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Ultrastructural study of *Sarcocystis muriviperae* development in the intestine of its snake hosts

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Abstract. The ultrastructure of the endogenous stages – merozoites, microgamonts, macrogamonts and oocysts, of *Sarcocystis muriviperae* from the snakes *Vipera palaestinae* and *Coluber jugularis* is described. Snakes were infected via white mice fed on sporocysts obtained from naturally infected snakes of the same species. Snakes examined 4 days post-infection contained only young and premature gamonts. Infection in snakes sacrificed on day 7 post-infection consisted predominantly of mature microgamonts and macrogamonts; snakes examined on day 10 post-infection revealed only oocysts. The fine structure of the endogenous stages from the two snakes, including size and contents of the wall-forming bodies, was identical, confirming their suggested conspecificity. Observed endogenous stages also conformed in their major details with the same developmental stages of other *Sarcocystis* species studied from other snakes and mammalian definitive hosts and from *in vitro* culture. However, they differed from the latter in size and contents of the wall-forming bodies. The observed fertilization process was reminiscent of that described earlier in *S. bovicanis.*

Sarcocystis muriviperae Matuschka, Heydorn, Mehlhorn, Abd-El-Al, Diesing et Bichler 1987, has been described from *Vipera palaestinae* from Israel. Of the several rodent species fed on its sporocysts, only laboratory white mice have proven to be suitable intermediate hosts (Matuschka et al. 1987). Sporocysts of *Sarcocystis* obtained from an additional five species of snakes from Israel induced sarcocyst infection in white mice, with primary walls identical to that reported in *S. muriviperae*, and similarly failed to develop in other rodents. *Sarcocystis* spp. from all these snakes were considered to be conspecific with those recovered from *V. palaestinae*. Sarcocysts with similar primary walls were found in wild house mice (*Mus musculus*). These developed to *S. muriviperae* when fed to *V. palaestinae* (Finkelman and Paperna, unpublished). The present communication provides an account of the fine structure of the entire endogenous development, via gamogony and oogony stages, of *S. muriviperae* in experimentally infected *V. palaestinae* (viper) and *Coluber jugularis* (black snake).

