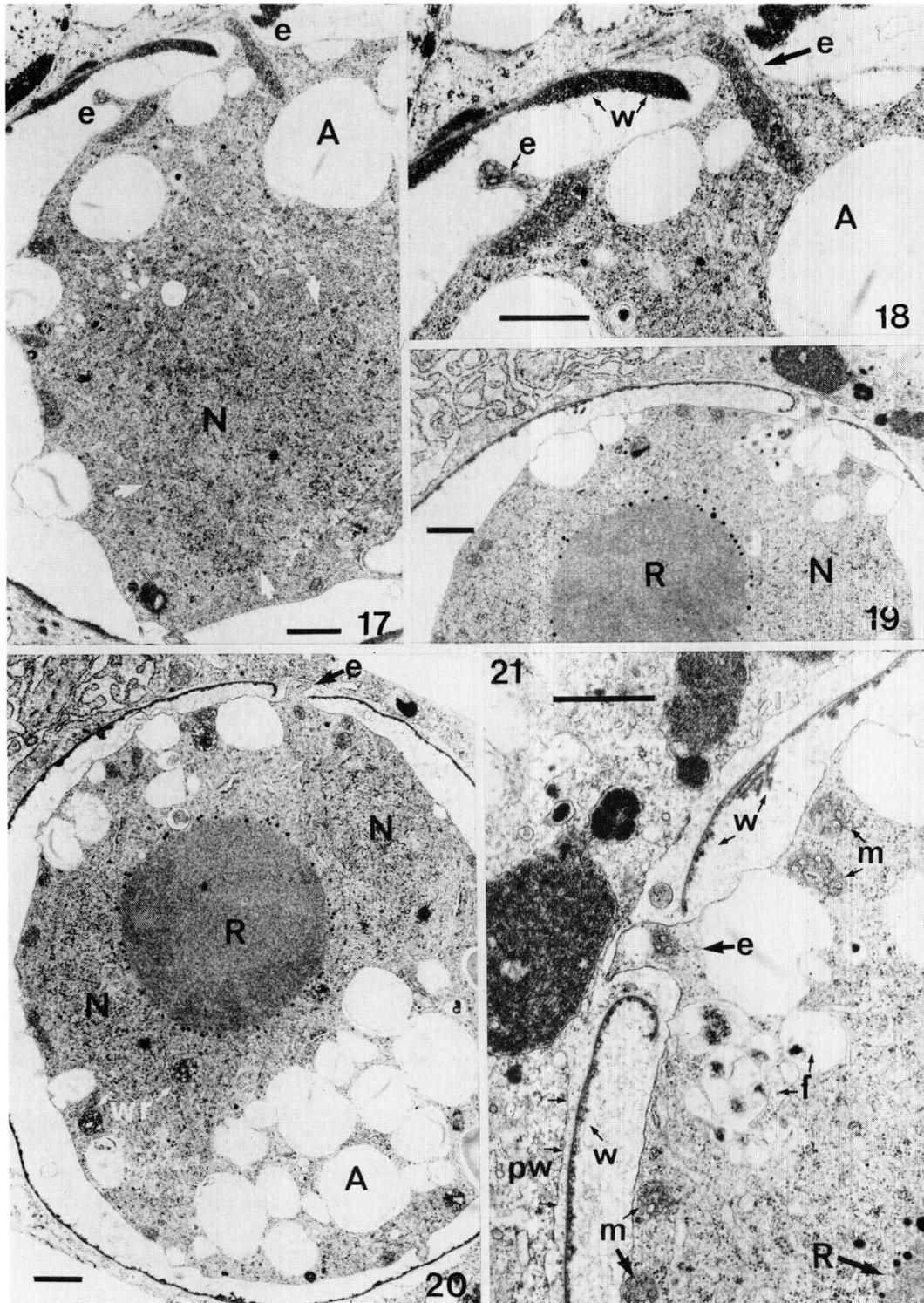
numbers of heavily vacuolated late-stage zygotes and early oocysts were found in smears of most tissues (Figs 1-5). Histological sections (Figs 4, 5) showed the parasites to be located in the endothelial cells of the blood vessels: they were particularly abundant in the liver, spleen, kidney and lungs, but were also found in the muscles, intestinal wall, ovary and the envelopes of the brain. Occasional parasites were seen in thin blood films (Fig. 3), doubtless following their release from ruptured blood vessels when obtaining the blood from a severed toe. The parasites, which measured from 19.0–22.0 \times 12.0–14.0 μm were bounded by a prominent and relatively rigid membrane.

