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Demonstration that *Sarcocystis montanaensis* has a Speckled Kingsnake–Prairie Vole Life Cycle

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ABSTRACT: Sporulated oocysts and free sporocysts of a *Sarcocystis* species were obtained from the feces of a naturally infected speckled kingsnake (*Lampropeltis getula holbrooki*) collected in northwestern Arkansas. Twenty sporocysts were 11.7 × 8.9 μm. Sporocysts lacked a Stieda body, had a granular sporocyst residuum, and contained 4 sporozoites. Sporocysts were inoculated orally into laboratory mice (*Mus musculus*), white-footed mice (*Peromyscus leucopus*), and prairie voles (*Microtus ochrogaster*). Sarcocysts were found only in prairie voles. Precystic stages occurred in the liver. Severe gross and microscopic lesions were observed in the livers of voles examined 7 and 8 days postinoculation (PI) of 5,000 sporocysts. Sarcocysts were present in the tongues of voles examined 117 days PI. Sarcocysts were thin-walled, divided into compartments by septa, and had osmiophilic, electron-dense, knoblike projections on the primary cyst wall. Metrocytes divided by endodyogeny and were present both centrally within groups of compartmentalized bradyzoites and at the periphery of the sarcocysts. Sarcocysts of the parasite examined in this study were similar to those of *Sarcocystis montanaensis* Dubey, 1983, and a *Sarcocystis* species Lindsay, Upton, Blagburn, Toivio-Kinnucan, McAllister, and Trauth, 1991, with a southern copperhead–prairie vole life cycle. It was concluded that the parasite was *S. montanaensis* and that it may use several species of snakes as definitive hosts.

KEY WORDS: *Sarcocystis montanaensis,* sarcocyst, prairie vole, *Microtus ochrogaster,* speckled kingsnake, *Lampropeltis getula holbrooki,* transmission, life cycle, pathogenesis, ultrastructure.

Little is known about the life cycles and pathogenicity of *Sarcocystis* species utilizing snakes as definitive hosts. Dubey et al. (1989) listed 10 species of *Sarcocystis* that had snakes as definitive hosts; 9 had rodent intermediate hosts and 1 had a lizard intermediate host. The present study was undertaken because we are interested in defining the host range and pathogenicity of snake-transmitted *Sarcocystis* species that occur in the United States. We herein report intermediate host transmission studies and sarcocyst ultrastructure of a *Sarcocystis* species isolated from a speckled kingsnake. The results indicate that this parasite is *Sarcocystis montanaensis* Dubey, 1983, and that at least 2 species of snakes are definitive hosts for the parasite.

Materials and Methods

Source and preparation of inocula

Feces containing sporulated oocysts and free sporocysts were collected from a naturally infected female speckled kingsnake (*Lampropeltis getula holbrooki*) (Arkansas State University Museum of Zoology [ASUMZ 13094], State University, Arkansas) collected in mid-May 1989 from Benton County, Arkansas (36°18′N, 94°33′W). Feces were placed in 2.5% (w/v) potassium dichromate solution for 8 days before they were used in transmission studies. Feces were washed free of potassium dichromate solution by centrifugation in phosphate-buffered saline (pH 7.2) (PBS), sporocysts were counted in a hemacytometer, and stored at 4°C in PBS for 6 days prior to use for inoculations into experimental intermediate hosts.

Inoculation and examination of rodents

Two experiments were conducted. In experiment 1, sporocysts were inoculated orally into 2 ICR laboratory mice (*Mus musculus*) (2,000 or 3,500 sporocysts/mouse), 2 white-footed mice (*Peromyscus leucopus*) (600 or 1,200 sporocysts/white-footed mouse), and 2 prairie voles (*Microtus ochrogaster*) (1,200 or 2,400 sporocysts/vole). All experimental hosts were obtained from commercial sources or were from laboratory colonies maintained at Kansas State University. The laboratory mice were killed 115 days postinoculation (PI), the white-footed mice were killed 110 days PI, and the voles were killed 117 days PI and examined at necropsy. Squash preparations were made of the brain, tongue, diaphragm, and heart of each animal and examined with Nomarski interference-contrast microscopy for sarcocysts. Portions of tissues were also fixed in 10% (v/v) neutral buffered formalin solution for his-

