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Brachiola gambiae sp. n. the Microsporidian Parasite of *Anopheles gambiae* and *A. melas* in Liberia

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Summary. *Brachiola gambiae* sp. n., former *Nosema* cf. *stegomyiae,* infected natural and insectary colonies of *Anopheles gambiae* and *A. melas* in Liberia and reduced susceptivity of the mosquito to development of malaria parasites and their transmission to man. It infects most tissues of adult male and female mosquitoes, destroying the midgut, Malpighian tubules, the fat body, muscles, hypoderm and connective tissues. It is usually transmitted with feces released by mosquitoes during feeding on cotton swabs with honey water. In sporogony the oval binucleate spores, 2.5-3 × 1.5-2 µm, have an anisofilar polar filament coiled in 9 coils in one row. Five anterior coils are of larger diameter than the posterior three to four. Macrospores 3-4 × 2 µm form a low percentage of mature spores (1:30). Tubulo-vesicular secretions are present.

Key Words: *Anopheles gambiae, A. melas, Brachiola gambiae* sp. n., life cycle, *Nosema stegomyiae,* spore morphology.

INTRODUCTION

The recent identification of *Brachiola vesicularum* Cali, Takvorian, Lewin, Rendel, Sian, Wittner, Tanowitz, Koehne et Weiss, 1998 as a new genus and species of microsporidia infecting man associated with AIDS and the transfer (Lowman *et al*. 2000) of *Nosema algerae* Vávra and Undeen 1970 to the same genus brought up the question of the systematic position of other mosquito born microsporidia with direct infectivity. In 1959 Fox and Weiser published a case of a *Nosema* identified at that time as *Nosema stegomyiae,* isolated from rearing of *Anopheles gambiae* in Liberia, preventing the development of malaria parasites in infected mosquitoes. The microsporidian caused destructive epizooties in mosquito rearing in the insectary. The pathogen with minute oval binucleate spores 2.5-3 × 1.5-2 µm (Fox and Weiser 1959) was a very aggressive pathogen attacking all tissues of adult mosquitoes. Present were also macrospores 3 × 5 µm. It was transmitted in food in mosquito colonies and released with the feces of all infected animals. The main source of infection in colonies was infected cotton feeding wads with sugar water or honey where infected animals defecated during feeding. This microsporidian infected beside *A. gambiae* also *A. melas.*

Former mentions of microsporidian infections of mosquitoes in rearing and nature were of *Plistophora*

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stegomyiae by Marchoux *et al.* 1903, *N. stegomyiae* Lutz and Splendore 1908, *N. stegomyiae* Simond 1903 in *Aedes aegypti* and *N. anophelis* Kudo 1924 in *Anopheles quadrimaculatus*. The microsporidia involved had all oval spores 3.5-7 × 2-2.5 µm, therefore the microsporidia from Liberia were identified as belonging to this group. Relations of these infections were not clear, but the general pathology of such infections was rather similar and very typical. They often caused spectacular massive mosquito mortality in rearing and therefore all were considered as conspecific. In 1970 Vávra and Undeen described another microsporidian, *N. algerae* from a colony of *Anopheles stephensi*, and this organism, causing numerous epizooties in mosquito rearing, was studied further repeatedly in many cases but its relation to *N. stegomyiae* was not investigated. In all cases reported after 1970 the spore size was identical, 3.5-6 × 2.5-3 µm. The difference in size of mature spores (2.5 µm in *N. stegomyiae* Fox et Weiser, 1959 and 4.5-5 µm in *N. algerae* Vávra et Undeen, 1970) were overlooked due to the fact that both species have elongate sporoblasts about of the same size (4.5-5 µm) and these were in fresh material and section preparations difficult to distinguish from mature spores. Later it was shown that *N. algerae* survives under temperatures close to 37°C in tissue culture and to some extent also in vertebrate hosts. Recently *Brachiola vesicularum* infecting man was established as a type for a genus of microsporidia surviving under higher temperatures, with specific surface secretions produced during late schizogony. *N. algerae*, with the same type of secretions, was transferred to the newly formed genus *Brachiola*. Both species have also some similar ultrastructural details in the organization of the polar filament (terminal part thinner). *B. vesicularum* is not known to appear in rearing of mosquitoes. It is a pathogen of immunodeficient man. The *N. stegomyiae* of Fox and Weiser (1959) isolated from an infected mosquito colony caused a similar pathology as *B. algerae*, but its spores were of the size of *B. vesicularum*. Therefore the old material from Liberia was used for comparison and new evaluation.

