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The ultrastructure of cultured *Plasmodium gallinaceum* ookinetes: a comparison of intact stages with forms damaged by extracts from blood fed, susceptible *Aedes aegypti*

R. F. Gass

Summary

The ultrastructure of intact, mature and immature ookinetes of *Plasmodium gallinaceum* is compared with corresponding stages which have been damaged by *Aedes aegypti* extracts prepared during blood digestion. The study reveals some new details of ookinete ultrastructure. In particular the composition, development and mode of formation of the pellicle of plasmodial ookinetes is shown to be similar to that of other sporozoans. The pellicle is composed of three membranes, develops in the growing protrusion of the retort-shaped early ookinete and its inner layer is probably formed by fusion of peripheral vesicles. Staining with ruthenium red indicates the presence of a surface coat. Furthermore Golgi-like structures and lysosomes can be observed.

After exposure to *A. aegypti* extracts the parasites exhibit signs of severe cell damage and degeneration, such as disintegration and vacuolization of the cytoplasm. Damage of the plasma membrane is demonstrated by its permeability for ruthenium red. Cell damage is particularly prominent in those cell parts which are not covered by the pellicle and therefore suggests a protective function for this structure.

Key words: Plasmodium gallinaceum; ultrastructure; ookinete; pellicle; surface coat; ruthenium red; *Aedes aegypti;* blood digestion.

Introduction

Gass and Yeates (1979) demonstrated by means of light microscopy marked in vitro damage of cultured *Plasmodium gallinaceum* ookinetes after

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incubation of the parasites with crude *Aedes aegypti* extracts obtained during blood digestion. The factors responsible for damage were neither present in unfed mosquitoes nor in blood alone and damage was not a result of osmotic stress. The main effective agents were identified as digestive proteinases with a trypsin-like enzyme as major component. Incubation throughout the period of ookinete development led at high concentrations of the mosquito extracts to lysis of the parasites. At lower concentrations ookinete development was observed, although the cells were clearly damaged. Typical characteristics of damage were interruption of the development at the stage of an immature, retort shaped ookinete and irregular Giemsa staining.

In this paper ultrastructural observations on these damaged forms are presented. For comparative purposes the study includes a partial reinvestigation of non-damaged ookinetes of *P. gallinaceum* both at the mature and the immature stages. Quite a number of observations on the fine structure of mature ookinetes of haemosporidians have been published (Garnham et al., 1962; 1969; Desser and Wright, 1968; Desser, 1970; 1972; Aikawa, 1971; Canning and Sinden, 1973) while less work has been done on early ookinete stages (Davies, 1974; Gallucci, 1974b).

Materials and methods

The parasite *P. gallinaceum* (strain obtained 1969 by Prof. Maegraith, Liverpool) was used in this study. Cultivation of the ookinetes and the preparation of crude extracts from blood fed *A. aegypti* (Rockefeller strain) were carried out according to Gass and Yeates (1979). Intact immature and mature ookinetes were obtained 11 h and 22 h respectively after the beginning of the culture (TC 199, pH 7.7, 27° C). Damaged ookinetes were produced by incubating the parasites for 24 h in crude *A. aegypti* extracts (50 ♀♀/ml TC 199) prepared 25 h after blood meal.

For electron microscopy cultured ookinetes were washed in culture medium, pooled in a mixture of medium and chicken serum (1:1) and collected in a melting point capillary which was

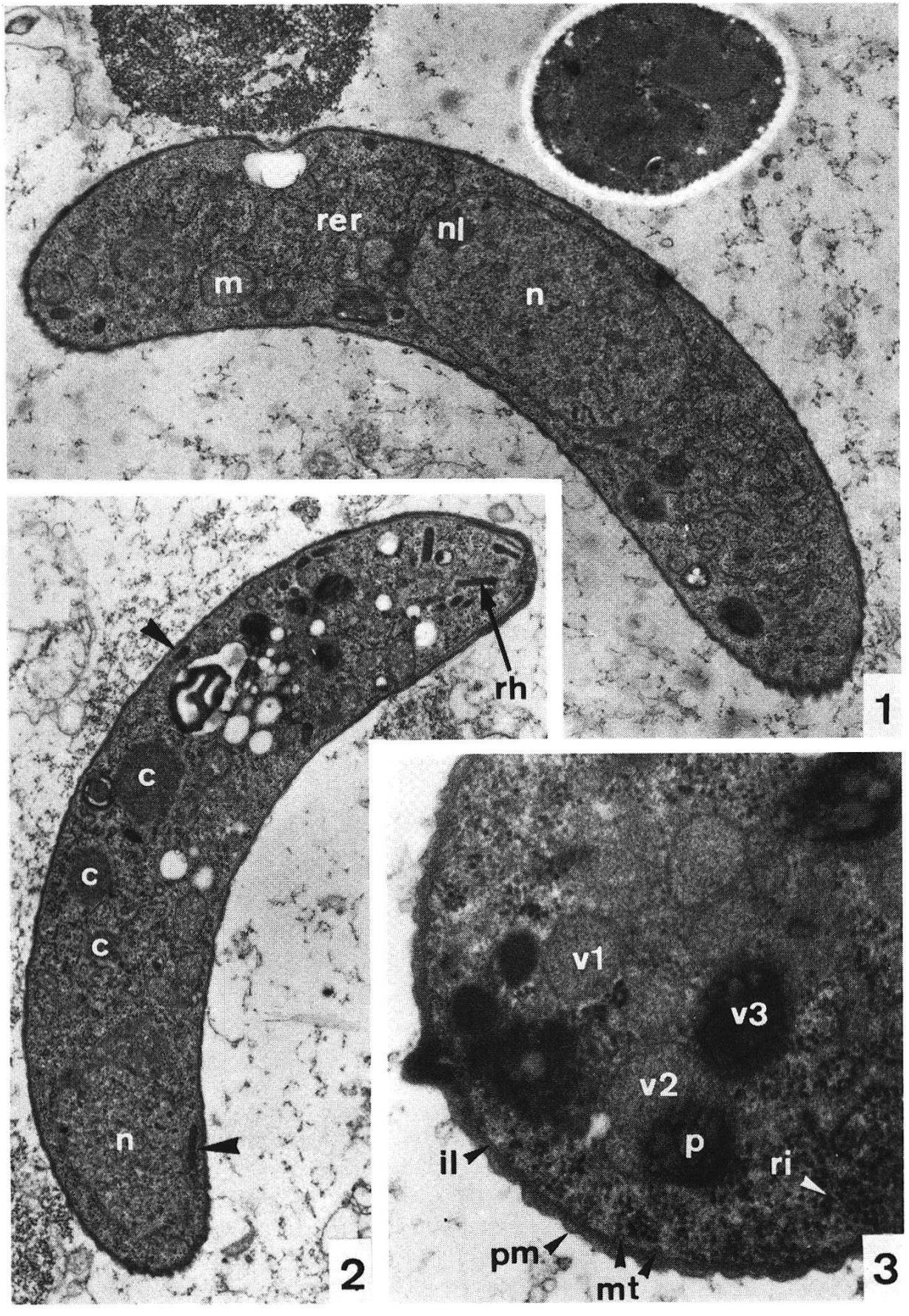
Abbreviations (Figs. 1–19)

