Concurrent Presence of *Sarcocystis Neurona* Sporocysts, *Besnoitia Darlingi* Tissue Cysts, and *Sarcocystis Inghami* Sarcocysts in Naturally Infected Opossums (*Didelphis Virginiana*)

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Concurrent presence of *Sarcocystis neurona* sporocysts, *Besnoitia darlingi* tissue cysts, and *Sarcocystis inghami* sarcocysts in naturally infected opossums (*Didelphis virginiana*)

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Abstract. Opossums (*Didelphis virginiana*) are exposed to a wide range of coccidia through feeding on a variety of foods, including, but not limited to, carrion, insects, and nestling birds. Abundant *D. virginiana* populations in urban and suburban areas can be important reservoirs of parasitic infection because of their profuse and prolonged excretion of the sporocysts of several species of *Sarcocystis,* their omnivorous diet, and their relatively long life span. This report describes 2 adult female opossums found to be simultaneously infected with the tissue cysts of *Besnoitia darlingi*, sarcocysts of *Sarcocystis inghami*, as well as with the intestinal sporocysts of *S. neurona.* Cysts typical of *B. darlingi* based on gross, histological, and ultrastructural characteristics were disseminated throughout the visceral organs, musculature, ears, and skin. The *S. neurona* and *B. darlingi* infections were confirmed by comparative sequence analysis of polymerase chain reaction–amplified diagnostic genetic loci. Sarcocysts of *S. inghami* are also described. Such examples of multiple parasitic infections show that concurrent infections occur naturally. The propensity for species to coexist should be considered in the differential diagnosis of tissue cyst–forming coccidian protozoa and may have important epidemiological and evolutionary implications.

The role of the North American Virginia opossums (*Didelphis virginiana*) in the epidemiology of coccidian parasites is not fully understood. Heteroxenous apicomplexans of the family Sarcocystidae generally require a herbivorous or omnivorous intermediate host and a carnivorous definitive host.[14] How extensive a role opossums play in disseminating specific coccidians has yet to be fully defined. Because opossums consume a wide variety of foods, they would appear vulnerable to ingesting tissue cysts of many coccidian species. In addition, opossums ingesting sporocysts excreted by other carnivores risk becoming intermediate hosts of other coccidian species.[15] Such is the case for *Besnoitia darlingi*, for which cats serve as definitive hosts and shed oocysts that are ingested by opossums, which serve as intermediate hosts. Because opossums have few marsupial relatives in the New World, and none throughout much of their North American range, they represent an interesting model to understand the capacity of phylogenetically unrelated hosts to exchange parasites as a consequence of shared feeding behavior. Although most experimental studies to elucidate the life history of parasites are restricted to inoculation with a single species, wildlife often simultaneously harbor multiple parasitic infections. *Besnoitia darlingi* infections are prevalent in opossums (*D. virginiana*) of the United States.[5] Also, opossums are of interest because they serve as intermediate hosts for *Sarcocystis greineri* sarcocysts[1,3] and definitive hosts for several species of *Sarcocystis,* including *S. neurona, S. falcatula, S. speeri, S. lindsayi,* and other unnamed species.[4,6,13]

Routine necropsy of opossums (n = 137) collected from central Michigan between January and December, 2002, identified 3 protozoal infections: intestinal sporocysts of *S. neurona,* tissue cysts of *B. darlingi*, and sarcocysts of *S. inghami.* Using histopathological, ultrastructural, and genetic diagnostic methods, 2 cases of triple infection of *B. darlingi* cysts, *S. neurona* sporocysts, and *S. inghami* sarcocysts are reported in adult female opossums from Michigan.

Opossums were collected and examined using a standardized protocol. They were either killed by automobiles on the roadways or livetrapped by permit and humanely killed. Data on age, sex, and location of collection were gathered, and each animal was assigned an identification number. At necropsy, numerous macroscopic, white, firm, glistening cysts measuring up to 1 mm in diameter were observed throughout the abdominal and skeletal musculature (Fig. 1A). In addition, the ears, salivary glands, skin, pleura of lungs, serosa of gastrointestinal tract, heart, tongue, kidney, and capsule of liver had these cystic structures. Tissues of affected opossums containing cysts suggestive of *Besnoitia* spp. were fixed in 10% buffered formalin and routinely processed for histopathological examination. These cysts consisted of a single severely hypertrophied cell surrounded by a thick outer hyaline wall (4–20 μm) and a scant rim of eosinophilic host cell cytoplasm with elongated host cell nuclei surrounding a noncompartmentalized area containing hundreds of slender bradyzoites. Most cysts were surrounded by eosinophilic collagen without leukocytes or other inflammatory cells (Fig. 1B). Occasionally, cysts in lung sections were partially or totally collapsed and calcified. They contained few visible bradyzoites and were surrounded by a dense cellular inflammatory reaction. Based on these mor-