MATERIALS AND METHODS

The type series for *Nosema* cf. *stegomyiae* Fox and Weiser, 1959 was prepared from 70% alcohol fixed infected adults of *Anopheles gambiae* from rearing at the Liberian Institute, Harbel, Liberia. There were no smears available. Adult mosquitoes were refixed in Bouin´s, embedded in paraffin and cut in sections 4-6 µm thick. Part of this material stained with Heidenhain´s iron hematoxylin and mounted in Canada balsam in 1957 was used for reprocessing for TEM. Slides were opened, balsam was removed and sections were transferred into alcohol and water. The sections were closed in 2% agar, refixed in 2% glutaraldehyde in cacodylate buffer, postfixed in 1% osmium tetroxide, washed in buffer and dehydrated in alcohol. The material was embedded in Epon and ultrathin sections were contrasted with uranyl acetate and lead citrate and inspected in a Philips EM 300 TEM.

RESULTS

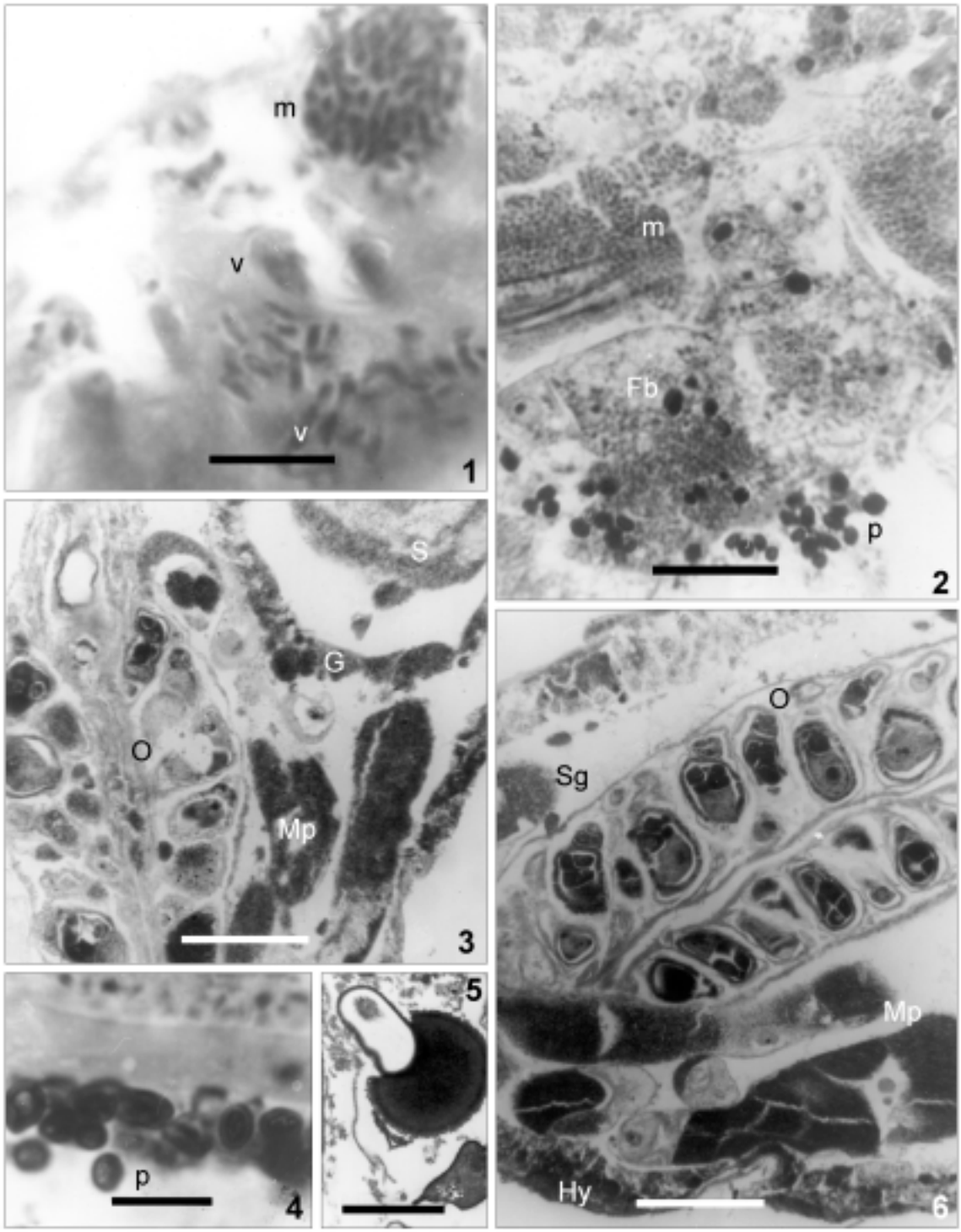
Optical microscopy and pathology

