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Life Cycle and Description of *Amblyospora camposi* n. sp. (Microsporidia: Amblyosporidae) in the Mosquito *Culex renatoi* (Diptera, Culicidae) and the Copepod *Paracyclops fimbriatus fimbriatus* (Copepoda, Cyclopidae)

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ABSTRACT. The life cycle of *Amblyospora camposi* n. sp. is described from the mosquito *Culex renatoi* and the copepod *Paracyclops fimbriatus fimbriatus* collected in the leaf axils of the plant *Eryngium cabrerae* in Argentina. Meiospores of *A. camposi* (5.8 × 4.1 μm) were infectious per os to female adults of the copepod *P. f. fimbriatus*. All developmental stages in the copepod had unpaired nuclei, with sporulation involving the formation of a sub-persistent, sporontogenic, interfacial envelope and the production of a second type of uninucleate spore. These spores, formed in the ovaries of *P. f. fimbriatus*, were large, pyriform, and measured 10.70 × 3.85 μm. When ingested they infected *C. renatoi* larvae to initiate a sequence that involves schizogony and gametogony and ends with plasmogamy and nuclear association to form diplokaryotic meronts. Oblong ovate binucleate spores (7.86 × 2.96 μm) are formed in the adult mosquito and are responsible for vertical transmission to the filial generation. This is the first report of an *Amblyospora* species from a mosquito that inhabits the small-water bodies held in parts of terresterial plants (phytotelmata).

Key Words. *Eryngium cabrerae*, host specificity, phytotelmata, taxonomy, ultrastructure.

PHYTOTELMATA ("plant pool") is a term used for the small caches of water in parts of plants that are inhabited by diverse and complex biological communities. Mosquitoes are very common inhabitants in many phytotelm communities and have been the subject of numerous entomological and ecological studies (Frank and Lounibos 1983). Relatively little attention has been given to diseases of organisms that inhabit phytotelmata. During an ecological study on the mosquito *Culex renatoi* Lane and Ramalho (Campos and Lounibos 1999), a phytotelmatic species that breeds in the plant *Eryngium cabrerae* (Family Umbelliferae) (Casal and García 1967), we discovered a microsporidium infecting the larval fat body. Initial observations revealed the presence of eight oval spores within a sporophorous vesicle implicating the species as a member of the genus *Amblyospora* Hazard and Oldacre, 1975. We describe this new species and its development in larvae and adults of the mosquito host and document the role of the copepod *Paracyclops fimbriatus fimbriatus* (Fischer, 1853) in the life cycle.

MATERIALS AND METHODS

Collection site. The sampling area was located in Punta Lara, (34°51′53″S, 57°52′23″W) near the Rio de La Plata in Buenos Aires province, Argentina. The site consists of open fields fringed by forest habitats that border the river. Mosquitoes and associated microcrustacea were found in water-holding leaf axils of the plant *Eryngium cabrerae*, which were located only in the open-field areas.

Collection and processing of mosquitoes. Water, containing mosquito larvae and associated organisms, was extracted from the leaf axils of plants with an aspirator composed of a tube connected to a 125-ml trap flask to which a rubber bulb was attached. The axils were flooded 3 times with distilled water to ensure extraction of all the organisms (Lounibos 1983). The samples were placed in separate containers and the total number of immature stages from each plant was determined. Samples were collected throughout the year and larvae identified using a key by Darsie and Mitchell (1985).

Larvae of *C. renatoi* infected with a new *Amblyospora* sp. were isolated based on the chalky white appearance of the fat body. Meiospores from some infected larvae were used as inoculum for transmission experiments to copepods found in the breeding sites. Other infected larvae were smeared on slides, air-dried, fixed in methanol, and stained with 10% Giemsa stain buffered at pH 7.4. For ultrastructural studies, infected tissues were fixed for 2 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.1% $CaCl_2$ and postfixed in 1% aqueous OsO_4. These tissues were dehydrated through an ascending ethanol and acetone series and embedded in Epon-Araldite. Thin-sections were post-stained with methanolic uranyl acetate followed by lead citrate and examined and photographed with a Hitachi H-600 electron microscope at 75kV.