c	= crystalloid	p	= pigment
cl	= collar (apical complex)	pm	= plasma membrane
g	= Golgi-like structure	pr	= polar ring (apical complex)
il	= inner pellicular layer	r	= ring (apical complex)
ly	= lysosomes	rer	= rough endoplasmic reticulum
m	= mitochondria	ri	= ribosomes
mt	= microtubules	rh	= rhoptries
n	= nucleus	rr	= ruthenium red
nl	= nucleolus	v	= vacuoles

Figs. 1–9. Intact, mature ookinetes of *P. gallinaceum.*

Figs. 1, 2. Longitudinal sections showing the general morphology. Fig. 2 demonstrates three crystalloids (c) and rhoptries (rh) extending to the posterior cell part (arrows). Fig. 1, ×14,000; Fig. 2, ×12,000.

Fig. 3. Transverse section. Note the pellicular structure and the vacuoles which exhibit different types of contents (v_1–v_3). ×44,500.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then sealed with plasticine at one end. By centrifugation (10 min, 200 *g*) a plug-like pellet was obtained which was fixed for 1 h in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4° C. The preparations were then washed overnight in 0.2 M cacodylate buffered 3% sucrose at 4° C, treated for 2 h in 0.2 M cacodylate buffered 2% (w./v) OsO_4 at 4° C and dehydrated in acetone series and propylene oxide before being embedded in Epon. Pre-staining was carried out during dehydration for 1 h in 1% (w/v) uranyl acetate in 70% acetone. Before examination in a Philips EM 300 the thin sections were stained with lead citrate.

Some samples were stained with ruthenium red. Commercial ruthenium red (Merck, Darmstadt) was purified by NH_3 extraction and included into the fixation procedure under the conditions described by Luft (1971a). These preparations were examined without further staining.

Results

Intact ookinetes

The mature ookinetes of *P. gallinaceum* (Figs. 1, 2) are enclosed in an envelope consisting of a bilayered pellicle and a subpellicular layer of 50–60 longitudinal microtubules (Fig. 3). The outer pellicular layer, the plasma membrane, is corrugated and separated from the inner layer by a relatively electron-transparent space. This inner layer is composed of two tightly appressed membranes (Figs. 7, 15). It is interrupted at the apical cell pole where the two membranes join together (Fig. 9).

The apical complex in the anterior part of the ookinete (Figs. 7–9) is composed of (i) a circular gap in the inner pellicular layer beneath the cap-like projecting cell pole, (ii) 3 annular structures: a polar ring, a collar and a third, innermost, electron-pale ring, and (iii) rhoptries which are present in the form of convoluted tubes and which seem to extend into the posterior part of the cell (Fig. 2).

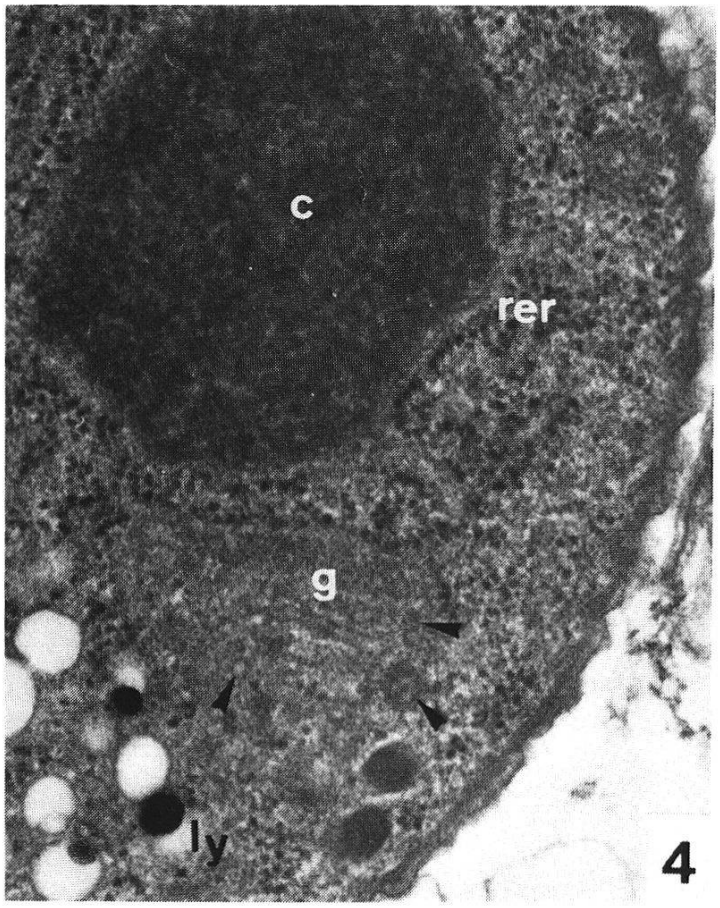
In the nucleus up to 3 nucleoli have been observed (Fig. 6). The cytoplasm is rich in ribosomes (Fig. 3) and contains mitochondria with tubular cristae within a matrix which is sometimes rather dense (Figs. 1, 10). Crystalloids, up to three in number (Fig. 2), are surrounded by rough endoplasmic reticulum (Fig. 4). Numerous vacuoles are present. These either (i) contain fine granular material, sometimes together with pigment of crystalloid appearance (Fig. 3), or (ii) are fully loaded with pigment (Figs. 3, 5) or (iii) contain amorphous, electron dense material (Fig. 5). In one section a Golgi-like zone was found (Fig. 4)