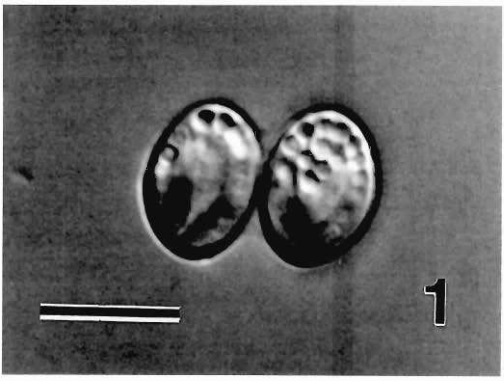


Figure 1. Nomarski interference-contrast photomicrograph of an oocyst of *Sarcocystis montanaensis* from the feces of a naturally infected speckled kingsnake. Bar = 10 μm.

tologic examination. Portions of tissues positive for sarcocysts were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for transmission electron microscopy (TEM).

In experiment 2, 2 voles were inoculated orally with 12,000 sporocysts and killed 3 or 5 days PI, and 3 voles were inoculated orally with 5,000 sporocysts and killed 7, 8, or 15 days PI. Portions of the lungs, liver, spleen, pancreas, kidneys, and adrenal glands were removed from the voles and processed for histologic examination to detect precystic stages. Portions of the liver from each vole were also fixed and processed for TEM. Impression smears were made from each liver, air dried, and fixed in 100% methanol.

Histologic processing and transmission electron microscopy

Tissues fixed in 10% neutral buffered formalin were embedded in paraffin, sectioned at 8 μm, and duplicate sections were stained with Giemsa or hematoxylin and eosin. The impression smears were stained with Diff-Quick® blood stain (Giemsa type) (Scientific Products, McGraw Park, Illinois).

Tissues in glutaraldehyde solution were postfixed in 1% osmium tetroxide, dehydrated in ethanols, embedded in Spurr's plastic, sectioned, and stained with uranyl acetate and lead citrate. Thin sections were examined in a Philips 301 transmission electron microscope operating at 60 kV.

Measurements of developmental stages and sarcocyst structures are expressed in micrometers.

Comparison to *Sarcocystis montanaensis* Dubey, 1983, and *Sarcocystis* species (Lindsay et al., 1991)

The sarcocysts observed in this study were structurally similar to *S. montanaensis* Dubey, 1983, described from meadow voles (*Microtus pennsylvanicus*) and long-tailed voles (*Microtus longicaudatus*) from Montana, U.S.A. (Dubey, 1983a) and a *Sarcocystis* species that has a southern copperhead (*Agkistrodon contortrix contortrix*)–prairie vole cycle (Lindsay et al., 1991); therefore, we reexamined our original photomicrographs of these parasites.

Results

Description of fecal stages of *Sarcocystis* species from *Lampropeltis getula holbrooki*

Fully sporulated oocysts and free sporocysts were present in the feces of the speckled kingsnake (Fig. 1). Twenty sporocysts were 11.2–12.6 × 8.6–9.2 (mean, 11.7 × 8.9). The shape index was 1.2–1.4 (mean, 1.3). The sporocyst wall was about 0.5 thick, lacked a Stieda body, and enclosed 4 sporozoites and a granular sporocyst residuum.

Transmission studies

Sarcocysts were not demonstrated in laboratory mice examined 115 days PI or in white-footed mice examined 110 days PI. Sarcocysts were observed in impression smears of the tongues from both voles examined 117 days PI. Grossly visible sarcocysts were not observed.

Lesions and developmental stages seen in voles

Tissue sections from laboratory mice and white-footed mice in experiment 1 were not examined. No lesions were seen in the tissues from voles in experiment 1 (both examined 117 days PI).