Mosquito larvae without gross lesions were reared in laboratory containers and the pupae were placed in cages for adult emergence. A chicken was provided as a blood source for 24 h and then all adults (males and females) were transferred into individual vials. The sex of the adults was determined and then each was individually smeared, stained with Giemsa, and examined for the presence of stages and spores of *Amblyospora*.

Collection and processing of copepods. Two species of copepods were found associated with mosquito larvae in the leaf axils of *E. cabrerae*. The copepods were identified according to the descriptions of Dussart (1969), Reid (1985), and Ringuelet (1958). Individual copepods were smeared and stained with Giemsa and examined for evidence of infections with microsporidia. Gravid females were isolated and colonies of both species were established in the laboratory for transmission studies.

Mosquito to copepod transmission. Infected mosquito larvae were triturated in water with a glass tissue grinder and then filtered through cotton. The filtrate was centrifuged for 10–30 min at 4000 *g*, the supernatant discarded, and the meiospores re-suspended in distilled water. Spore concentration was determined with a haemocytometer.

Adult copepods of each species were divided by sex and separated into groups of 15 individuals in 10 ml of water. Each group was inoculated with meiospores (at a final concentration of 1×10^3 spores per ml) and transferred, 24 h post-exposure, to 100 ml of water plus an appropriate amount of fish food. Control groups were prepared in a similar manner but without the addition of meiospores. The test was replicated 3 times.

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Table 1. Summary by plant of *Culex renatoi* infected with the microsporidium *Amblyospora camposi* collected from the leaf axils of *Eryngium cabrerae* in Argentina.

Plant #	Date collected	Number of larvae	% infection in larvae	Infections in male adults	Infections in female adults	% infection in adult
1	11/22/95	18	5.5	1/3	1/6	22.2
2	05/13/96	22	45.5	0/3	0/3	0
3	11/05/96	12	16.7	1/2	0/8	10
4	11/05/96	17	5.9	0/1	0/5	0
5	11/12/96	17	5.9	0/2	0/9	0
6	11/12/96	16	18.75	0/4	0/4	0
7	11/12/96	16	25.0	0/5	0/4	0

Individuals were examined daily for signs of patent infections and the presence of spores. Infected individuals were processed for light and electron microscopy as described above.

Copepod to mosquito transmission. Spores from the exposed, infected copepods were used as inoculum. Fifty field-collected, first-instar larvae of *C. renatoi* were placed in Petri dishes containing 10 ml of water with one or three dead, infected copepods. After 24 h exposure, the larvae were transferred to containers with 750 ml of water plus fish food. Control groups were handled in a similar manner but without the addition of spores. This test was replicated 10 times. Individuals were smeared as larvae and pupae, stained with Giemsa, and examined for the presence of vegetative stages and spores.

RESULTS

Field studies. *Culex renatoi* and *Culex castroi* were routinely collected from the axils of *E. cabrerae*. Infections with the *Amblyospora* sp. were restricted to *C. renatoi* found in November 1995 and May and November 1996. In November of both years, 31 plants were examined and there was an average infection level in larvae of 4.45% with the *Amblyospora* sp. (11/247). The percent patent infection in larvae by plant was variable ranging between 5.5% and 45.5% (Table 1).

Two species of copepods (*Ectocyclops* sp. and *P. f. fimbriatus*) were found associated with mosquito larvae in the plant axils. There was no evidence of microsporidia infections in either species of copepods from field collections.

Development in *C. renatoi* larvae involving meiosis. This sequence involved meiosis followed by octosporoblastic sporogony and the production of meiospores. Diplokaryotic meronts (Fig. 1) multiplied in fat body cells of the mosquito. The sporont was identified by the formation of a sporontogenic interfacial vesicle and the presence of synaptonemal complexes in each of the two nuclei of the diplokaryon (Fig. 2). Three sporogonic divisions (Fig. 3–4) gave rise to eight sporoblasts and finally eight spores within a sporophorous vesicle. Electron-dense amorphous materials accumulated in the episporontal space and as sporogony progressed, tubular material appeared (Fig. 3–4). Mature spores were uninucleate with a lamellate polaroplast and a large posterior vacuole (Fig. 5). The spore wall was composed of a thick structureless endospore and a laminate exospore of Larsson's type IIIC (Larsson 1986), characteristic for *Amblyospora* (Fig. 6). The polar filament was anisofilar with 3–4 broad proximal and 4 1/2 (range = 4–5) narrow distal coils (Fig. 6). These meiospores were infectious per os to the copepod intermediate host. Living meiospores measured 5.8 × 4.1 ± 0.4 μm (n = 32). Preserved meiospores measured 4.56 ± 0.54 × 3.11 ± 0.47 μm (n = 32).