Fig. 4. The micrograph shows a Golgi-like structure (g) with surrounding vesicles (arrows). Close to this structure are some dense spherules, perhaps primary lysosomes (ly). × 44,500.

Fig. 5. A further type of vacuole (compare Fig. 3) which contains amorphous, dense material. × 26,000.

Fig. 6. Transverse section showing a nuclear profile containing three nucleoli (arrows). × 11,000.

Figs. 7–9. Apical complex. The polar ring (pr) is clearly visible when it is cut tangentially (Fig. 8). The inner pellicular layer (il) which is composed of two appressed membranes is interrupted at the apical cell pole. Fig. 9 shows that at this interruption the two membranes join together (arrows). On one side this structure has been artificially intensified with drawing-ink (double arrow). Fig. 7, × 56,000; Fig. 8, × 39,000; Fig. 9, × 96,000.



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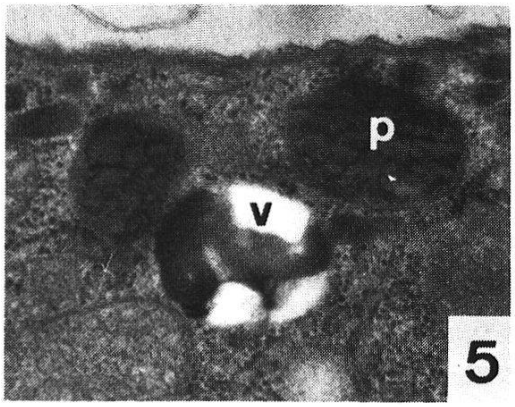

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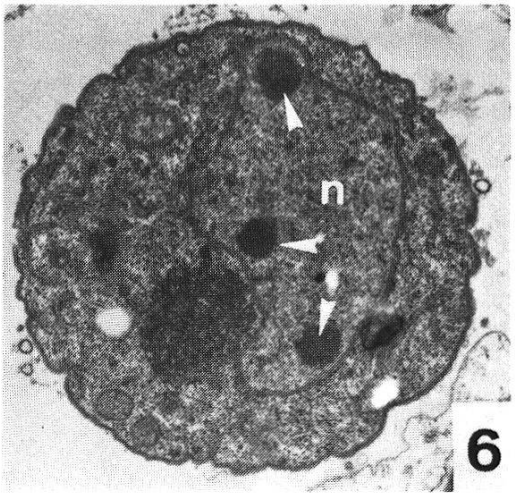

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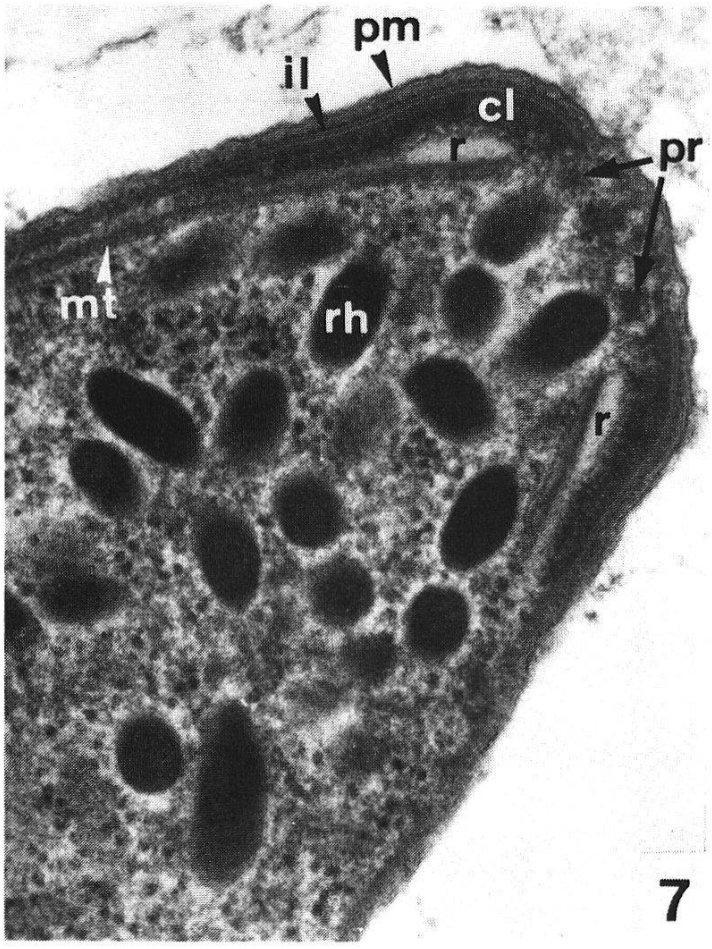