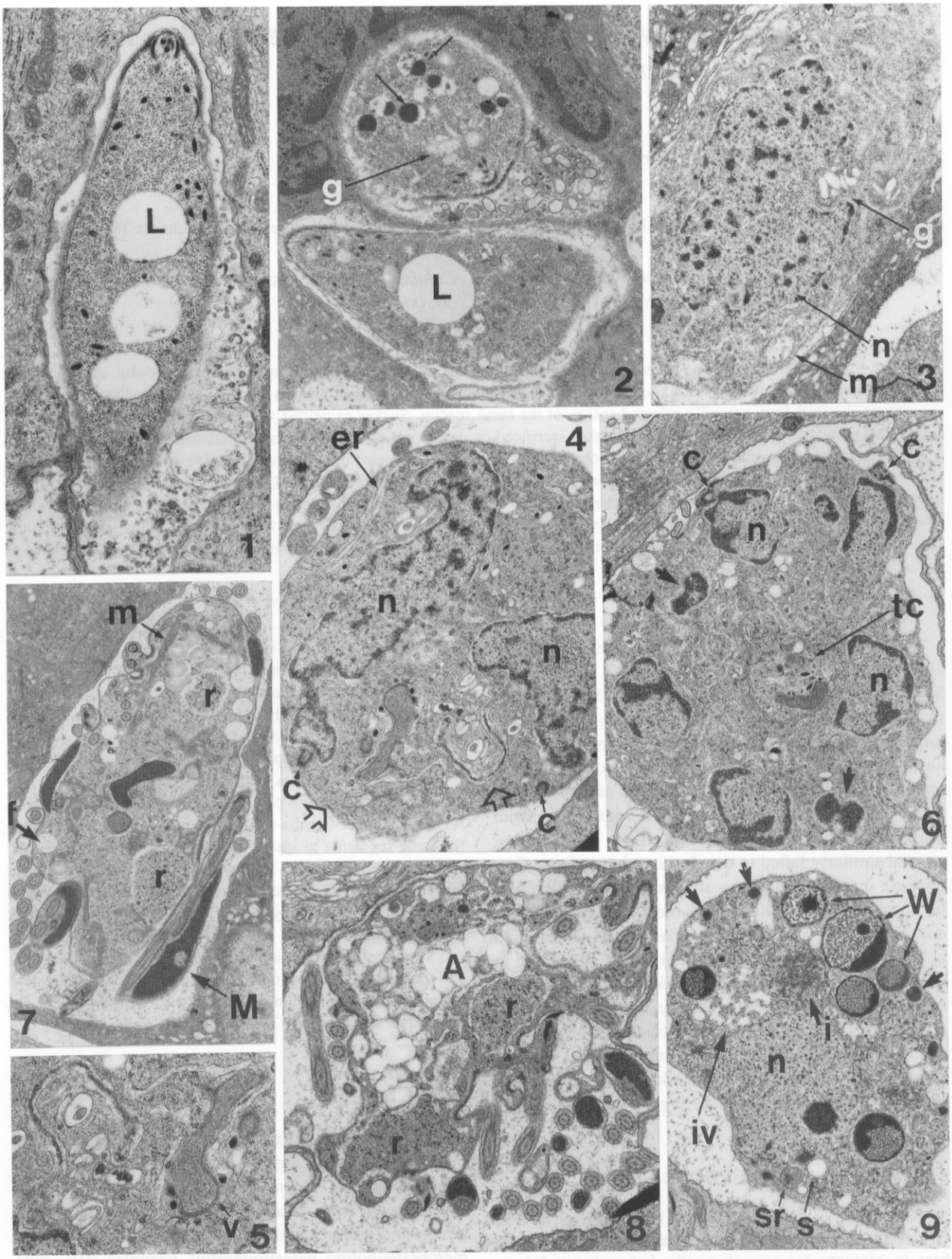
MATERIALS AND METHODS

Young, wild-caught snakes, which were too small to feed on rodents, were used for experimental infection. They were kept in captivity for several months before being fed on infection-free new born mice. Freedom from infection was further confirmed by standard fecal examination by floatation on concentrated sugar. Vipers were infected with sarcocysts from mice previously fed on sporocysts from wild-caught naturally infected vipers, and similarly, black snakes were infected with sarcocysts from mice fed on sporocysts obtained from naturally infected black snakes. Infected snakes were sacrificed on days 4, 8 and 10 post-infection (p.i.).

For electron microscopy, the intestine was divided into five segments. Tissue samples from each were fixed in 2.5 % glutaraldehyde, buffered to pH 7.4 with 0.1 M cacodylate buffer, for 24 h at 4°C. Giemsa-stained smears were prepared from the same segments to identify the sectors of the intestine, in which infection occurred. Following several rinses in the same buffer, the selected segments were postfixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer for 1 h. After rinsing in the same buffer, material was dehydrated in graded alcohols, and after treatment in propylene oxide embedded in Agar 812® medium. Thin sections cut on a Reichert Ultracut with a diamond knife were stained on-grid with uranyl acetate and lead citrate and examined in a Jeol 100CX TEM.

RESULTS

Infection was located in the first and second segments of the anterior gut. The developmental schedule was the same in vipers and black snakes. Gamogony and oogony took place in the mucosal epithelium; young and mature gamonts were located within single membrane-bound parasitophorous vacuoles (PV, Figs. 1, 4–5, 10). By day 4 p.i., infection was comprised only of merozoites in transition to gamonts. Snakes sacrificed on days 7 and 8 p.i. contained mainly mature



Figs. 1–9. *Sarcocystis muriviperae.* **Fig. 1.** Merozoite within parasitophorous vacuole (PV) containing deposits of diverse substances, from viper, × 16,200. **Fig. 2.** Merozoite (bottom) and early macrogamont (top), within individual PVs in the same gut host cell; early macrogamont contains large Golgi complex (g) and wall-forming bodies (WF)-like electron-dense granules (arrows), from black snake, × 12,800.

gamonts. From day 10 p.i. on, infection was comprised entirely of sporulating oocysts, located in the lamina propria.