According to R. Fox (personal comm.), at the Harbel insectary the adult mosquitoes, males and females were infected. There was no evidence of larval infection. Infected females did not produce progeny. Most infections of adults started only after feeding. Spores were released in infected feces and adult mosquitoes in rearing were infected with contaminated sugar solution offered in wads to males and used also by females. Besides fecal pellets also saliva contained spore material released from bursting cells in the pharyngeal pump of the mosquitoes. After observations by R. Fox (personal comm.) during dissections of living animals, the pharyngeal pump and the foregut were the primary location of the infection in every mosquito before spread of infection to all other organs.

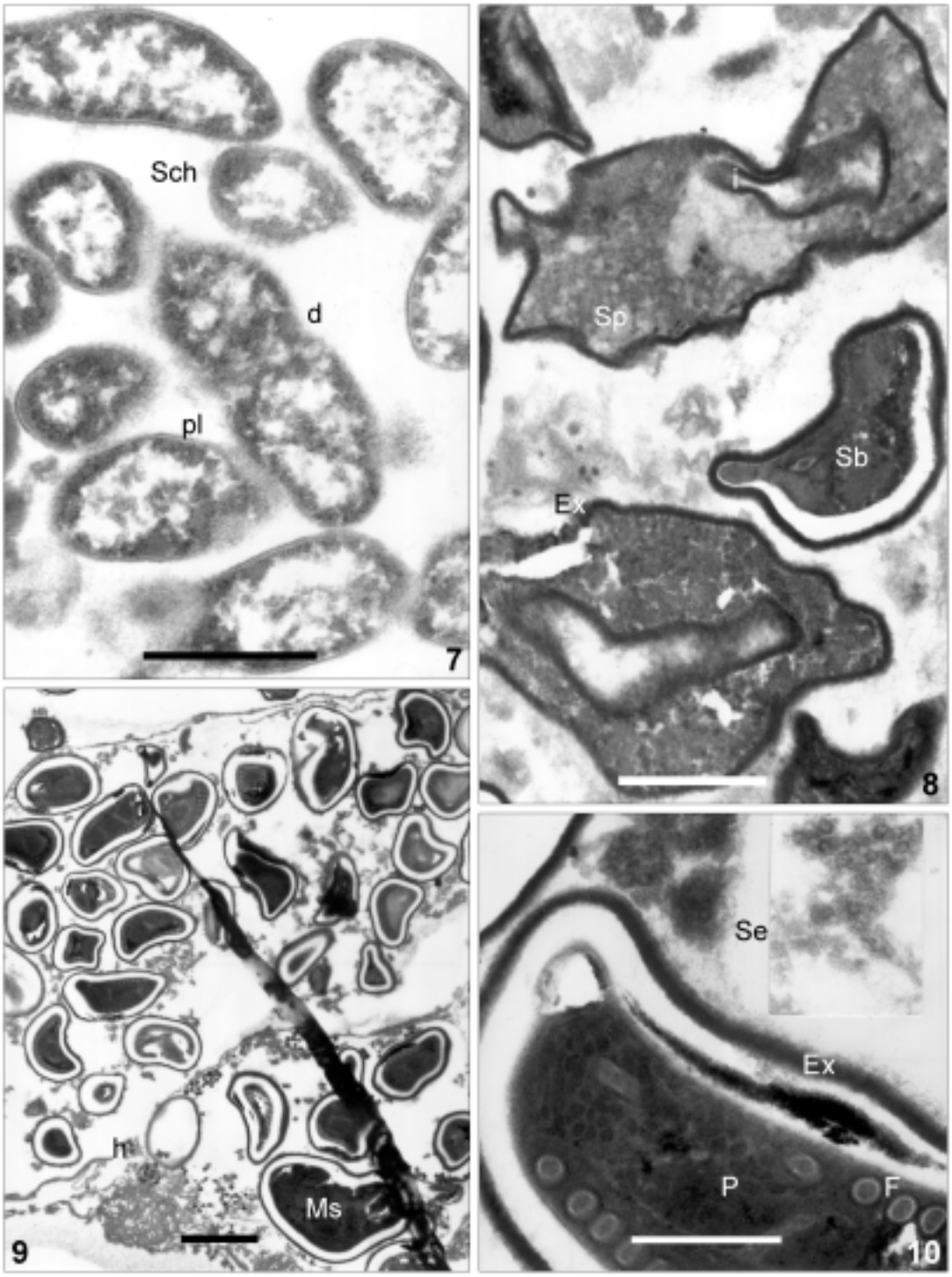
After entry of some vegetative stages in the host tissue (Fig. 1) sporogonial stages filled the centre of the lobe with stages of the same degree of development (Figs 3, 5), destroyed the midgut, the Malpighian tubules and invaded the hypoderm and fat body. They infected the connective tissues of the ovary and muscles (Fig. 2).