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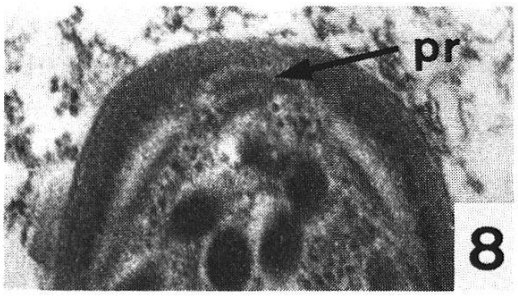

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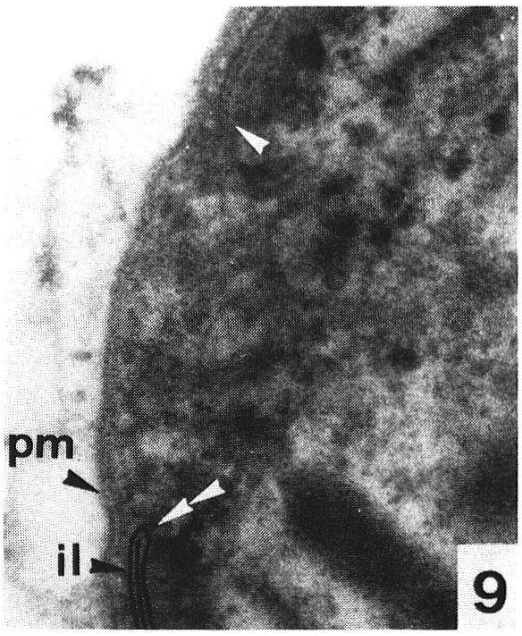

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which appears to be composed of a series of parallel membranes or parallel flattened cisternae free of ribosomes and which is surrounded by small vesicles. Close to this structure dense spherules are observed which might perhaps be primary lysosomes (Fig. 4).

Immature ookinetes are roughly retort-shaped. They consist of a posterior spherical part – the initial zygote – and a protrusion which develops and grows with the age of the ookinete (Fig. 10). A pellicle of similar composition to that of mature ookinetes and subpellicular microtubules are only differentiated in the protrusion, while the spherical part is only covered by the plasma membrane.

Damaged ookinetes

Zygotes of *P. gallinaceum* were exposed for 24 h to crude extracts from blood fed *A. aegypti.* Under these conditions ookinete development was interrupted at the stage of a retort-shaped, immature form and this showed irregular Giemsa staining (Gass and Yeates, 1979). Electron micrographs demonstrate that both effects are a result of severe cell damage. The cells have undergone swelling and heavy vacuolization. The ground substances of the cytoplasm and nucleoplasm look disintegrated and flocculent (Figs. 11, 12, 18). As a consequence all membranous structures are more clearly visible, particularly the pellicle (Fig. 15), the endoplasmic reticulum (Fig. 12) and the nuclear envelope, which in certain ookinetes exhibits swellings (Fig. 14). Vacuoles seem to fuse into larger membrane-bound units (Figs. 11, 18). Although the plasma membrane is directly exposed to the enzymes of the mosquito extracts, no apparent damage is observable.

The most prominent signs of damage are concentrated at the sites where the ookinete is not covered by the pellicle – always in the spherical part of the cell. Here the plasma membrane appears strongly folded, often with distinct invaginations into the cell body (Fig. 11, 12). On the other hand the cell protrusion, particularly when large, is relatively insensitive to damage (Fig. 11). In contrast to intact ookinetes, the pellicle extends beyond the protrusion and partially covers the spherical section of the cell (Figs. 12, 18).

To investigate the state of the plasma membrane damaged ookinetes were

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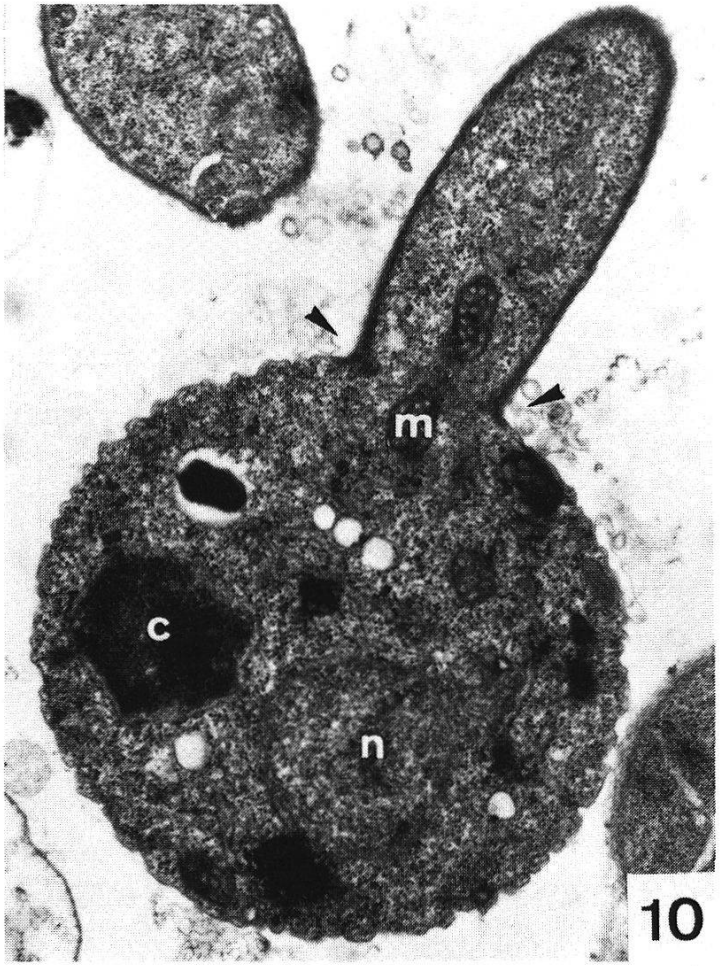
Fig. 10. Intact, immature ookinete of *P. gallinaceum*
Note the pellicle which covers only the cell protrusion and ends at the margin of the spherical cell portion (arrows). × 14,000.