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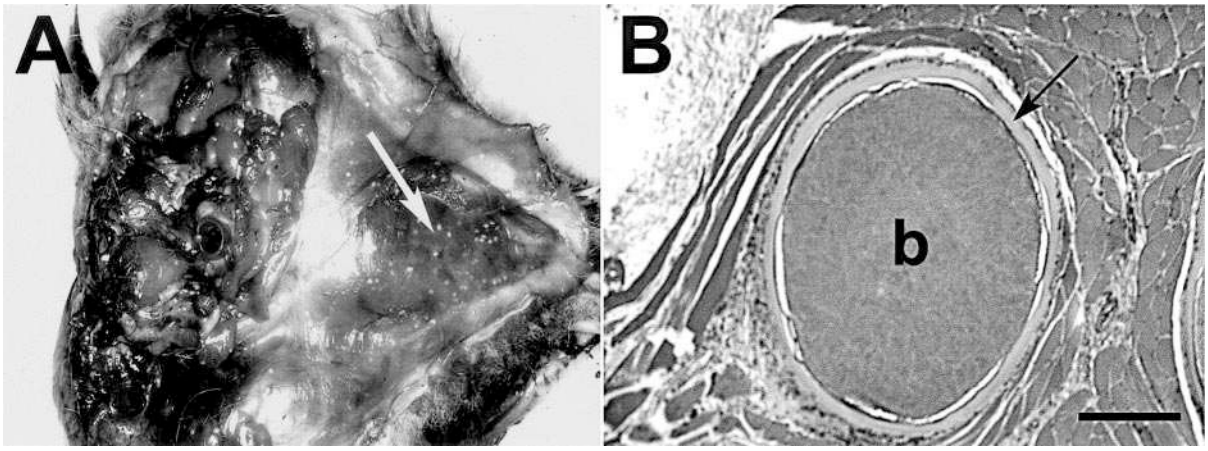

Figure 1. **A,** carcass of a female opossum (*Didelphis virginiana*) naturally infected with *Besnoitia darlingi*. Note the presence of numerous white tiny cysts of *B. darlingi* distributed throughout the intermandibular musculature (arrow). **B,** *Besnoitia darlingi* cyst in a longitudinal section from tongue of an opossum (*D. virginiana*). Note the presence of a thick capsule (arrow) surrounding the cyst that contains a myriad of bradyzoites (b). HE stain. Bar = 100 μm.

phological features, the cysts were tentatively classified as *B. darlingi*, and histological sections of cysts from the liver and heart of these opossums were deposited in the U.S. National Parasite collection, Beltsville, Maryland, as USNPC accession no. 092443.

Samples from the salivary gland that had *B. darlingi* cysts were postfixed in 1% osmium tetraoxide–1.5% potassium ferrocyanide and routinely processed for transmission electron microscopy (TEM). Examination of ultrastructural features showed a thick layer of host connective tissue surrounding the tissue cyst (Fig. 2A). The host cell cytoplasm contained occasional mitochondria, a few Golgi complexes, and abundant rough endoplasmic reticulum. Bradyzoites measured 7.8–11 μm (9 ± 0.92) in length (L) by 1.17–2 μm (1.5 ± 0.24) in width (W). These bradyzoites contained numerous randomly arranged micronemes located mainly posterior to the nucleus, and a few were observed toward the conoidal end. The nucleus was located centrally or toward the posterior half of the bradyzoite (Fig. 2B). Most of the enigmatic bodies were located toward the posterior end of the bradyzoite. Attributes of the cysts and bradyzoites of the species of *Besnoitia* corresponded to those previously reported for *B. darlingi* on gross, histological, and TEM examination.[5,11,15]

Infections with various species of *Besnoitia* generally induce little inflammation in their intermediate hosts.[11] Their location within the privileged site of a hypertrophied host cell may account for this lack of leukocyte response. Other related species within this family Sarcocystidae that also form large intracellular cysts containing bradyzoites, including *Sarcocystis* spp., *Toxoplasma gondii*, *Hammondia* spp., and *Frenkelia* spp., lack the thick connective tissue capsule that surrounds *Besnoitia* cysts.[9]