Pellicle-bound stout and elongate merozoites with a distinct apical complex, microtubules, micronemes and several large lipid vacuoles occurred inside the gut epithelial cells till day 7 p.i. (Figs. 1–2). They were enclosed within an expanded PV which contained heavy deposits of globular and membranous substance. An early stage gamont contained a large Golgi apparatus and osmiophilic globules reminiscent of wall-forming bodies (WF, Fig. 2).

Premature microgamonts were found only in the vipers. In the developing microgamonts, the nucleus expanded and ramified before division (Fig. 3). The two daughter nuclei also expanded prior to division (Fig. 4). These enlarged daughter nuclei were already accompanied by centrioles. The dividing microgamont cytoplasm contained large expanded endoplasmic reticulum (*vide* ER) and a complex of tubuli and vesicles containing dense granular deposits (a Golgi-like apparatus?) adjoining a membrane-bound granular vesicle (Figs. 4–5). The latter tubular complex and the adjunct vesicle could also be seen in later-stage microgamonts containing up to 5 (up to 12 in smears examined by light microscope) small nuclei. Large electron-dense aggregates occurred alongside the nuclei, possibly defunct nuclei (Fig. 6). Cytoplasm of these premature microgamonts revealed extensive ER and cisternae with granular deposit, and contained a variable number of amylopectin granules. It also contained a few micronemes. Cytoplasm of mature microgamonts (Figs. 7–8) contained a similar membrane-bound granular body, variable amounts of amylopectin granules, small food vacuoles with contents identical to that of the PV, many elongated mitochondria, and large and small residual nuclei. Microgametes revealed an elongate dense nucleus, an adjunctive mitochondrion and three evident flagella (Figs. 7–8).

The macrogamont PV contained regularly dispersed electron-dense flakes (Figs. 9–10). Macrogamonts were bound by a two-unit membrane wall (Fig. 11) and contained a large homogeneous nucleus with a conspicuously dense nucleolus, limited amounts of amylopectin granules, two or more lipid vacuoles, a number of variable-size food vacuoles, mitochondria, extensive rough ER, a few smooth-walled cisternae, and an array of expanded involuted vesicles (Figs. 9–10). The apical complex persisted in the macrogamonts to the stage until WF appeared (Fig. 11). The conspicuously large WF contained initially electron dense homogeneous substance, which gradually disaggregated into osmiophilic flocculent particles (Figs. 9–10, 15). Smaller electron-dense bodies were also found both in pre- (Fig. 9) and post-fertilized macrogamonts (Fig. 15). Both the small bodies and the large WF were embedded within the cytoplasm. In late zygotes, fully developed WF seemed to be enclosed within a cytoplasmic halo or enclave (Fig. 15). Aggregates of flocculent electron-dense material in the cytoplasm appeared to be WF primordia (Fig. 9). Canaliculi occurred only in late-stage macrogamonts, during and after fertilization (Figs. 13, 15).

Wall formation began before fertilization (Figs. 12–14): the flaky particles in the PV lumen (Fig. 10) condensed into elongate particles (Fig. 15) which became deposited on the surface of the macrogamonts forming a dense layer, or a veil, with randomly spaced projections or hips (Figs. 12–13, 15). Beneath, the fine wall of the macrogamont had transformed into a three-unit-membrane envelope (Figs. 11–14). Microgamonts entered between the macrogamont's two innermost wall membranes (Fig. 12). The nucleoplasm of the microgamete penetrated into the macrogamont through a special thickened electron-dense pore (micropyle) in the macrogamont's wall, leaving behind the mitochondrion (Fig. 13) and the flagella (Fig. 14). The process of zygote maturation to a walled oocyst coincided with the PV's disintegration, with its contents spilling into the surrounding tissues (Fig. 21). Zygotes contained a variable number of amylopectin granules, some large lipid vacuoles, canaliculi and dense mitochondria. WF seemed to degenerate (Figs. 15–16, 22). The enveloping membrane beneath the veil duplicated (Figs. 17–18) and then separated (Figs. 11, 19), and electron-dense wall material was deposited between the outermost and the second membrane (Fig. 20). The oocyst wall ultimately consolidated into a thick dense boundary (Fig. 22).