Figs. 11–15. Ookinetes of *P. gallinaceum*, damaged after one day of exposure to extracts from blood fed *A. aegypti.*

Figs. 11, 12. General view of damaged ookinetes. The signs of damage, such as disintegration and vacuolization of the cytoplasm, are particularly prominent in the spherical cell part which is not fully covered by the pellicle. Here the plasma membrane appears strongly and irregularly folded (arrows). Note that in Fig. 12 the pellicle covers to some extent also the spherical cell part (double arrow) (compare Fig. 10). Fig. 11, × 10,000; Fig. 12, × 11,000.

Fig. 13. Peripheral vesicles (arrows) which might form by fusion the inner pellicular layer. × 26,000.



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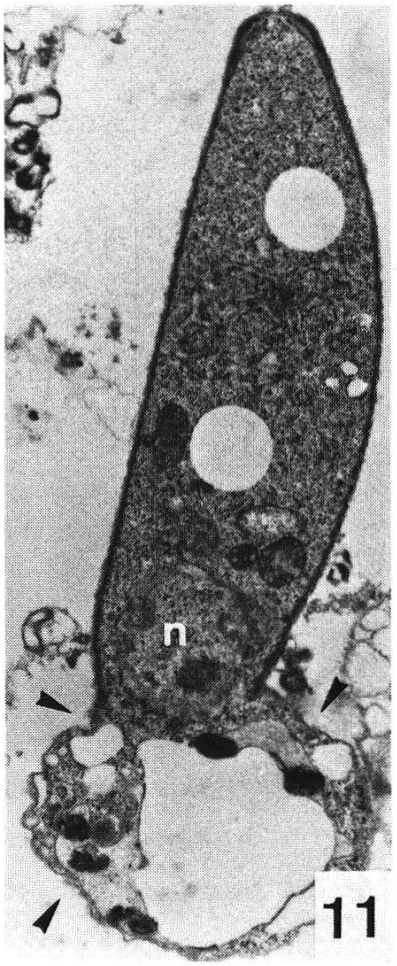

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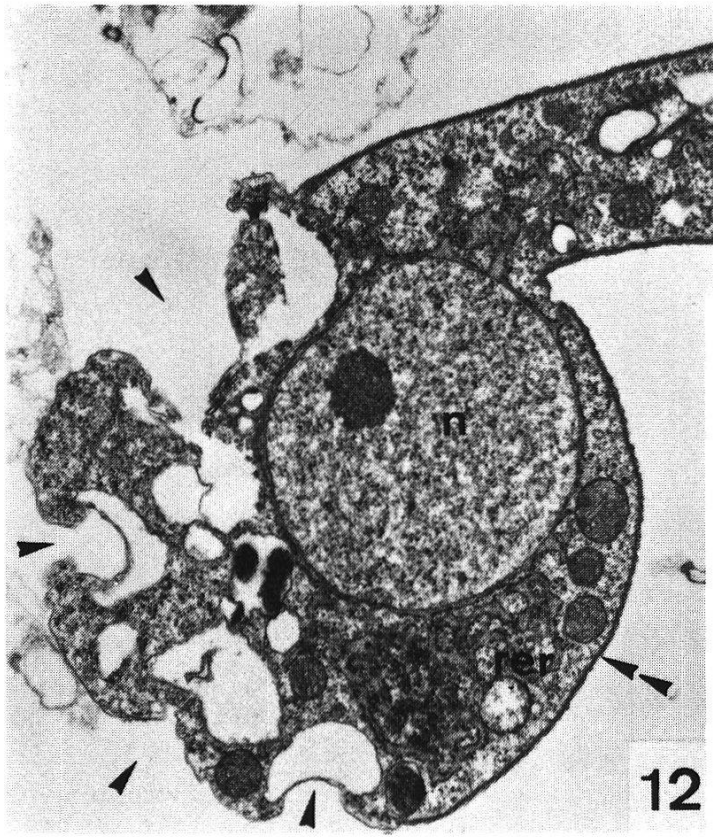

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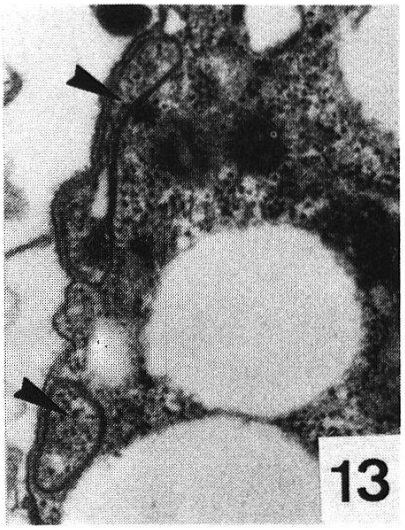

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stained with ruthenium red and compared to intact cells. This dye reacts with the acidic mucopolysaccharide surface coat found on most cells and is usually excluded by plasma membranes of intact cells (Luft, 1971b; Jensen and Hammond, 1975).

Figs. 16 and 17 demonstrate that intact mature ookinetes exclude the dye. The cytoplasm, which has not been stained with uranyl acetate or lead citrate, remains uncontrasted and apart from pigment granules its structures are poorly defined. The plasma membrane exhibits rather strong affinity for the dye, leading to a distinct layer of extracellular ruthenium red (Fig. 17). In contrast to intact ookinetes ruthenium red enters damaged forms and clearly stains all cytoplasmic structures (Fig. 18). Aggregation of the dye at the plasma membrane is not observed (Fig. 19).