Sporocysts of *Sarcocystis* spp. were isolated from the small intestine of opossums and purified as described.[8] The morphology of isolated sporocysts was typed as previously

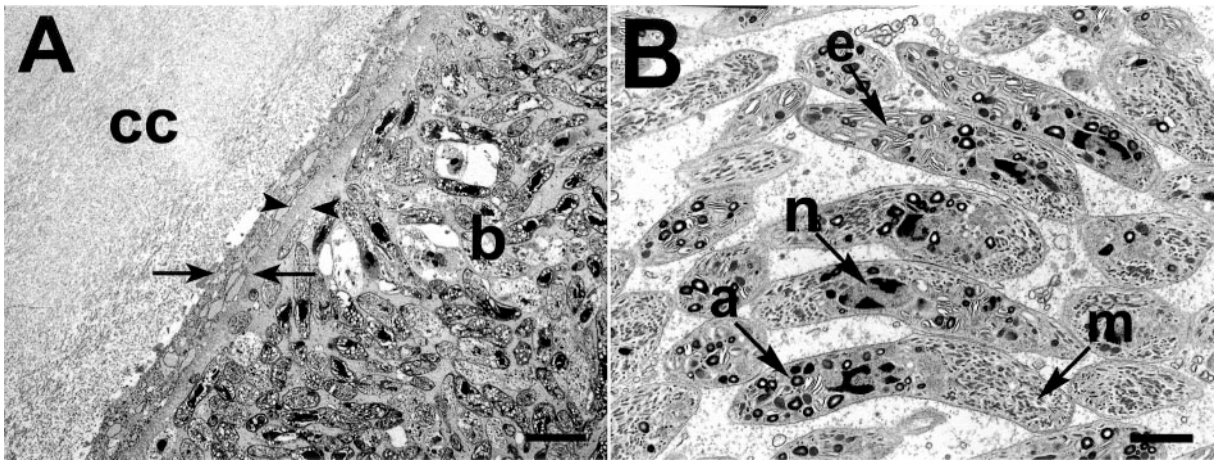

Figure 2. TEM of *Besnoitia darlingi* tissue cyst from salivary gland of a naturally infected opossum. **A,** a thick cyst capsule (cc) surrounding the compressed host cytoplasm (opposing arrows), and the parasitophorous membrane (opposing arrowheads) enclosing bradyzoites (b) that fill the cyst cavity. Lead acetate stain. Bar = 5 μm. **B,** longitudinal section of bradyzoites, showing numerous micronemes (m) posterior to the nucleus (n), amylopectin (a), and enigmatic bodies (e). Lead acetate stain. Bar = 2 μm.

described.[3] At least 25 sporocysts from each infected animal were examined and photographed using a laser scanning microscope[a] with 488-nm laser line. *Sarcocystis* sporocysts were ovoid to round and 9.4–11.5 μm (10.6 ± 0.56) in L by 6.4–8.3 μm (7.23 ± 0.49) in W, with L/W ratio of 1.2-1.5. The dimensions and morphology of the sporocysts isolated from these 2 opossum's intestines in this study are typical of several species of *Sarcocystis* excreted by opossums.[4] Therefore, representative samples of the parts of small intestine of these sporocyst-infected opossums were fixed in 10% buffered formalin and routinely processed for histopathological examination. *Sarcocystis* sporocysts within the small intestine of opossums were confirmed histologically. The majority of sporocysts were found in clusters within the lamina propria of the luminal upper two-thirds of the intestinal villi mainly in the upper half (duodenum and proximal jejunum) of the small intestine. Mild plasmacytic infiltrates (essentially Russell's bodies) were observed throughout the lamina propria of the villi, particularly in areas of sporocyst concentration. Small numbers of eosinophils and lymphocytes were present as deep as the lamina muscularis mucosa. However, no evidence of developing sexual stages of *S. neurona* was seen at this location.