Ripe oocysts in the lamina propria already containing young sporocysts were enclosed in a distinctly thick, multilayered wall (as evidenced by its resistance to

← **Fig. 3.** Early microgamont with expanded and ramified nucleus (n); g, Golgi complex, m, mitochondria, from viper, × 16,200. **Fig. 4.** Young microgamonts showing two expanded nuclei (n), accompanied by centrioles (c), with prominent endoplasmic reticulum (er) and a tubular complex adjoining a large granular vesicle (between arrows), from viper, × 9,500. **Fig. 5.** Detail from Fig. 4 to show the tubular complex and the vesicle (v), × 16,900. **Fig. 6.** Premature microgamont with peripherally located nuclei (n) accompanied by centrioles (c), seemingly residues of defunct nuclei (arrows) and the tubular-vesicular complex (tc), from viper, × 11,050. **Fig. 7.** Mature microgamonts with microgametes (M); f, food vacuoles; m, mitochondria; r, nuclei residues; from black snake. × 8,600. **Fig. 8.** Mature microgamont from viper (A, amylopectin granules; r, residual nuclei), × 10,900. **Fig. 9.** Mature macrogamonts from a viper with a nucleus containing a conspicuous nucleolus (n), large WF (W), small electron dense-bodies (arrows), presumed primordium of the WFs (i), an array of convoluted vesicles (iv) and cisternae, empty (s) and with granular contents (sr), × 6,820.