Infections in adult mosquitoes. *Culex renatoi* adults (from field-collected larvae and pupae) did not take blood meals when offered and therefore vertical transmission could not be verified. However, of 59 adults examined, binucleate spores were found in two males and one female (Table 1). The binucleate spores were oblong ovate and measured 7.86 ± 0.68 × 2.96 ± 0.34 μm (fresh, n = 10). In addition to the binucleate spores, groups of meiospores were also present in each of the infected adults examined.

Copepod infection experiments. *Amblyospora* sp. was successfully transmitted to only females of *P. f. fimbriatus* with infection rates of 33% (5/15), 66% (10/15), and 93% (14/15). The infections were restricted to the ovaries and required 10–12 d for the development of mature spores.

Development in P. f. fimbriatus. Uninucleate cells (schizonts) were the first stages observed in ovaries of the copepod (Fig. 7). These stages divided by binary fission (Fig. 8). Plasmodia with 4–8 nuclei were observed in Giemsa-stained preparations, which became rosette-like stages (Fig. 9). Complete cytokinesis produced uninucleate sporonts that developed into sporoblast and uninucleate spores (Fig. 10, 11). Sporulation involved the production of a sub-persistent sporontogenic interfacial envelope around each sporont formed by a duplication of the plasmalemma. The uninucleate spore was pyriform, often curved in shape (Fig. 11, 12), and measured 10.7 ± 0.35 × 3.85 ± 0.1 μm (fresh, mean ± SE, n = 15) and 9.2 ± 0.49 × 3.4 ± 0.4 μm (fixed, n = 15). The mature spore was uninucleate, with an isofilar polar filament with 7–8 turns and a large posterior vacuole (Fig. 13). The polaroplast was an extensive and highly compartmentalized organelle that occupied two-thirds of the spore (Fig. 12, 13). The spore wall was thin with the endospore approximately 3 times the thickness of the rugose, unlayered exospore (Fig. 14).

Mosquito infection experiments. Experimental transmission with spores from *P. f. fimbriatus* to larval *C. renatoi* revealed developmental stages of *Amblyospora* in Giemsa-stained smears of larvae and pupae indistinguishable from those in previous reports (Andreadis 1988; Becnel 1992; Sweeney et al. 1988). Transmission was successful in only one of the trials with 2 of 30 larvae infected. Stages were commonly observed in the cytoplasm of host cells of unknown origin. The earliest stages were small, uninucleate, and round with relatively large nuclei. Later stages (gametes) were pyriform with the single nucleus located at the broad end of the cell. Gametes came together in pairs and underwent cytoplasmic fusion (plasmogamy) to form cells with 2 nuclei in a common cytoplasm. These nuclei did not fuse but became associated to form a diplokaryon. Because *C. renatoi* has not been cultured, it was not possible to obtain progeny from exposed female adults to verify vertical transmission in the lab.

TAXONOMIC SUMMARY

Amblyospora camposi n. sp. Micieli, García and Becnel (Fig. 1–14)

Type definitive host. *Culex renatoi* Lane and Ramalho (Diptera, Culicidae).

Type intermediate host. *Paracyclops fimbriatus fimbriatus* (Fischer, 1853) (Copepoda, Cyclopidae).

Transmission. Transovarially to filial generations of *C. renatoi* via binucleate spore. Per os to *P. f. fimbriatus* via meiospores liberated into environment by death of infected mosquito larvae of filial generation. Per os to *C. renatoi* via uninucleate spores from *P. f. fimbriatus*.