Toad BX4, infected by the intraperitoneal injection of blood containing sporozoites, was sacrificed day 25 p.i. The infection was very much lighter than that of toad BX6, and most of the parasites were at the sporulated oocyst stage (Figs 6-8). In fresh, squash preparations of the tissues the oocysts were most readily detected in the lung and spleen and were relatively rare elsewhere. The number of sporozoites was variable and estimated to range from 12 to 30. These long, slim bodies measured from 7.0–9.0 \times 1.0–1.4 μm and were frequently arranged like a bunch of bananas, juxtaposed to a conspicuous and highly vacuolated oocyst residuum (Figs 6,7) of about 2.5 – 3.0 μm . Occasional unsporulated oocysts, similar to those seen in toad BX6, were found in the liver and spleen. Finally, clumps of morphologically similar parasites previously seen in smears of the small intestine of another juvenile and naturally infected toad (Fig. 9) were considered to represent groups of released sporozoites from ruptured *Lankesterella* oocysts.

Electron micrographs of late zygotes or early oocysts (Figs 10-12) showed them to be bounded by a thin, trilaminated wall (Fig. 12) which was apparently sufficiently firm or impermeable to induce a wrinkled appearance during fixation or sectioning. Many parasites at a similar stage of development in fact failed to be properly fixed, or infiltrated, and collapsed following sectioning and exposure to the electron beam. The dense, ribosome-filled cytoplasm contained a single nucleus of irregular shape and with scattered chromatin, and a great many amylopectin granules. The rough endoplasmic reticulum (ER) was filled with a finely granular substance of medium density. Two types of large, electron-dense granules were present, reminiscent of the wall-forming bodies of zygotes and early oocysts of the coccidia of higher vertebrates. Many of these were round and very dense (Fig. 11, Wa), while a smaller number, situated more to the periphery of the cell, had a loose, granular appearance (Fig. 11, Wb). Neither of these two types of granules were located in an ER cisterna, though those of the loose type were surrounded by ER.



Figs 10-16. – Ultrastructure of the undivided zygotes (early oocysts) of *Lankesterella petiti* n. sp., in an experimentally infected toad, *Bufo marinus*. Fig. 10. Low magnification of entire parasite. A. Amylopectin granules; N. Nucleus. Fig. 11. Higher power view to show the wall-forming-like bodies (Wa,Wb) and details of the parasitophorous vacuole (pv) wall, rich in ER (er). Fig. 12. Enlarged view of the zygote cell-wall. Figs 13,14. Enlarged view of the oocyst periphery, host-cell cytoplasm (H) and the parasitophorous vacuole (pv), and between the bold arrows), showing the material (d) deposited on the parasitophorous vacuole wall. Fig. 15. Whole parasite, and remnants of a degenerate oocyst (RO)(for other abbreviations see 16). Fig. 16. Enlarged view of a zygote, showing the nucleus, a refractile body (R), lipid vacuole (L), Golgi-complex (g), degenerative wall forming bodies (wr) and a micropore (small, black arrow); in the background is the host erythrocyte (E). Scale bars = 1.0 μ m in Figs 10,11,13, and 16; 0.5 μ m in Fig. 12; 3.0 μ m in Fig. 15. Magnification of Fig. 14 is the same as in Fig. 13.



Figs 17-21. - Ultrastructure of *Lankesterella petiti* n. sp., in an experimentally infected toad, *Bufo marinus*. Fig. 17. An undivided zygote or early oocyst, showing the single nucleus (N) and the parasite protrusions (e) passing through perforations in the oocyst wall. A, amylopectin granules. Fig. 18. Enlarged view of the same parasite, showing details of the protrusions (e) with their contained mitochondria, and the substance deposited on the parasitophorous vacuole membrane to form a wall (w) around the oocyst. Figs 19-21. A sporulating oocyst, with its wall (w) within the parasitophorous vacuole and its delicate membrane (pw). N, divided nucleus; R, large, central refractile body; wr, residual wall-forming-like bodies; e, parasite protrusions, with mitochondria; f, associated food vacuoles; m, peripherally located mitochondria. Scale bars = 1.0 μ m.

Other stages, seen in the same material, were either oval, with a single nucleus, a moderate number of amylopectin granules and a few lipid vacuoles (Figs 13-18), or rounded, with several nuclei and fewer (but larger) amylopectin granules (Figs 19-21). These bodies were considered to be young oocysts prior to sporulation, and older ones in the process of sporulation, respectively. Both stages contained one to several refractile bodies which were round, granular and bounded by a coat of electron-dense droplets. Both the undivided and the sporulating oocysts were bounded by a single membrane containing a few micropores.

A flocculent, electron-dense substance becomes deposited on the single-layered membrane of the parasitophorous vacuole (Figs 13, 14) and is subsequently consolidated into hollow rodlets which form a wall surrounding the oocyst (Figs 17-21). This wall is interrupted in several places, allowing extensions of the parasite to protrude out to the margin of the parasitophorous vacuole. It was not possible, however, to detect any evidence of desmosomes or any other form of junction between the parasite protrusions and the membrane of the parasitophorous vacuole. The host cytoplasm above the point of contact contained aggregates of lysosomal bodies (Fig. 21). Each of the parasite extensions contained a mitochondrion, and additional ones were also evident at the periphery of the organism, on both sides of the protrusion (Figs 18, 21). Food vacuoles were accumulated in the vicinity of the parasite projections (Fig. 21). The cytoplasm of the young and dividing oocysts contained a dense network of smooth ER, with many cisternae (Figs 13, 14, 16, 18, 21) and peripherally located Golgi complexes (Fig. 16). A few small bodies with electron-dense, flocculent material were interpreted as being the remnants of wall-forming-like organelles (Figs 16, 20).