In most regions of the fat body adjacent to the body cavity there are broad oval brown bodies, in their final size 6 × 8 µm (Figs 2, 4). The brown mass is clear, not stained with haematoxylin, without any granulation and in the centre of the body is one single spore of the microsporidian (Figs 4, 6). This brown mass (melanin) - ensheeted spores are solitary, do not form coherent masses and have no connection with any period of the invasive process of the microsporidian in tissues. They are mainly in the hypoderm and adjacent layers of the fat body.

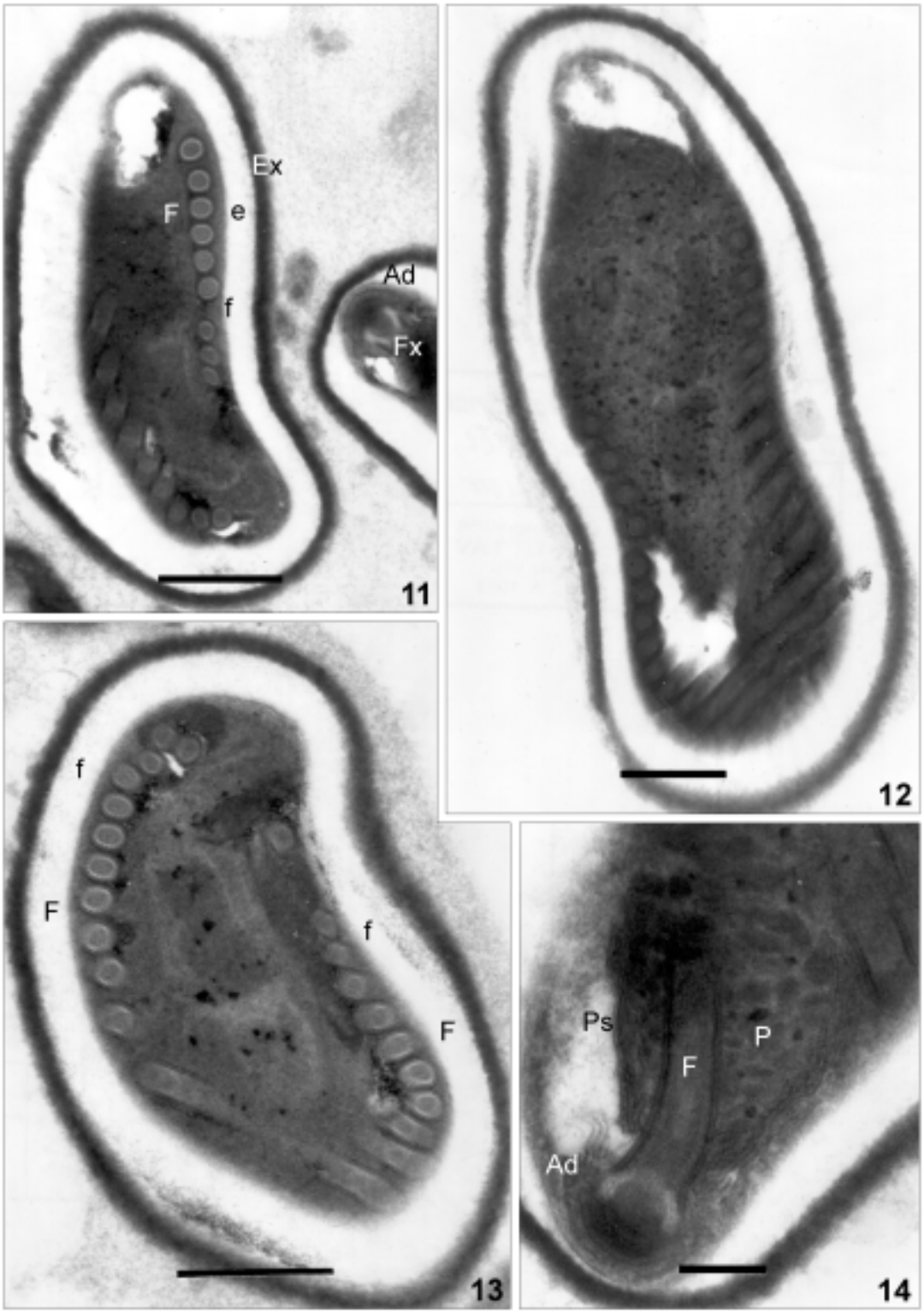
The center of the midgut inside the peritrophic membrane is filled with remains of red blood cells and groups of spores. These spores have their wall impregnated with brown coagulate. This explains some early obser-