Site of infection. Adipose tissue and ovary of *C. renatoi*. Ovary of *P. f. fimbriatus*.

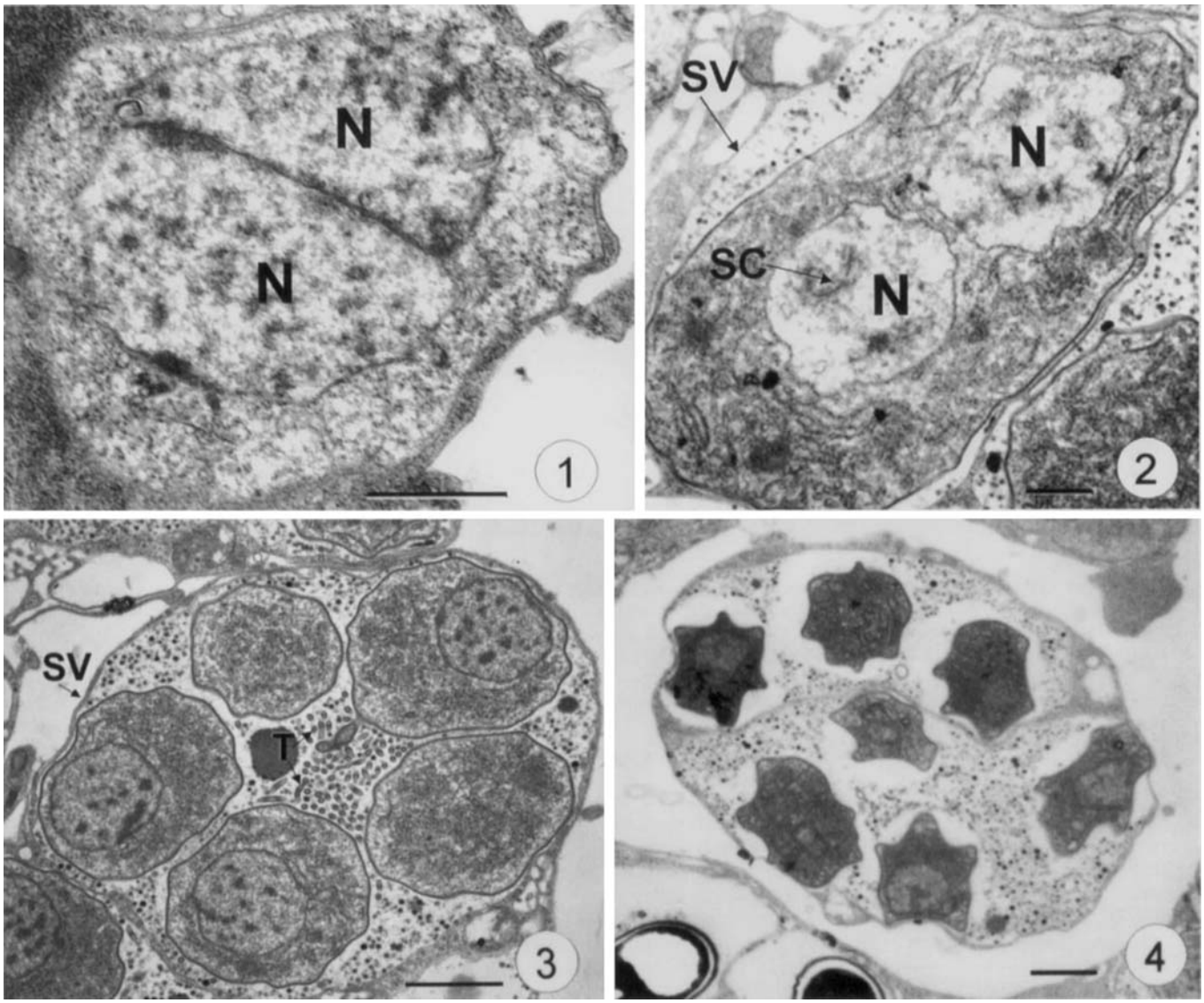

Fig. **1–4**. Electron micrographs of developmental stages of *Amblyospora camposi* in larvae of *Culex renatoi* during the sporulation sequence involving meiosis. **1.** Meront, bar = 1 μm. **2.** Sporont undergoing meiosis, bar = 1 μm. **3.** Sporogonial plasmodium, bar = 2 μm. **4.** Octonucleate sporogonial plasmodium, bar = 2 μm. N, nucleus; SC, synaptonemal complexes; SV, sporontogenic sporophorous vesicle; T, tubules.