Sections from tongue and other skeletal muscles were scanned for the presence of sarcocysts using a dissecting microscope at 20 and 40× magnifications. Whenever a sarcocyst was detected, the cyst-containing section was processed for light and electron microscopies. All measurements are made from fixed and stained sections mounted on glass slides using light microscopy and a calibrated ocular micrometer. Microscopic fusiform sarcocysts were detected mainly in the tongue of opossums. This site is preferred by many species of *Sarcocystis*, with the notable exception of the macroscopic *S. greineri* reported from skeletal muscles of opossums in Florida.[1,3] Sarcocysts measured up to 700 μm long and 110 μm wide (Fig. 3). The sarcocyst wall was 7 μm thick. Villar protrusions (VP) were approximately 4.9–6.2 μm (5.5 ± 0.46) in L by 0.9–1.54 μm (1.21 ± 0.151) in W. Sarcocysts of 3 species of *Sarcocystis* have been described from skeletal muscles of other opossum species: *S. didelphidis* and *S. garnhami* in the South American opossum (*D. marsupialis*) and *S. marmosae* in *Marmosa murina*.[3] *Sarcocystis inghami* can be readily differentiated from these other species by the characteristic morphology of the VP on the cyst wall, which have a narrow base, are wider in the middle, and are rounded off distally. In addition, the bifurcation of some VP in the primary cysts wall was a unique feature of this species. The prevalence and ultrastructural description of this species of *Sarcocystis* were discussed in a recent report.[7]

Besnoitia darlingi cysts isolated from opossums were gently squashed between 2 microscopic glass slides under sterile conditions. The released bradyzoites were washed twice with sterile 1× phosphate-buffered saline plus 10,000 U ml^{-1} of penicillin, 10,000 μg ml^{-1} of streptomycin, and 1.25 μg ml^{-1} of fungizone by centrifugation[b] at 1,500 × *g* for 5 minutes at 4 C. They were inoculated directly onto 25-cm^2 tissue culture flask with 60–80% confluent monolayer of bovine turbinate (BT) cells[c] and Eagle minimal essential medium with Earle's salt supplemented with 5% fetal calf serum, L-glutamine, and antibiotic–antimycotic mixture.[d] Parasites harvested from the culture flask were directly inoculated onto 12-mm^2 glass coverslips containing a monolayer of BT cells positioned at the bottom of a 24-well tissue culture plate. Coverslips of infected BT cells were fixed in 10% buffered formalin for 30 minutes, placed in 100% methanol for 10 minutes at room temperature, and stained with Giemsa. Stained coverslips of infected BT cells were attached to glass microscopic slides with Permount[e] and examined by light microscopy[f] to observe the development of *B. darlingi* infection from 1 to 30 days after infections. Intracellular *B. darlingi* tachyzoites were first visible on the second day postinoculation (DPI). Organisms were successfully passaged into BT cells and were cryopreserved in liquid nitrogen for future use. In formalin-methanol–fixed smears, culture-derived intracellular parasites were located in a vacuole, usually near the host cell nucleus, and measured 4.5–6.5 μm (5.6 ± 0.63) in L by 1.7–2.8 μm (2.1 ± 0.26) in W at 4 DPI.

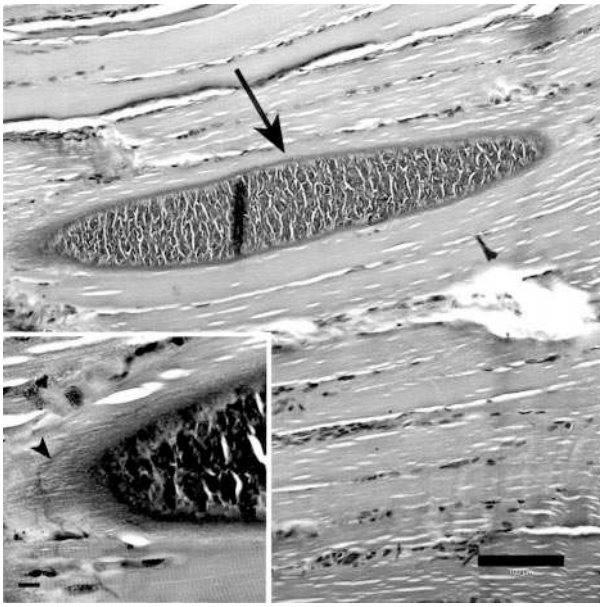

Figure 3. Differential interference contrast photomicrograph of a sarcocyst (arrow) of *Sarcocystis inghami* in a longitudinal section from the tongue of a naturally infected opossum. Inset is a close-up of the sarcocyst showing hair-like VP (arrowhead) at the posterior end. HE stain. Sarcocyst, Bar = 100 μm; inset, Bar = 10 μm.