Figs 1-6. 1 - *Brachiola gambiae* sp. n. (Heidenhain); **2** - infected muscle of *Anopheles gambia*, ensheathed spores with melanin; **3** - body of a female mosquito infected with *B. gambiae* with eggs degenerating. In the gut content are released spores in part closed in a brown sheath of melanin; **4** - spores ensheathed with melanin and muscle filled with spores; **5** - spore with melanin cover; **6** - heavy infection of female mosquito Malpighian tubules, salivary glands and hypoderm while ovary is not infected. Fb - fat body, G - dark spores fill cells of the midgut epithel, Hy - hypoderm, m - spores in muscle, Mp - Malpighian tubules, O - ovary, p - ensheathed spores with melanin, S - spores, Sg - salivary glands, v - schizonts in the fat body. Scale bars 3 μm (5); 10 μm (4); 15 μm (1) - 25 μm (2, 3, 6).



Figs 7-10. 7 - *Brachiola gambiae* sp. n. schizonts with destroyed structures of nuclei and smooth plasmalemma. Schizonts of the same stage, formed by synchronous binary fission. The grouping is not closed in any sac or pansporoblast. **8** - crenate sporonts with deep invaginations of their wall, plasmalemma is dissolved. The smooth cell wall is thick and formed of a layer of fine electron-dense granulations. Sporoblast with forming polar filament. **9** - group of mature spores in the remains of the membrane of the host cell. Spore wall not rigid, twisted, on the side a macrospore. **10** - vesiculo-tubular structures produced during sporogony, damaged by other fixation, not connected with the exospore of the spore. In its interior cross sections of the polar filament and the polaroplast. d - dividing stage, E - electron-dense granulations, Ex - exospore wall, F - polar filament, h - host cell, i - invaginations of wall, MS - macrospore, P - polaroplast, pl - smooth plasmalemma, Sb - sporoblasts, Sch - schizonts, Se - vesiculo-tubular structures, Sp - crenate sporonts, Scale bars 500 nm (8, 10); 1 µm (7); 2 µm (9).



Figs 11-14. 11 - *Brachiola gambiae* sp. n. mature spore with finely granular exospore and lucent endospore, six larger coils of polar filament and three with minor diameter. Other spore with anchoring disc and the electron lucent fixation of the polar filament. **12** - macrospore with 13 coils of polar filament, without apparent anisofilarity. **13** - normal spore with 9 large and 3 reduced coils and two nuclei. **14** - apical spore end with anchoring disc, fixation of the polar filament and polaroplast closed in the polar sac. Ad - anchoring disc, e - endospore wall, Ex - exospore wall, f - polar filament of minor diameter, F - polar filament, P - polaroplast, Ps - polar sac. Scale bars 200 nm (13, 14); 500 nm (11, 12).