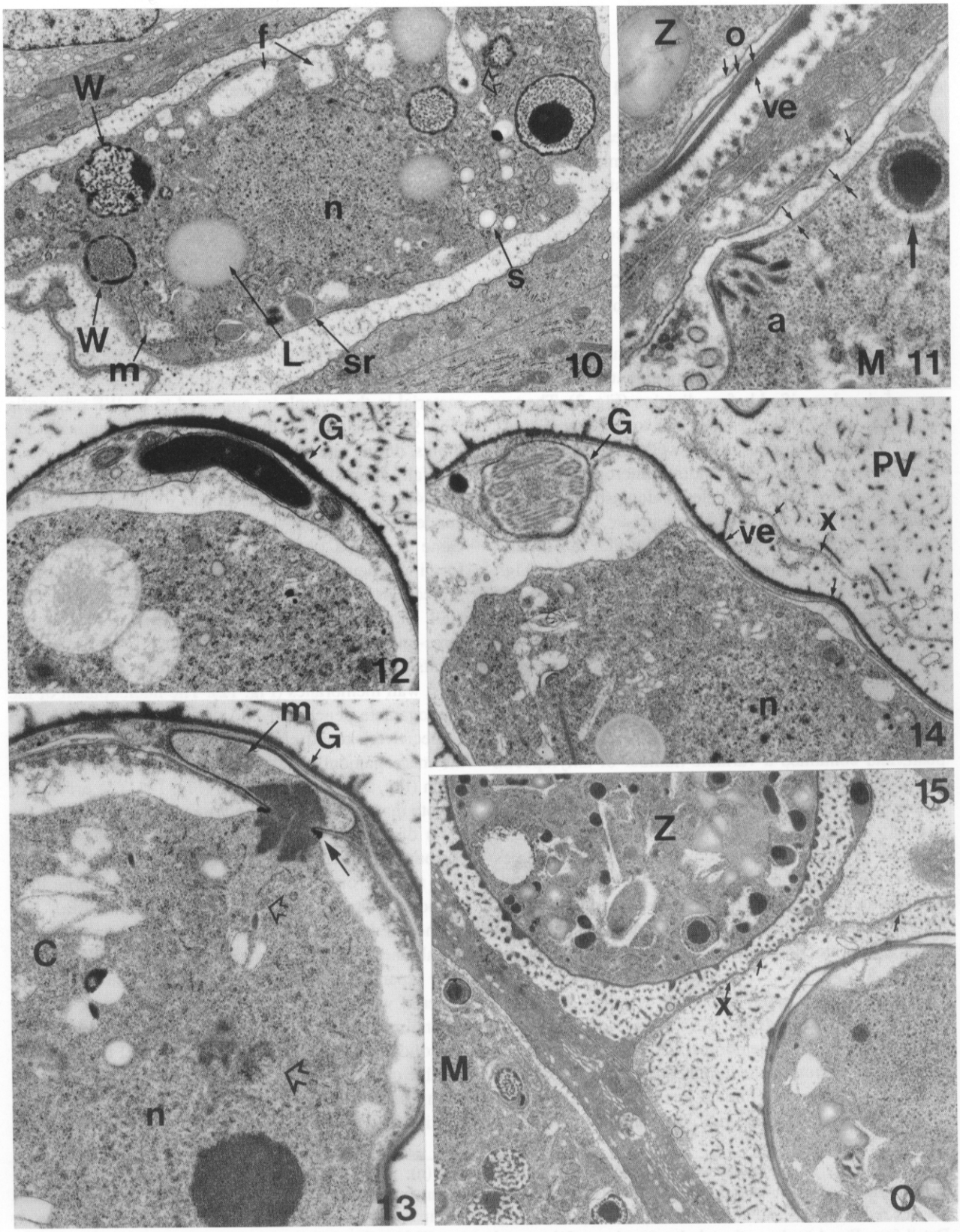


Fig. 10. Mature macrogamont from viper showing nucleus (n), wall-forming bodies (WF) (W), food vacuoles (f), feeding invagination (empty arrow), lipid vacuole (L), and empty (s) and granule filled (sr) cisternae, × 8,100. **Fig. 11.** A macrogamonts (M) with a prominent apical complex (a) showing WF (bold arrow) and bound with a two-membrane wall (small arrows), sharing host cell with a presumed zygote (Z), bound by a veil (ve) two membranes and a plasmalemma (o & small arrows), from the black snake, × 26,900. **Fig. 12.** Microgamont (G) located between the macrogamont's two innermost wall membranes, from viper, × 17,300.

processing and sectioning – Figs. 23–24. The sporocyst wall was hard but thinner. Sporocysts seemed to contain more amylopectin granules than their preceding stages.

DISCUSSION

According to Matuschka et al. (1987) data, the development of *S. muriviperae* in *V. palaestinae* lasted somewhat longer than that seen in our experiments, in the former sporulation was only observed in 10 % of the oocysts on day 14 p.i. and complete sporulation was only observed in snakes sacrificed on day 19 p.i. Other notable variations between our and Matuschka et al.'s (1987) data were differences in the location of the infection (anterior vs. posterior gut), and some discrepancies in the range of oocyst dimensions, to be discussed elsewhere (Finkelman and Paperna, unpublished). In spite of these differences, it is unlikely that the presently studied species, which demonstrated a remarkably low host specificity, differs from that described by Matuschka et al. (1987) from the same hosts and geographical region.