Interface. Sporophorous vesicle produced by the sporont during the sporulation sequence involving meiosis in the mosquito host. A non-persistent sporontogenic interfacial envelope is produced by the sporont in the copepod host. All other stages in direct contact with the host cell cytoplasm.

Development. Uninucleate spores from *P. f. fimbriatus* ingested by mosquito larvae initiate schizogonic series that ends with gametes. Gametes unite in pairs (gametogony) and nuclei associate as diplokarya to form meronts. Sporulation in the female ends with binucleate spores that transovarially infect filial generations (presumed). Sporulation in filial generation involves meiosis, and ends with the production of meiospores. Meiospores ingested by copepod initiate a schizogonic sequence leading to sporulation and production of the uninucleate spores responsible for horizontal transmission to a new generation of *C. renatoi.*

Spores from *P. f. fimbriatus*. 10.7 ± 0.35 × 3.85 ± 0.1 μm (fresh, mean ± SE, n = 15), 9.2 ± 0.49 × 3.4 ± 0.4 μm (fixed, n = 15). Pyriform, curved. Polar filament isofilar with 7–8 turns, extensive polaroplast compartmentalized and vesiculate. Endospore thicker than the rugose exospore. **Binucleate spore:** Oblong ovate, 7.86 ± 0.68 × 2.96 ± 0.34 μm (fresh, n = 10). **Meiospore:** 5.8 × 4.1 ± 0.4 μm (fresh, n = 32). 4.56 ± 0.54 × 3.11 ± 0.47 μm (fixed, n = 32). Mature spores were uninucleate with a thick undulating exospore, a lamellate polaroplast, and a posterior vacuole. The polar filament is anisofilar with 4 broad proximal and 4 1/2 (range = 4–5) narrow distal coils.

Type locality. Punta Lara, (34°51′53″S, 57°52′23″W) Buenos Aires province, Argentina, in axil of the plant *Eryngium cabrera* (Family Umbelliferae).

Deposition of type specimens. Type slides have been deposited in the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, DC, USA (USNM numbers Holotype # 51491 and Paratype # 51492). Type specimens embedded in plastic resin are also in the collection of the authors.