vations of Marchoux *et al.* (1903) and Simond (1903). Spores in the area between infected epithelial cells and the peritrophic membrane were not stained. The spread of the microsporidian in the host is mediated by hemocytes containing spores. In the mass of spores in different tissues there are two types of spores: such with intensive staining with Hematoxylin and others, which are less stained. On sections there is no perfect evidence of empty spores. Vegetative stages (Fig. 1) are visible only as empty areas in the infected tissue, usually on the border of a large group. In narrow muscle bands (Fig. 4) the spores are arranged in rows, in dense tissues such as midgut muscles or Malpighian tubules (Figs 3, 6) the spores are in dense groups, in the fat body (Fig. 2) they are distributed in the cytoplasm around fat droplets. In sections of the ovary the infection appears late and infects mainly the connective tissue (Fig. 6) and nutrient cells of the ovary. Eggs without nutrition degenerated (Fig. 3). In other cases developing eggs were not infected (Fig. 6). Fresh spore material and smears were not available and the exact determination of sizes and shapes of spores (Figs 1, 2) was difficult (missing exact contours in dried smears). Therefore, supported by identical symptoms of the mosquito attack in rearing, the organism was indicated as identical with *Nosema stegomyiae* Marchoux, Salimbeni et Simond, 1903. The diagnosis was supported by dense groupings of mature spores in infected tissues and the brown impregnation of spores in the gut content. Some details in descriptions of Marchoux *et al.* (1903) and Simond (1903) indicate a possibility of a further species involved (plasmodial stages, reniform spores), but could be connected with the different host.

Ultrastructure

In the re-fixed and stained ultrathin sections we find stages of the end of schizogony, early and late sporonts and mature spores. Late schizonts are in groups of twenty and more (Fig. 7) seen as oval bodies, 2.5-3 × 0.8 μm, some constricted in the centre. The nucleus (diplokaryon?) is evident due to fixation of paraffin blocks only as an irregular empty space without nuclear membrane in the centre of the cell with content in irregular clumps. The schizont is closed in a fine continuous smooth coat of electron dense material (Fig. 7). The outer surface of this coat is covered by a thin layer of fine electron dense granulation, without irregular secretory processes. Evidently the schizonts are formed by synchronous nuclear division and binary fission. In the interspace are fine electron dense concretions without direct connection with any stage (Fig. 10). The group is not closed in any sac or pansporoblast.

The next step, thick walled sporonts with smooth walls (Fig. 8) are rather rare in the studied material. Where present they are in groups of the same age. Usually they measure 2.5 × 3 μm. Their wall is of fine granular electron-dense material, 37-40 nm thick, without a smooth layer on their outer or inner surface. There is no evidence of any system of gaps or blisters in this wall. There is no electron lucent layer between the wall and the cytoplasm. The cytoplasm is a dense granulated mass without distinct diplokaryotic nucleus, its eventual location visible only as confluent grouping of empty areas in the centre. Internal structures were destroyed during the fixation with Bouin's in 1957. Eventual surface deposits on the sporonts were dissolved. Tiny membranous wicks and minute surface granulations are coiled in the interspace (Fig. 10). Evaginations of the plasmalemma were absent, but there are deep invaginations (Fig. 8) connected with formation of crenate sporonts with first signs of formation of the polar filament.

Spores are oval to pyriform, with deformations and compressions from fixation. Fixed for TEM they measure 2.2-2.5 × 1.4-1.6 μm. They appear in dense groups, all of the same stage of development (Fig. 9). The electron-dense exospore (Figs 11, 13) is 70 nm thick, the electron-lucent interspace, the endospore, is 170 nm thick (Figs 11, 13). The anterior pole is attenuated, 50 nm. The plasmalemma is very thin and indistinct. In the centre are two electron dense nuclei. The polar filament is fixed in the anterior hemispheric knoblike anchoring disc 280-300 × 120 μm (Figs 11, 14). The polar sac encloses the polaroplast with irregular remains of its lamellar structure. In it is fixed the polar filament with its manubrium part with a broadened end 150-180 nm in diameter, with a distinct electron lucent ring. The filament has a system of 20 electron-lucent slightly twisted longitudinal columns. The coiled part of the filament is anisofilar, has 6 broader (diam. 100 nm) and 3 narrow (diam. 70 nm) turns (Fig. 11). Besides spores with 9 (6+3) turns there are some with up to 12 turns (8+4) (Fig. 13). The fixing umbrella is adhering to the plasmalemma in a collar 150 nm broad.