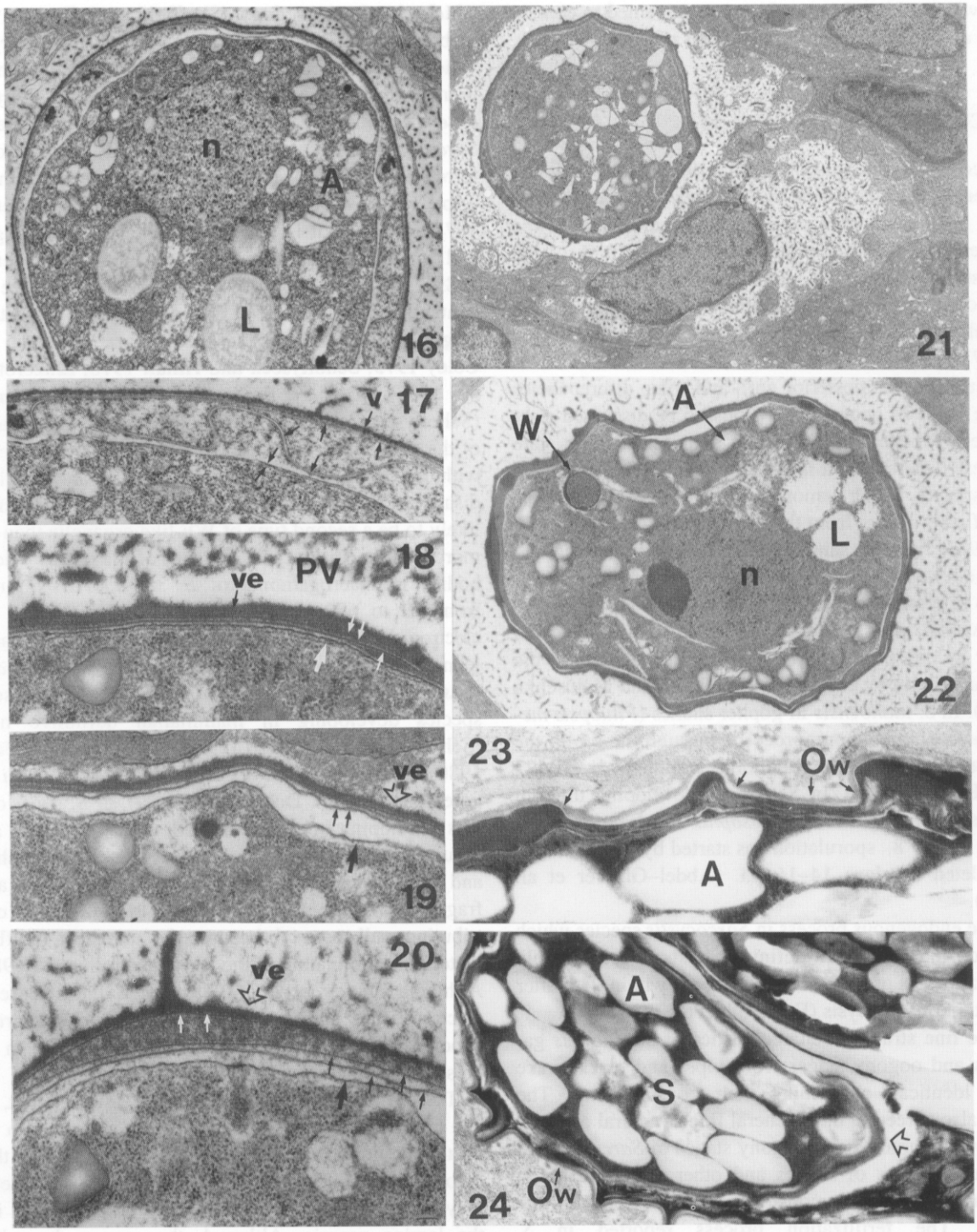
The time taken by other species of *Sarcocystis* to develop in reptilian definitive hosts also varies: in *Elaphe longissima*, young oocysts of *S. clethrionomyelaphis* occur by day 6 p.i. and fully sporulated oocysts in the lamina propria by day 10 p.i. (Mehlhorn and Matuschka 1986). In *S. idahoensis* in gopher snakes (*Pituopsis melanoleucus*), gamogony proceeds through the 5th to the 9th day p.i., whereas sporulation is completed by day 23 p.i. (Bledsoe 1980). In *S. gongyli* developing through skinks to the snake *Spalerosophis diadema*, zygotes occur by day 8, sporulation has started by day 10 and is completed by days 14–16 p.i. (Abdel-Ghaffer et al. 1990).