In experiment 2, no gross or microscopic lesions were seen in the voles examined 3 or 15 days PI. Gross lesions consisting of multiple 1–2-mm areas of white discoloration were observed on the serosal and cut surfaces of the livers of voles examined 7 and 8 days PI. Voles examined 5, 7, and 8 days PI had microscopic lesions in the liver (Fig. 2) but not the other tissues examined. Multifocal small discrete areas of coagulative necrosis of hepatocytes and portal infiltrates of mononuclear cells were observed in the vole examined 5 days PI. Lesions were more severe in the voles examined 7 and 8 days PI. They included coalescing areas of coagulative hepatocellular necrosis with moderate infiltration of the portal regions and occasionally central veins with mononuclear cells.

Precystic developmental stages were identified only in the livers of the voles examined 5, 7, and 8 days PI. Schizonts were few in number and were within hepatocytes. Schizonts were not present directly in lesions but were in areas of the liver that had not undergone necrosis (Fig. 2). A single immature schizont was observed 5 days PI; it was 14 × 11 and had a bilobed nucleus. Mature schizonts were seen 7 and 8 days PI. They were 15–25 × 14–24 (mean, 21.5 ×

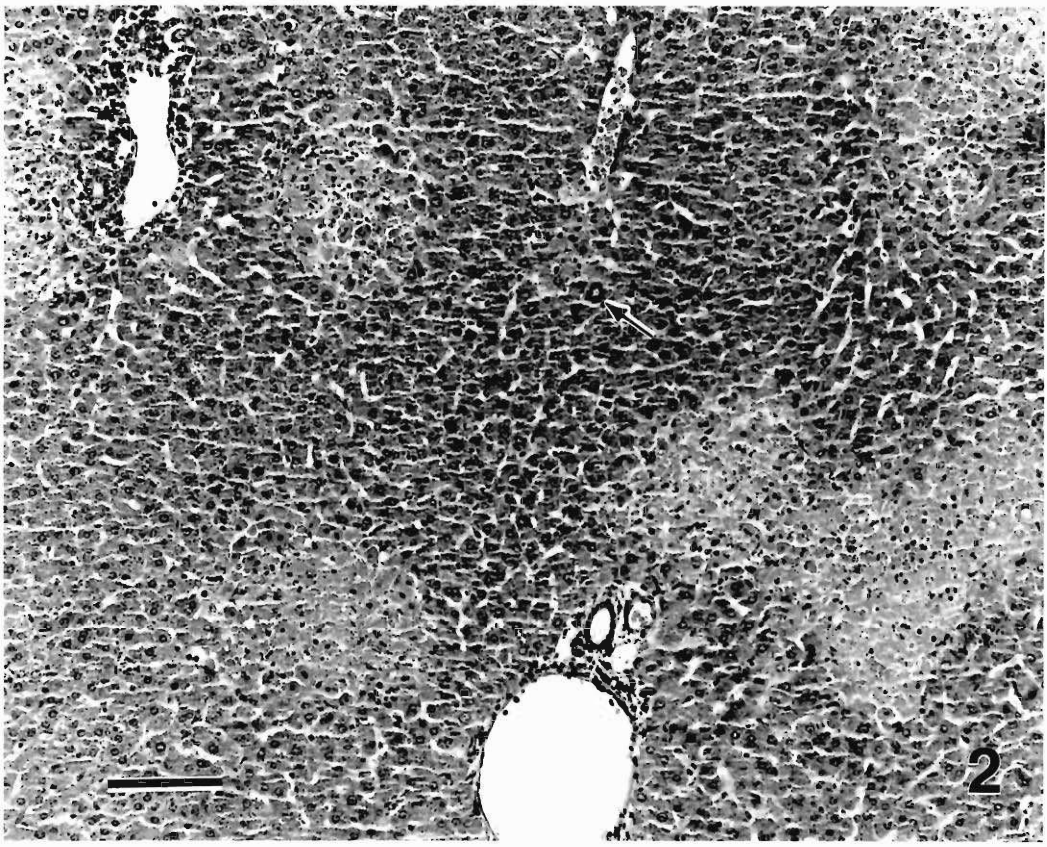


Figure 2. Photomicrograph of lesions caused by *Sarcocystis montanaensis* in the liver of prairie vole orally inoculated 7 days previously with sporocysts isolated from a speckled kingsnake. Hematoxylin and eosin stain. Note the areas of necrosis and a single schizont (arrow). Bar = 100 μm.