Discussion

This study was carried out to investigate at an ultrastructural level the damage of ookinetes of *P. gallinaceum* caused by digestive proteinases of their vector *A. aegypti* (Gass and Yeates, 1979). For comparative purposes intact ookinetes were reinvestigated. Although the ultrastructure of plasmodial ookinetes is largely known (Garnham et al., 1962; 1969; Canning and Sinden, 1973; Davies, 1974) a brief account of their morphology is given here. This is considered to be justified as this study has revealed additional information on some morphological features.

In particular the composition of the pellicle has now been demonstrated to be similar to that in merozoites, sporozoites and gametocytes (Sterling and Aikawa, 1973; Aikawa, 1977). It is composed of three membranes, the outer plasma membrane and an inner double-membrane layer. Although such a pellicle is typical for motile stages of sporozoans (Scholtyseck, 1979), it has so far not been described for plasmodial ookinetes, except *P. berghei*, where remnants of a third innermost membrane have been found (Davies, 1974). There are also similarities to other sporozoa in the development of the pellicle (Gallucci, 1974b; Mehlhorn and Schein, 1977; Mehlhorn et al., 1978). The inner membrane complex of the macrogamete disintegrates, initially perhaps at the

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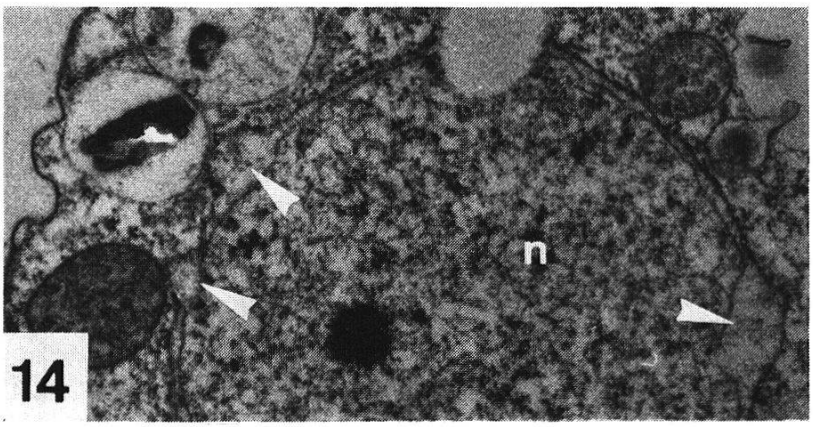
Fig. 14. Swellings of the nuclear envelope in damaged ookinetes (arrows). × 15,000.

Fig. 15. Elements of the pellicle: plasma membrane (pm), inner layer formed by two appressed membranes (il) and subpellicular microtubules (mt). × 135,000.

Figs. 16–19. Ookinetes of *P. gallinaceum* stained with ruthenium red.

Figs. 16, 17. Intact, mature ookinete which excludes ruthenium red (rr). The cytoplasm remains unstained while aggregation of the dye is observed at the plasma membrane. This aggregation suggests the presence of a surface coat (Fig. 17). Fig. 16, × 29,000; Fig. 17, × 88,000.

Figs. 18, 19. In damaged ookinetes ruthenium red (rr) penetrates the plasma membrane and leads to intracellular staining. No aggregation of the dye is observable at the cell surface (Fig. 19). Fig. 18, × 12,000; Fig. 19, × 40,000.



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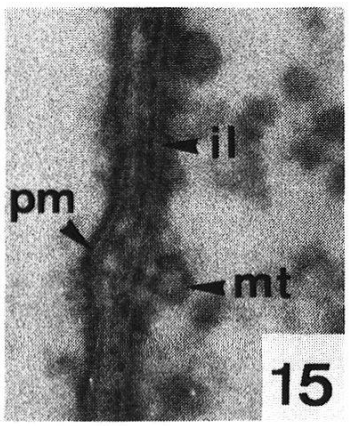

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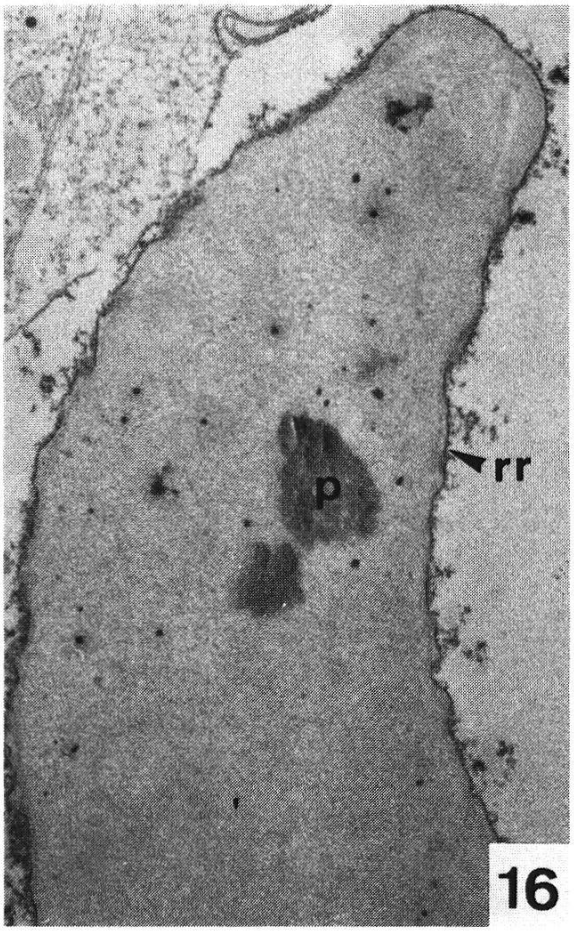