The endogenous process in *Sarcocystis* species which develop in mammalian definitive hosts is considerably faster: oocysts of *S. muris* in cats occur within 24 h after ingestion of sarcocysts (Entzeroth et al. 1985).

The fine structural characteristics of *Sarcocystis* gamonts and oogony stages from viper and black snake were identical, confirming their conspecificity. The latter also agreed in their general fine structural organization with gamonts and oogony stages of *Sarcocystis* in different species of snakes and other definitive hosts, although they did demonstrate several evident differences. The fertilization process reported in *S. muriviperae* is similar to that of *S. cruzi* (= *S. bovicanis*) which develops in dogs (Sheffield and Fayer 1980, Dubey et al. 1989). Unlike most species of *Sarcocystis* which are enclosed in a PV bound by a multiple-membrane wall (Entzeroth et al. 1985), the PV of *S. muriviperae* was bound by a single membrane. Most speeies of *Sarcocystis* possess a single-type of WF, often also referred to as dense granules or bodies reminiscent of Type 1 WF of eimeriid coccidia (Scholtyseck et al. 1971), although it is uncertain if they are homologous and functionally identical to the latter (Dubey et al. 1989). The single-type WF-like bodies differ in size and contents among species of *Sarcocystis* (compared to *S. singaporensis, S. fusiformis,* or *S. muris* – Zaman and Coley 1975, Scholtyseck and Hillali 1978, Entzeroth et al. 1985), as they do among species of *Eimeria* (Scholtyseck et al. 1966). Even less certain is the nature of the small electron-dense bodies which occur together with the large bodies in macrogamonts and similarly disappear in the differentiating oocyst. They are more likely to be a second type of WF-like organelle than primordia of the larger type. The size of the electron dense bodies in macrogamonts of *S. singaporensis* ranges from 90 to 650 μm (Zaman and Coley 1975). The electron-dense flakes seen here in the PV of the macrogamonts and oocysts are absent from PV of *S. singaporensis* in pythons (Zaman and Coley 1975) and some species developing in mammalian definitive hosts (Scholtyseck and Hillali 1978, Sheffield and Fayer 1980), whereas they do occur in the PV of *S. cruzi* zygotes (in Fig. 51A, Dubey et al. 1989), of *S. muris* oocysts developing in cats (Entzeroth et al. 1985) and also around oocysts of *S. clethrionomyelaphis* (Mehlhorn and Matuschka 1986). It has been suggested that after fragmentation the WF-like bodies, in addition to contributing wall material, become the source of the flaky material accumulating in the PV (Entzeroth et al. 1985). Although Dubey et al. (1989) noted the release of electron-dense particles in *S. cruzi* via a characteristic exocytosis pore, such a pore was not revealed in the presently studied macrogamonts.

Toward the later part of oogonous development, the PV borders collapsed and the flakes spread into the host-cell cytoplasm. The way they spread suggests their cytolytic effect. These changes in host-cell consistency may mark the onset of the oocyst's displacement into the lamina propria. Although the process by which

← **Fig. 13.** Onset of the fertilization process: the microgamont's nucleoplasm penetrates into the macrogamont's cytoplasm via a special micropyle (arrow), leaving behind the microgamont's mitochondrion (m); empty arrows point to textural changes noticed in the macrogamont cytoplasm, possibly in relation to this process (C, canaliculi), from viper, × 22,600. **Fig. 14.** End of fertilization, flagella remain inside the microgamont's residues (G) among the macrogamont's wall layers (n, nucleus, x, degrading border between two adjacent parasitophorous vacuoles (PV), ve, veil), from viper, × 13,000. **Fig. 15.** Three adjoining parasitophorous vacuoles (x, common PV wall) in the same host cell containing a macrogamont prior wall formation (M), presumably a zygote stage (Z), enclosed in a veil, containing both large WF and small electron-dense granules (WF-like) and young walled oocyst (O), from black snake, × 9,200.