18.8; $N = 11$) and contained from 30 to 34 radially arranged merozoites and a residuum. A single 23 × 22 schizont with 33 radially arranged merozoites was seen in the impression smears from the vole examined 7 days PI. Both intracellular and extracellular merozoites were present in impression smears from voles examined 7 and 8 days PI. The merozoites were 7–11 × 2–3 (mean, 8.6 × 2.3; $N = 24$). Merozoites usually occurred singly in monocyte-like cells; one merozoite was observed in a polymorphonuclear neutrophil.

Schizonts were not observed with TEM. A few extracellular merozoites were seen in hepatic capillaries in the vole examined 7 days PI. They were structurally consistent with merozoites of other *Sarcocystis* species.

Sarcocysts were observed in tissue sections from both voles examined 117 days PI. Sarcocysts were thin-walled and measured 45–84 (mean, 67.9; $N = 7$) in diameter. All were seen in the tongues with the exception of a single sarcocyst seen in the diaphragm of 1 vole.

Transmission electron microscopy revealed that the sarcocyst wall could be classified as a type 1 sarcocyst wall using the classification of Dubey et al. (1989). The primary cyst wall was composed of the parasitophorous vacuole (PV) membrane. The PV membrane was ornamented with numerous knoblike structures (Fig. 3). The knoblike structures were 0.1–0.2 and had an electron-dense upper portion and a lower portion that was composed only of the unit membrane of the PV. Because of the plane of section, some knoblike structures of some sarcocysts appeared to blend together to form flattened projections (Fig. 4) or there appeared to be holes in the primary cyst wall (Fig. 5). The entire sarcocyst wall was 0.5–1.1 thick and was composed of the primary cyst wall and the underlying electron-dense