Etymology. Named after Raul E. Campos for without his

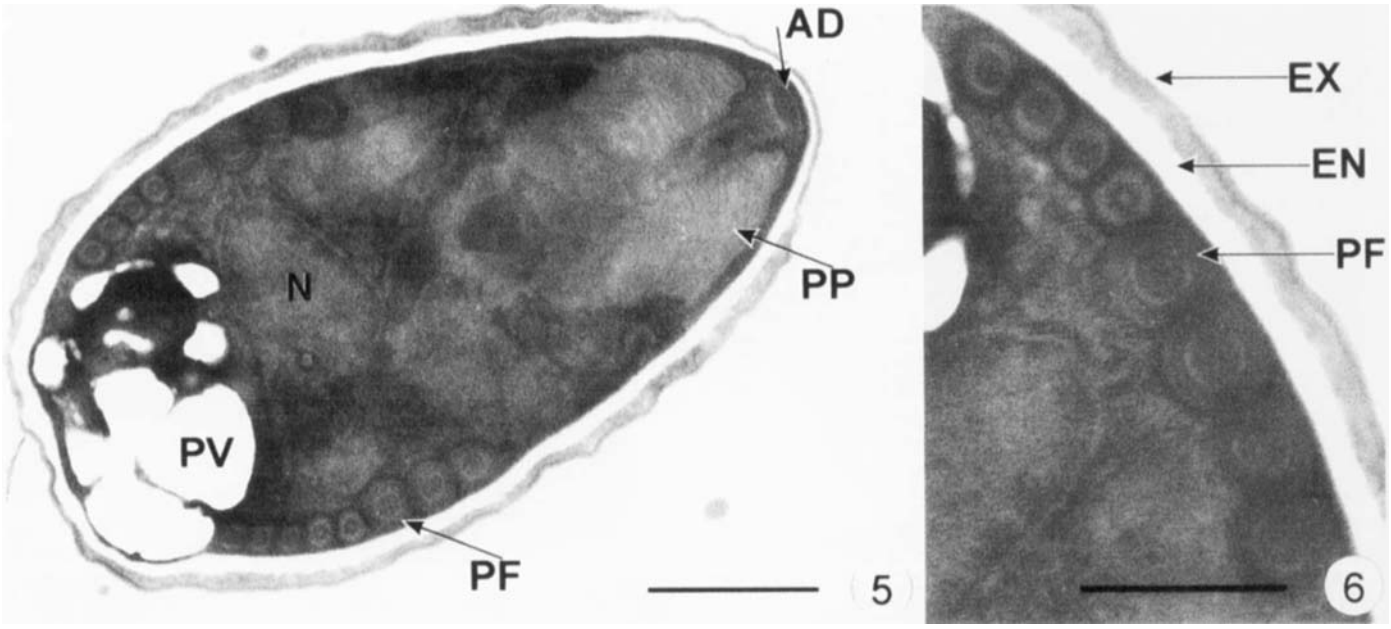

Fig. **5–6**. Electron micrographs of meiospores of *Amblyospora camposi* in larvae of *Culex renatoi*. **5.** Mature spore, bar = 1 μm. **6.** Details of the spore wall and polar filament coils, bar = 0.5 μm. AD, anchoring disk; EN, endospore; EX, exospore; N, nucleus; PF, polar filament; PP, polaroplast; PV, posterior vacuole.

dedicated study of mosquitoes in Argentina, this species of microsporidia would not have been found.

DISCUSSION

It has been estimated that at least 400 mosquito species in 15 genera occur in phytotelmata (Fish 1983), but pathogens in general and microsporidia specifically have been rarely reported. This is the first report of an *Amblyospora* species from a mosquito that inhabits phytotelmata. Other species of microsporidia that have been reported from phytotelm mosquito species are *Pilosporella fishi* from *Wyeomyia vanduzeei* (Hazard and Oldacre 1975), *Pilosporella chapmani* from *Aedes triseriatus* (Hazard and Oldacre 1975), *Parathelohania* sp. from *Anopheles bradleyi* (Hazard and Oldacre 1975), *Pleistophora* sp. and *Stempellia magna* from *Aedes sierrensis* (Sanders and Poinar 1976; Clark and Fukuda 1967), *Plistophora* sp. from *Orthopodomyia signifera* (Chapman et al. 1967) and *Plistophora* sp. from *Toxorhynchites rutilus septentrionalis* (Chapman et al. 1967). Each of these species occurs in treehole habitats except for *P. fishi* in *W. vanduzeei,* which is found in bromeliads (Frank 1983).

Amblyospora spp. have been reported mostly from mosquitoes and are characterized by intricate life cycles involving multiple spore types responsible for horizontal and vertical transmission (Becnel and Andreadis 1999; Sprague et al. 1992). They affect two generations of the mosquito and involve an obligate intermediate host. The complexity of the development of these parasites together with the difficulties involved with the culture of both the mosquito and the appropriate intermediate hosts has hindered life-cycle studies. To date (including this study), the complete life cycles for species of Amblyosporidae have been determined for 11 *Amblyospora* spp., from