At the periphery of a group of normal spores are some larger (macro-) spores (Figs 9, 12), usually 1in 30.

They are larger in size, 4.5-5 × 2 µm, with all structures equal to normal spores, but with up to 13 turns of the polar filament.

DISCUSSION

It is interesting that all published cases of infections of mosquito colonies after 1970 were ascribed to *Nosema algerae* with typical oval spores 3-4.5 × 2-2.5 µm. Possibly in some cases the real size of spores was not measured due to the typical spectacular massive invasion of mosquito rearing and differences in spore size were estimated as natural variability.

The *Brachiola* definition is based on thermophily of the members of the genus, proliferation and sporulation at temperatures close to 37°C. Schizogony with diplokaryotic schizonts dividing by simple fission without multinucleate plasmodia. Stages are not closed in any membrane. Late schizonts with a layer of secretion on their plasmalemma producing rows of blisters and ridges and free vesiculo-tubular electron dense secretions. They are connected with different proliferations on the surface of late schizonts. Starting with early sporogony, binucleate sporonts are covered with a thick layer of fine granular electron-dense material replacing the plasmalemma and forming later the exospore. Groups of vesiculo-tubular secretions are fixed to the exospore or remain as appendages fixed to the spore. Sporogony is disporoblastic, sporonts produce two diplokaryotic spores with fine granular exospore. Spores with short polar filament with up to 10 coils, of which the final three (*B. vesicularum)* or one *(B. algerae)* are narrower. In *B. algerae* the principal host is an insect (mosquito, caterpillar), in *B. vesicularum* the host is the immunodeficient human patient.

Some parts of this definition need further verification. The separation of the genus *Brachiola* is characterized mainly based on its survival in warm-blooded animals at 37°C. This characteristic seems to be in *B. (N.) algerae* achieved artificially. In Lowman *et al*. (2000) evidently spores in figures 31-34 are not normal and may not be able to infect a normal warm-blooded host. But after conjunctival instillation (Vávra personal comm. 2002) *B. algerae* appears in liver and other organs without degeneration of spores. In the survival of *B. vesicularum* there is no experience with development of the pathogen in normal human hosts and its entry of the pathogen into the host and its circulation. With this experience the requirement of development of *Brachiola* in warm-blooded hosts for definition of the genus is rather weak and may depend very much of qualities of strains and resistance of hosts. If we consider myositis of left leg muscle of the patient without considering the way of entry and tissue affinity under support of AIDS, we should not base the definition of the genus on such details.

A comparison of ultrastructures of *N. stegomyiae* Fox et Weiser, 1959 with *B. vesicularum* brings interesting similarities. The development of both microsporidia is very similar. Schizogony (proliferate forms) have a thin smooth plasmalemma and the formation of sporonts is signalized by thickening of the outer wall composed of electron-dense granular material. On the surface of this wall there is in our material no sign of any adhering vesiculo-tubular secretion (eventually destroyed due to previous fixation for paraffin). There are groups of vesiculo-tubular material present in groupings of mature spores (Fig. 10).

In crenellated late sporonts there are deep invaginations of the wall in *N. stegomyiae* (Fig. 8). Almost identical is the ultrastructure of mature spores of *B. vesicularum* and *N. stegomyiae*: the structure of the spore wall and the size and shape of binucleate spores, the type of polar filament and its anisofilar formation with three narrow cross sections and 6 ordinary sections.

There is no experience with survival and development of *N. stegomyiae* under higher temperature but conditions of rearing of mosquitoes in tropical Africa offered temperatures close to 30°C. R. Fox (personal comm.), considered as possible a transmission of the microsporidian from cage to cage with the chicken used for feeding adults, with contamination of their skin with infective feces. The microsporidian with spores 2.5-3 × 2 µm has not been recognized again during the last 50 years. Nevertheless, evident differences of spore size in the Nigerian case and in *B. algerae* and the striking ultrastructural similarity of the (1957) material with members of the genus *Brachiola* invites to correct the old identification and to propose for the pathogen of *Anopheles gambiae* and *A. melas* in Liberia, interactive with transmission of malaria parasites in mosquitoes described above, the new name, *Brachiola gambiae* sp. n.

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