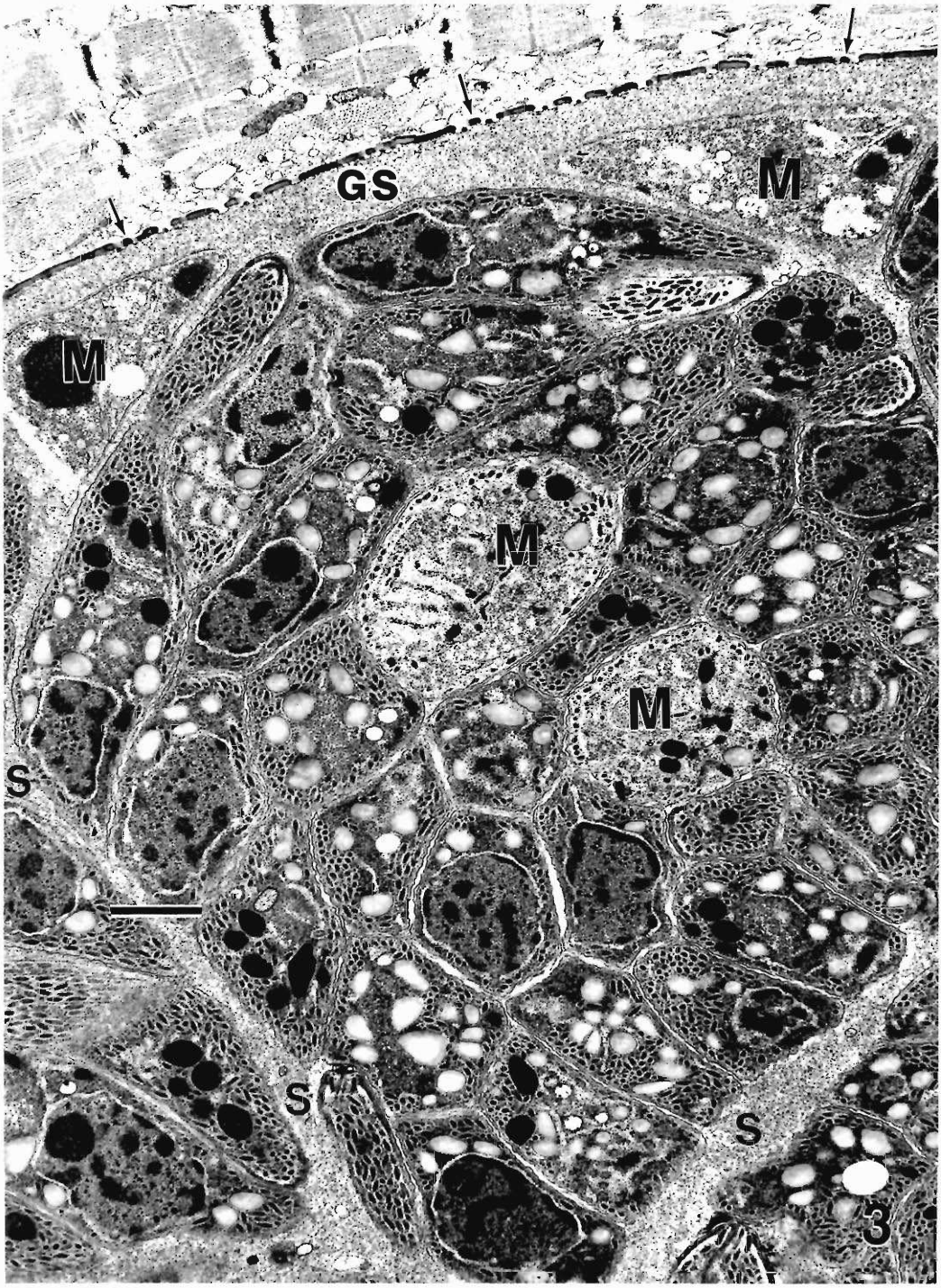
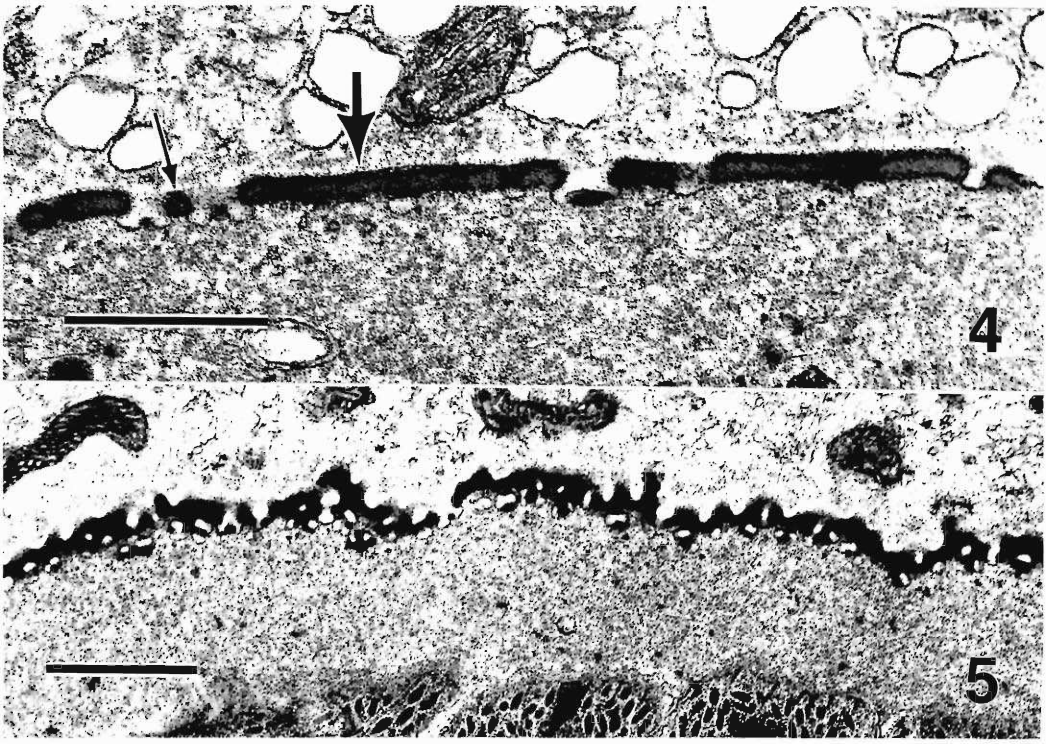