Figs. 16–24. *Sarcocystis muriviperae.* **Fig. 16.** Zygote or young oocyst with a single nucleus (n), large lipid vacuoles (L) and consumed amylopectin granules (A); WF absent. Wall envelopes separate from plasmalemma-bound cytoplasm, from viper, × 9,900. **Fig. 17.** Detailed view of the veil (ve) and the wall membranes (arrows) of the zygote shown in Fig. 16, × 15,000. **Figs. 18–20.** Stages in oocyst wall formation from black snake: veil (ve) deposition and duplication of membranes (arrows) (18, × 29,300); separation of the wall membranes from the plasmalemma and veil (19, × 16,000); deposition of wall material between the two outermost membranes (20, × 19,200). **Fig. 21.** Disintegration of the PV toward the completion of oocyst maturation within the gut epithelial host cell in black snake, × 5,400. **Fig. 22.** Oocyst with a consolidated wall, nucleus with prominent nucleolus (n), residual WF (W), lipid vacuole (L) and few amylopectin granules (a), in black snake, × 8,900. **Figs. 23–24.** Hardened wall of ripe oocyst (Ow) containing sporocysts (S) in the lamina propria of a black snake, A, amylopectin granules, open arrow, sporocyst wall (× 32,000 and × 10,900, respectively).

oocysts reach the lamina propria remains unknown, it seems that the migration takes place after the collapse of the epithelial host cell. Transfer mediated by another host cell, lymphocyte, or epithelial crypt cell has been described for poultry coccidia (Lawn and Rose 1982, Pasternak and Fernando 1984). Recently examined histological sections of gecko gut infested with *Schellackia* suggest mediation of macrophages in the migration of oocysts from the epithelium to the lamina propria (Paperna and Finkelman unpublished). In the presently studied infections in snakes, migration of oocysts to the lamina propria seems to occur only after sporocysts' differentiation. The young *S. muriviperae* zygote in the micrograph shown by Matuschka et al. (1987, Fig. 5) seems, however, to already be located in the lamina propria. In other species of *Sarcocystis*, migration may take place prior to sporogony (Entzeroth et al. 1985, Dubey et al. 1989). Wall formation follows the general scheme seen in *Sarcocystis* of mammalian definitive hosts, although there are some differences: we were able to detect only four membranes, as compared to the five reported by Vetterling et al. (1973) in *in vitro*-cultured *Sarcocystis* (but the same number as reported for *S. muris* – Entzeroth et al. 1985). All described oocysts from reptilian definitive hosts (*S. clethrionomyelaphis* – Mehlhorn and Matuschka, 1986, *S. muriviperae* – Matuschka et al. 1987* and *S. gongyli* – Abdel-Ghaffar et al. 1990), non- sporulated during the process of wall formation and demonstrated a rather thick deposited layer of electron-dense material between the outermost and second wall envelopes. In mammalian definitive hosts, the formed wall was less conspicuous (Entzeroth et al. 1985, Dubey et al. 1989). In sharp contrast to the fragile oocyst wall enclosing sporocysts when discharged in the faeces, the wall of the sporulated oocysts from the gut's lamina propria as seen in our electron micrographs was even thicker than the underlying sporocyst wall. This suggests that the oocyst wall undergoes degradation during the process by which it is released into the gut lumen and defecated. Formation of a thick oocyst wall prior to its eventual degradation into a fragile thin envelope when oocysts sporulate, has been described in piscine coccidia (Paperna and Landsberg 1985).

*The electron micrograph of the oocyst-sporocyst wall of *S. muriviperae* presented by Matuschka et al., (1987, fig. 6) seems however, to be the same photomicrograph presented earlier for *S. clethronomyelaphis*. In the latter publication, oocyst wall components are identified as residues of the host cell.

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