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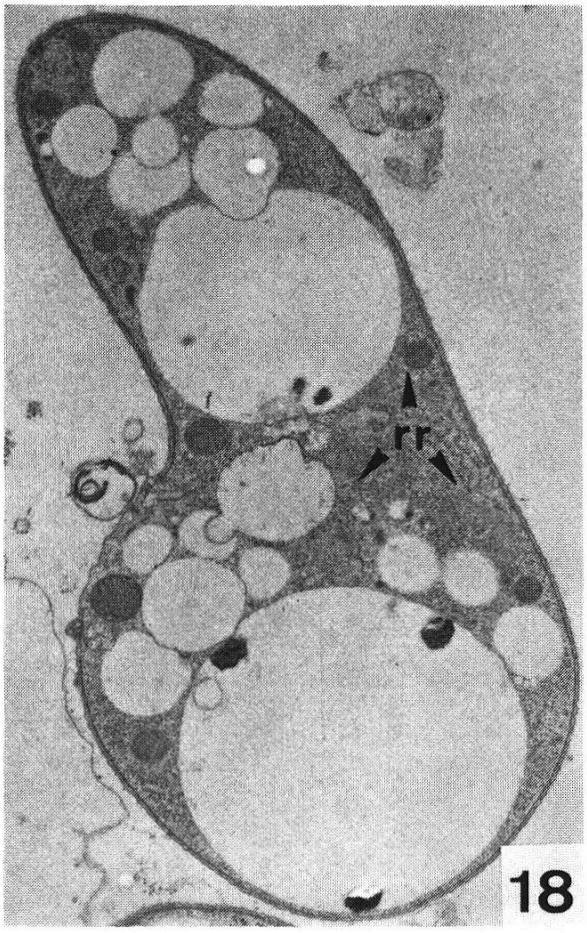

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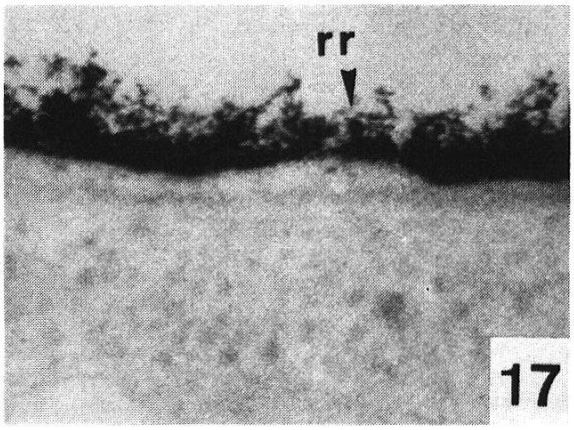

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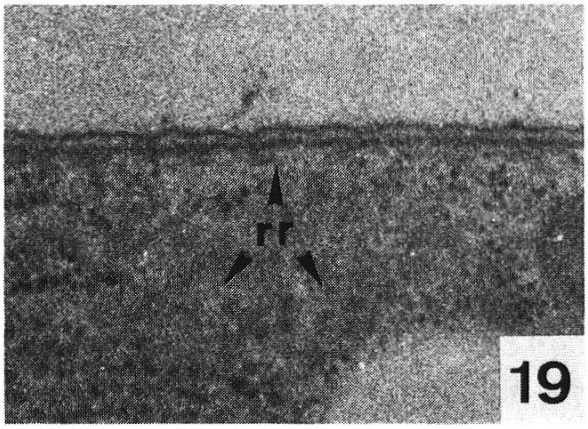

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site of fusion with the microgamete (Gallucci, 1974a) and reappears later in the protrusion of the early ookinete. It is only the mature, sickle-shaped ookinete which is again completely enclosed in a pellicle. As observed for other sporozoans (Dubremetz, 1975; Heydorn et al., 1975; Schein et al., 1979), the inner membrane complex seems to be formed by fusion of peripheral vesicles. This suggestion is compatible with the observed connection between the two appressed membranes of the inner pellicular layer found at the apical cell pole. Peripheral vesicles have however only been observed in damaged ookinetes (Fig. 13).

Staining of the ookinetes with ruthenium red leads to a prominent extracellular layer of the dye, indicating the presence of a surface coat (Luft, 1971b) as is known for other extracellular stages of plasmodium, such as merozoites and sporozoites (Aikawa, 1977). Without ruthenium red, however, the surface coat is not demonstrated by the preparation used. The apical complex is similar to that described for *P. berghei* (Canning and Sinden, 1973), but in *P. gallinaceum* the rhoptries are present not in the form of bulbous structures concentrated at the anterior cell portion, but rather in the form of tubes which extend up to the posterior part of the ookinete. This study has further pointed to the presence of Golgi-like structures in ookinetes. Close to these, dense spherules are found which are comparable to structures in the gametocytes of avian haemosporidians (Sterling and Aikawa, 1973) which have been supposed to be primary lysosomes (Aikawa et al., 1967).

Ookinetes which have been exposed for one day to extracts from blood fed *A. aegypti* exhibit distinct signs of cell damage and degeneration. This has been demonstrated to be caused by digestive proteinases, particularly trypsin, present in the extracts (Gass and Yeates, 1979). The death of the parasites is probably initiated by damage to the cell envelope, as this is directly exposed to the extracts. Evidence for this damage is provided by the observation that in contrast to intact ookinetes, the plasma membrane is permeable to ruthenium red (Luft, 1971b; Jensen and Hammond, 1975). As a result of the damaged plasma membrane the ookinete might become sensitive to osmotic stress and mosquito enzymes might be able to penetrate the interior of the cell. Both these factors could bring about the apparent signs of damage, such as disintegration and vacuolization of the cytoplasm. In the retort-shaped ookinete, cytoplasmic damage appears particularly prominent in the spherical portion of the cell, which is only partially enclosed by the pellicle. On the other hand, the damage appears relatively weak in the cell protrusion which is totally covered by the pellicle. This suggests the hypothesis that areas of the cell surface with an expanded pellicle are relatively resistant to attack by mosquito proteinases. Strong support for this hypothesis is provided by earlier work (Gass and Yeates, 1979) where it was demonstrated that mature ookinetes (complete pellicle) survive around 30 min in mosquito extracts without visible damage, while early stages (incomplete pellicle) were destroyed. However even with mature ookinetes

damage is only retarded, as after prolonged incubation they are destroyed. If the pellicular area is indeed in some way protected, one possible mechanism would be by means of chemical modifications of the plasma membrane which would render this structure less sensitive to proteolytic attack. Another possibility would be protection by means of the surface coat indicated here with ruthenium red. As protection is only of limited duration it is not surprising that this coat can no longer be demonstrated after prolonged incubation in mosquito extract (Fig. 19).