Figure 3. Transmission electron micrograph of a portion of a sarcocyst in the tongue of a prairie vole orally inoculated with sporocysts from a speckled kingsnake. Note the thin primary sarcocyst wall (arrows) that contains numerous knoblike projections, the underlying ground substance (GS), and septa (S) that divide the sarcocyst into compartments. Metrocytes (M) are present both centrally within groups of bradyzoites and at the periphery of the sarcocyst. Bar = 1.0 μm.



Figures 4, 5. Transmission electron micrographs of the sarcocyst wall of *Sarcocystis montanaensis* in prairie voles orally inoculated with sporocysts from a speckled kingsnake. 4. Note how the knoblike projections (small arrow) appear to be fused and give the primary sarcocyst wall a flattened appearance (large arrow) in areas. Bar = 0.5 μm. 5. Portion of the sarcocyst wall that appears to have holes in the primary cyst wall due to the angle of sectioning. Bar = 0.5 μm.

ground substance. The ground substance formed septa that divided the sarcocyst into compartments (Fig. 3). Metrocytes were present both at the periphery of the sarcocyst and centrally within groups of bradyzoites (Fig. 3). Metrocytes divided by endodyogeny. Bradyzoites contained all the organelles typical of this developmental stage and had micronemes that extended to the posterior of the parasite.

Comparison with *Sarcocystis montanaensis* and *Sarcocystis* species

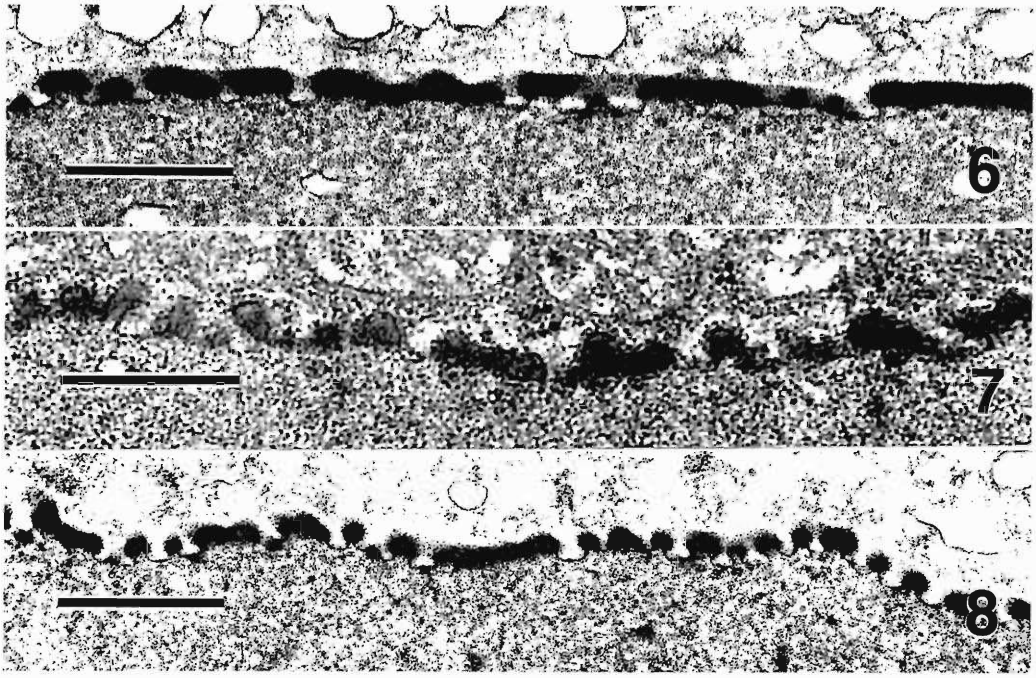
No differences were observed in the structure of the sarcocyst walls of the parasites examined using TEM (Figs. 6–8); therefore, we concluded that all were *S. montanaensis*. We were able to identify metrocytes at the periphery of some *S. montanaensis* sarcocysts in naturally infected meadow voles and *S. montanaensis* sarcocysts in prairie voles inoculated with sporocysts from a southern copperhead, but did not observe centrally located metrocytes in these cases.