Host tissues contained also residues of many degenerate oocysts at various stages of disintegration, and accompanied by lysosomes of host origin (Fig. 15).

DISCUSSION

This appears to be the first description of a lankesterellid from the toad, *Bufo marinus*, and we propose for it the name of *Lankesterella petiti* n. sp. in honour of Dr Gilles Petit, who first drew the senior author's attention to the blood forms of the parasite in *B. marinus* while working in his laboratory in Belém, in 1986.

We feel the new specific name justified for the following reasons. Evidence has been given indicating the

host-specificity of *L. hylae* for the Australian tree-frog *Litoria caerulea* (*Hyla caerulea*, in original report), the parasite being absent in other species of *Litoria* examined in the same locality (Stehbens, 1966b). The difference between *Rana esculenta*, host of the type species *L. minima* (and also *Rana catesbeiana*, the host for *Lankesterella* in Canada), and *Bufo marinus* is one of different families: Ranidae and Bufonidae. Finally, the geographic separation of these anurans: Europe (for type host of *L. minima*) and South America (or holarctic, to include Canadian host, versus neotropic zoogeographic regions) is very wide.

A thorough examination of the stained smears and histological sections prepared from most organs of the toads did not reveal the presence of any additional apicomplexan parasite, and although an *Eimeria* s.l. species has been found in the intestine of *Bufo marinus* from the same locality, its ultrastructure is very different (Paperna & Lainson, 1995). We are confident, therefore, that the different stages of development seen by TEM in the present material belong to the same organism and that, from their ultrastructural similarity to *L. minima* oocysts described by Desser *et al.*, (1990), we are dealing with a *Lankesterella* species.

The cystic, single-nucleated parasites loaded with amylopectin and with electron-dense wall-forming-like granules (Figs 10-18) are considered to be the late zygote or early oocyst stages seen by light microscopy (Figs 1-5). These precede, therefore, the multinucleated forms enclosed by the dense wall, deposited later (Figs 20, 21).

The pre-division stages superficially resemble those of *Schellackia* (Paperna, 1992), but the subsequent process of wall formation by the deposition of the electron-dense "shell" on the parasitophorous vacuole membrane, the development of parasite protrusions passing through micropores to that membrane and the refractile bodies surrounded by electron-dense droplets (the "paranuclear body" of Desser *et al.*, 1990) have not till now been recorded in other coccidia. They are features which are probably peculiar to *Lankesterella* and the present observations, added to those of Desser *et al.*, (1990) on the late oocyst ultrastructure, indicate a unique taxonomic status for the anuran lankesterellids.

The electron-dense granules (Wa, Wb) seen in the late zygote stages suggest at least an analogy to the "wall-forming bodies" of the coccidia of higher vertebrates, including those recorded in *Schellackia* (Ostrovská & Paperna, 1987); or, alternatively, to the "dense bodies" in similar stages of fish coccidia (Desser & Li, 1984). The degradation and final disappearance of these organelles in older oocysts suggest their pos-

sible contribution of material for the formation of both the oocyst wall (Wa granules ?) and the refractile bodies (Wb granules ?). While the role of wall-forming bodies is well documented for avian and mammalian coccidia (Ball & Pittilo, 1990; Chotobar *et al.*, 1980), that of similar organelles or analagous granules in the oocysts of the coccidia of lower vertebrates is uncertain (Desser & Li, 1984; Ostrovska & Paperna, 1987; Paperna, 1992, 1993; Kim & Paperna, 1992). On the other hand, a process by which Type II wall-forming bodies are transformed into the anlagen of the refractile bodies has been described for *Schellackia* (Ostrovska & Paperna, 1987).

The protrusions of the parasite passing through the oocystic micropores of *Lankesterella petiti*, n. sp., are possibly involved in acquisition of nutrients from the host cell. It is significant that mitochondria are present both in these extrusions and at their bases, where food vacuoles accumulate. Noteworthy, too, is the presence of many lysosomal bodies in the host-cell cytoplasm, at the point of contact with the protrusions.

The oocysts of *L. minima* in *Rana catesbeiana*, studied by Desser *et al.*, (1990), were supposedly older than those we have described here for *L. petiti* of *Bufo marinus*. It is interesting, therefore, that they still possess a thin, unconsolidated wall, and that there is no sign of the deposited thickening of the wall that was so conspicuous in the developing oocysts we have figured (Figs 13, 14, 18, 21) : neither does the oocyst of *L. minima* show any extrusions of the contained parasite into the parasitophorous vacuole. These variations may simply be the differences in morphology, or pace and sequence of development, exhibited by two different species of *Lankesterella*.

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