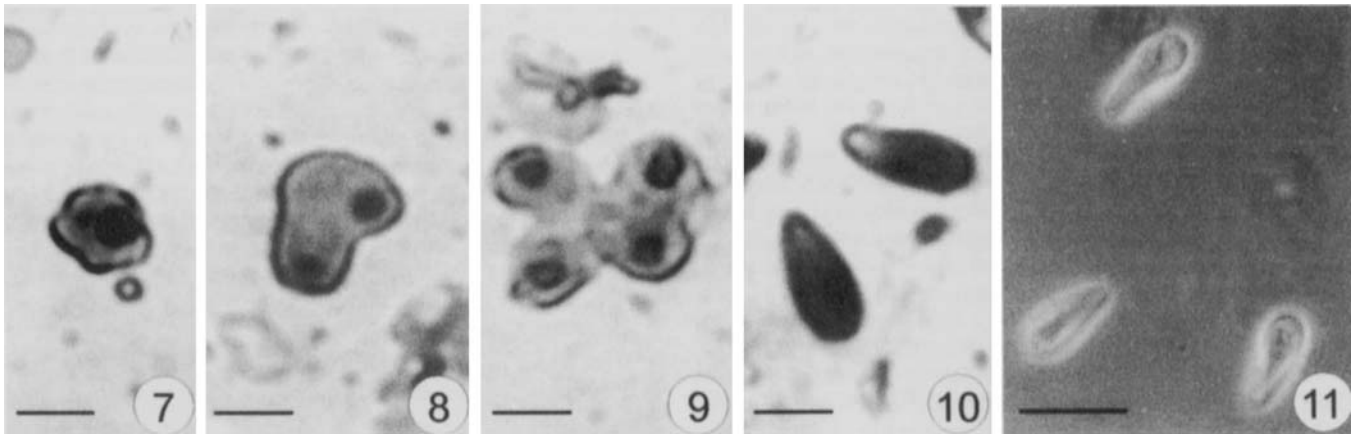

Fig. **7–11**. Light microscopy of developmental stages of *Amblyospora camposi* from the ovarian tissue of the copepod *Paracyclops fimbriatus fimbriatus*. Fig. **7–10,** Giemsa-stained. Fig. **11,** fresh, bar = 5 μm. **7.** Uninucleate schizonts, bar = 5 μm. **8.** Binary fission, bar = 5 μm. **9.** Multiple fission of sporogonial plasmodium with 4 nuclei, bar = 5 μm. **10–11.** Uninucleate spores, bar = 10 μm.