Discussion

The hepatic necrosis observed in the voles in this study was severe, appeared to be specific, and associated with the presence of schizonts. Hepatic necrosis was not observed in voles examined 3 and 15 days PI that did not have demonstrable hepatic schizonts. Direct destruction of hepatocytes by parasites did not appear to be the cause of the hepatic necrosis because so few schizonts were observed. The hepatic necrosis may be due to a host reaction to merozoites or to metabolites released from mature schizonts.

The presence of a thin sarcocyst wall in the *S. montanaensis* sarcocysts examined in our study distinguishes it from a thick-walled species of *Sarcocystis* that use snakes as definitive hosts and rodents as intermediate hosts (see Beaver and Maleckar, 1981; Matuschka, 1986; Mehlhorn and Matuschka, 1986; Matuschka et al., 1987; Munday and Mason, 1980).

The sarcocyst wall of *S. idahoensis* Bledsoe, 1980, is thin (<1.0 μm) and may contain villar-



Figures 6–8. Comparison of the sarcocyst walls of *Sarcocystis montanaensis* in voles. 6. Sarcocyst in a prairie vole orally inoculated with sporocysts isolated from a speckled kingsnake. Bar = 0.5 μm. 7. Sarcocyst in a naturally infected meadow vole. Bar = 0.5 μm. 8. Sarcocyst in a prairie vole orally inoculated with sporocysts isolated from a southern copperhead. Bar = 0.5 μm.

like processes (Bledsoe, 1980a; Dubey, 1983b). This species has a gopher snake (*Pituophis catenifer*) (syn. *Pituophis melanoleucus*)–deer mouse (*Peromyscus maniculatus*) life cycle (Bledsoe, 1980a, b). The ultrastructure of *S. idahoensis* sarcocysts has not been examined. The presence of villar-like projections and our inability to infect white-footed mice (*P. leucopus*) demonstrate that *S. montanaensis* and *S. idahoensis* are distinct species.

The ultrastructure of *S. montanaensis* sarcocysts seen in the present study closely resembles *S. crotali* Enzeroth, Chobotar, and Scholtyseck, 1985, that has a Mojave rattlesnake (*Crotalus scutulatus scutulatus*)–laboratory mouse (*M. musculus*) life cycle (Enzeroth et al., 1985). However, electron-dense projections of the *S. crotali* sarcocyst wall also have an electron-dense layer underlying the projections. This, and our inability to infect laboratory mice with sporocysts, indicate that the 2 are separate species.

The structure of the sarcocysts of *S. montanaensis* observed in naturally infected meadow and long-tailed voles and *S. montanaensis* in prairie voles inoculated with sporocysts from southern copperheads or speckled kingsnakes are similar, and the sporocysts isolated from southern copperheads (11.0 × 8.5) and speckled kingsnakes (11.7 × 8.9) are also similar. Minor differences such as sarcocyst size and positioning of metrocytes may be due to sarcocyst age (Dubey et al., 1989; Cawthorn and Speer, 1990). Sporocyst structure usually is not of importance in identifying *Sarcocystis* species (Dubey et al., 1989). The finding of posteriorly located micronemes in bradyzoites in all cases examined is a strong indication that the parasites are all the same species and is a major reason for our conclusion that the parasites are all *S. montanaensis.*

Acknowledgments

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1991 Professor of the Year

Helminthological Society of Washington member Harvy D. Blankespoor, Professor of Biology at Hope College, was named the 1991 Professor of the Year by the Council for Advancement and Support of Education. The award is to recognize undergraduate faculty members for their commitment to teaching, their contribution to students' lives, and their service to the teaching profession. With the award goes a $10,000 cash stipend from the Carnegie Foundation.

(Excerpted from the Chronicle of Higher Education)