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1 Aikawa M., Huff C. G., Sprinz H.: Fine structure of the asexual stages of *Plasmodium elongatum.* J. Cell Biol. *34*, 229–249 (1967).
2 Aikawa M.: Parasitological review. *Plasmodium:* The fine structure of malarial parasites. Exp. Parasit. *30*, 284–320 (1971).
3 Aikawa M.: Variations in structure and function during the life cycle of malarial parasites. Bull. WHO *55*, 139–156 (1977).
4 Canning E. U., Sinden R. E.: The organization of the ookinete and observations on nuclear divisions in oocysts of *Plasmodium berghei.* Parasitology *67*, 29–40 (1973).
5 Davies E. E.: Ultrastructural studies on the early ookinete stage of *Plasmodium berghei nigeriensis* and its transformation into an oocyst. Ann. trop. Med. Parasit. *68*, 283–290 (1974).
6 Desser S. S., Wright K. A.: A preliminary study of the fine structure of the ookinete, oocyst, and sporozoite formation of *Leucocytozoon simondi* Mathis and Leger. Canad. J. Zool. *46*, 303–307 (1968).
7 Desser S. S.: The fine structure of *Leucocytozoon simondi.* III. The ookinete and mature sporozoite. Canad. J. Zool. *48*, 641–645 (1970).
8 Desser S. S.: The fine structure of the ookinete of *Parahaemoproteus velans* = *Haemoproteus velans* Coatney and Roudabush) (haemosporidia: haemoproteidae). Canad. J. Zool. *50*, 477–480 (1972).
9 Dubremetz J. F.: La genèse des mérozoites chez la coccidie *Eimeria necatrix.* Etude ultrastructurale. J. Protozool. *22*, 71–84 (1975).
10 Gallucci B. B.: Fine structure of *Haemoproteus columbae* Kruse during macrogametogenesis and fertilization. J. Protozool. *21*, 254–263 (1974a).
11 Gallucci B. B.: Fine structure of *Haemoproteus columbae* Kruse during differentiation of the ookinete. J. Protozool. *21*, 264–275 (1974b).
12 Garnham P. C. C., Bird R. G., Baker J. R.: Electron microscope studies of motile stages of malaria parasites. III. The ookinetes of *Haemamoeba* and *Plasmodium.* Trans. roy. Soc. trop. Med. Hyg. *56*, 116–120 (1962).
13 Garnham P. C. C., Bird R. G., Baker J. R., Desser S. S., El-Nahal H. M. S.: Electron microscope studies on motile stages of malaria parasites. VI. The ookinete of *Plasmodium berghei yoelii* and its transformation into the early oocyst. Trans. roy. Soc. trop. Med. Hyg. *63*, 187–194 (1969).
14 Gass R. F., Yeates R. A.: In vitro damage of cultured ookinetes of *Plasmodium gallinaceum* by digestive proteinases from susceptible *Aedes aegypti.* Acta trop. (Basel) *36*, 243–252 (1979).
15 Heydorn A. O., Mehlhorn H., Gestrich R.: Licht- und elektronenmikroskopische Untersuchungen an Cysten von *Sarcocystis fusiformis* in der Muskulatur von Kälbern nach experimenteller Infektion mit Oocysten und Sporocysten von *Isospora hominis* Raillet & Lucet, 1891. II. Die Feinstruktur der Metrocyten und Merozoiten. Zbl. Bakt. I. Abt. Orig. *232*, 373–391 (1975).

16 Jensen J. B., Hammond D. M.: Ultrastructure of the invasion of *Eimeria magna* sporozoites into cultured cells. J. Protozool. *22*, 411–415 (1975).
17 Luft J. H.: Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanisms of action. Anat. Rec. *171*, 347–368 (1971a).
18 Luft J. H.: Ruthenium red and violet. II. Fine structural localization in animal tissues. Anat. Rec. *171*, 369–416 (1971b).
19 Mehlhorn H., Schein E.: Electron microscopic studies of the development of kinetes in *Theileria annulata* Dschunkowsky & Luhs, 1904 (sporozoa, piroplasmea). J. Protozool. *24*, 249–257 (1977).
20 Mehlhorn H., Schein E., Warnecke M.: Electron microscopic studies on the development of *Theileria parva* Theiler, 1904 in the gut of the vector ticks *Rhipicephalus appendiculatus* Neumann, 1901. Acta trop. (Basel) *35*, 123–136 (1978).
21 Schein E., Mehlhorn H., Voigt W. P.: Electron microscopical studies on the development of *Babesia canis* (Sporozoa) in the salivary glands of the vector tick *Dermacentor reticulatus*. Acta trop. (Basel) *36*, 229–241 (1979).
22 Scholtyseck E.: Fine structure of parasitic protozoa. An atlas of micrographs, drawings and diagrams. Springer-Verlag, Berlin/Heidelberg/New York 1979.
23 Sterling C. R., Aikawa M.: A comparative study of gametocyte ultrastructure in avian haemosporidia. J. Protozool. *20*, 81–92 (1973).