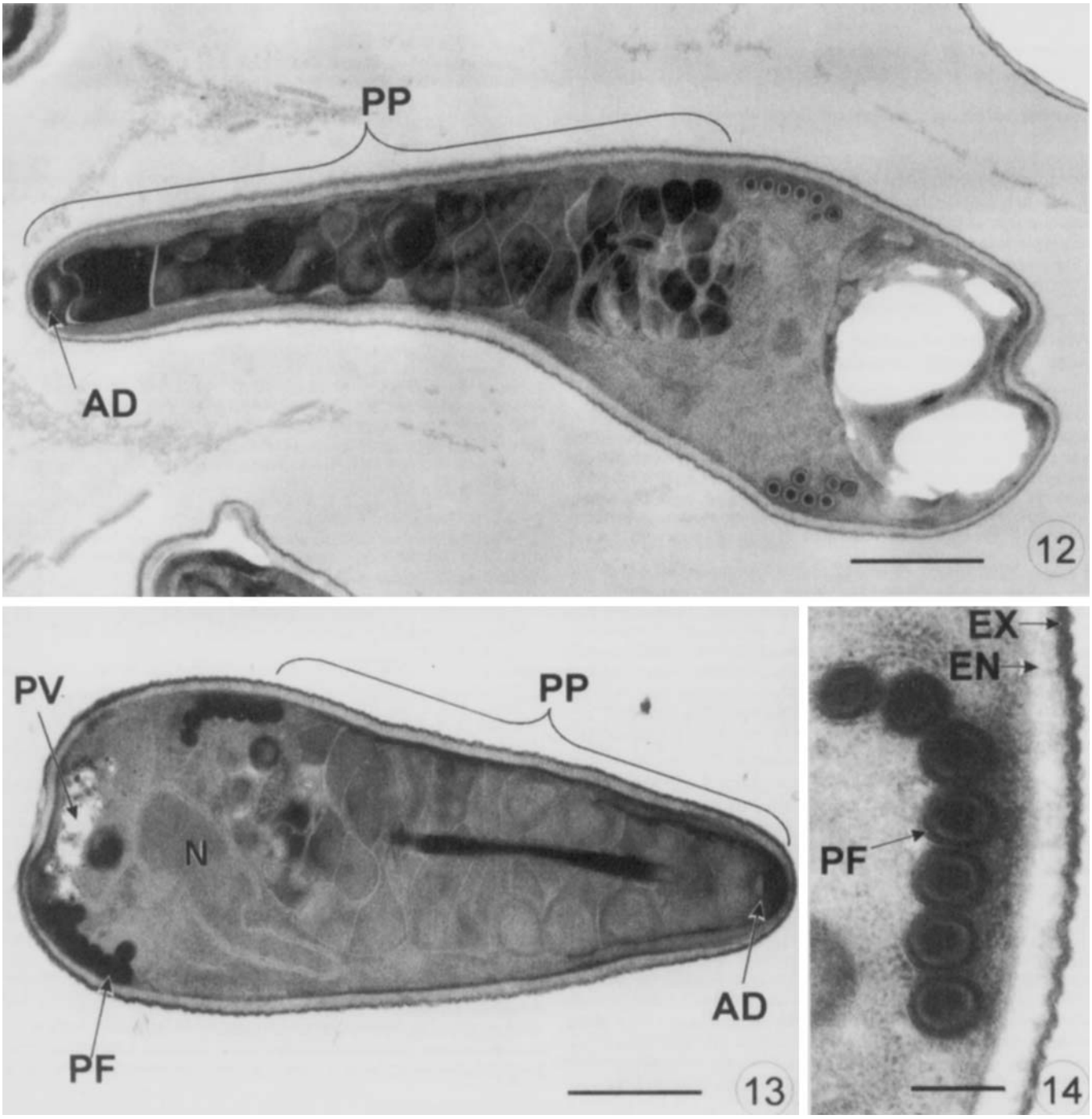

Fig. **12–14**. Electron micrographs of *Amblyospora camposi* spores in the ovarian tissue of the copepod *Paracyclops fimbriatus fimbriatus*. **12.** Immature spore with polar filament formation near completion and the genesis of the vacuolated polaroplast, bar = 1 μm. **13.** Mature spore demonstrating the extensive polaroplast divided into numerous compartments, bar = 1 μm. **14.** Details of the spore wall and polar filament coils, bar = 0.1 μm. AD, anchoring disk; EN, endospore; EX, exospore; N, nucleus; PF, polar filament; PP, polaroplast; PV, posterior vacuole.

Aedes and *Culex* mosquitoes (Andreadis 1985, 1988, 1990; Avery 1989; Becnel 1992; García and Becnel 1994; Micieli et al. 1998, 2000; Sweeney et al. 1985, 1988, 1990) two *Parathelohania* spp. (Avery and Undeen 1990), and one species of *Dubosqia* (Sweeney et al. 1993).

This study has provided evidence for the role of a copepod intermediate host in the life cycle of this *Amblyospora* sp. from *C. renatoi*. Meiospores infected the copepod *P. f. fimbriatus* (in the laboratory) and resulted in the production of uninucleate spores that are directly infectious to larvae of *C. renatoi*. Because this mosquito has not been cultured, the part of the life cycle that involves vertical transmission could not be determined in the laboratory. However, vertical transmission was implicated by the presence of binucleate spores in field-collected adults of *C. renatoi*. Therefore, the life cycle of this *Amblyospora* sp. is identical to that described for other species

of *Amblyospora* (Andreadis 1988; Becnel 1992; Micieli et al. 1998, 2000; Sweeney et al. 1988, 1990; White et al. 1994) and is characterized by 3 sporulation sequences involving 2 host species.

The present species has clear affinities with the genus *Amblyospora* based on spore morphology, development, and life cycle characteristics (Sprague et al. 1992). The meiospores of this *Amblyospora* sp. are of a similar size and polar filament structure as those reported for *A. ferocis* from Argentina (García and Becnel 1994) but is distinguished by differences in the spore wall, host, and habitat. Therefore, we have proposed the name *Amblyospora camposi* n. sp. for this microsporidium from *C. renatoi* and place it in the family Amblyosporidiae.

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