For cultivation of *Sarcocystis* spp. sporocysts, monolayers were infected with sporozoites obtained from KBr-purified sporocysts of *Sarcocystis* spp. as described.[8] Sporozoites were viable and maintained their infectivity after purification. Successful cultivation of the *S. neurona* isolates MIO11 and MIO12 from the 2 opossums in tissue culture cells was achieved.

Sporocysts isolated from the 2 triple-infected opossums were identified as *S. neurona* based on the polymerase chain reaction analysis with the diagnostic primers JNB25/JD396 and restriction fragment length polymorphism (RFLP).[16] Deoxyribonucleic acid was extracted from sporocysts using a commercial kit.[g] Analysis of the DNA extracted from *Sarcocystis* spp. sporocysts with JNB25/JD396 primers ampli-

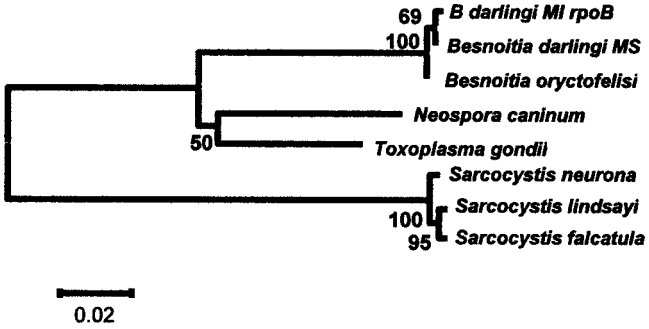

Figure 4. Midpoint rooted Neighbor joining tree of rpoB locus based on 1,000 bootstrap replicates using Kimura 2-parameter distances.

fied a 334-bp product. Subsequent RFLP analysis showed banding patterns characteristic of *S. neurona*, with bands at 180 and 154 bp with *Hin*fI digestion and a single band at 334 bp with *Hin*dIII digestion. For *Besnoitia* isolates, DNA was extracted from parasites derived from tissue cysts of 1 of the 2 naturally infected opossums and from peritoneal exudates of experimentally inoculated mice using a commercial kit.[g] Direct sequencing of a portion of the β subunit of the RNA polymerase (rpoB) gene, using previously described methods, was used to confirm the correspondence of representative *B. darlingi* isolates with exemplars reported from Mississippi.[5] The phylogenetic position of this locus was then estimated in relation to homologues from *S. neurona*, *Neospora caninum*, *Toxoplasma gondii*, and *B. oryctofelisi.* Comparative sequence analysis of the rpoB gene, encoded in the apicomplast, confirmed the identity of these parasite isolates as *B. darlingi* (Fig. 4).

Fisher's exact test was used to test whether infections with *B. darlingi* and with a species of *Sarcocystis* occurred with independent frequency. Of 137 opossums collected over the 2002 calendar year, 15 were infected with *B. darlingi* (10.9%) and 20 with *Sarcocystis* spp. (14.6%). Four opossums were coinfected with more than 1 parasite species: *B. darlingi* and a species of *Sarcocystis;* and 2 of these, both adult females collected during the summer, were triply infected with 3 sporozoan infections: intestinal sporocysts of *S. neurona*, tissue cysts of *B. darlingi*, and sarcocysts of *S. inghami.* Thus, the observed frequency of coinfection (4/137 = 2.9%) did not significantly differ from that expected, assuming independent acquisition and establishment of these infections (10.9% × 14.6% = 1.59%, or between 2 and 3 of 137 opossums) ($P = 0.235$, 2-tailed Fisher's exact test).

Frequent coinfections might indicate that certain opossums experience elevated risk of becoming parasitized with coccidia because of increasing cumulative risk as animals age, because the infections share common exposure routes, because poor immune status renders certain animals particularly vulnerable, or because infection with one parasite increases a host's susceptibility to subsequent heterologus infection. Indeed, variations in host behavior, in particular in exposure-related activities, are believed to be a main determinant of infection risk in wild animals including rodents.[2] Alternatively, especially infrequent coinfections may have suggested distinct exposure routes for each parasite or even possibly some form of competitive exclusion among parasite species. However, the current findings suggest no significant departure between the expected and observed frequencies of coinfection. Only modest power exists, however, to detect such a departure in a sample of this size, given the moderate infection rates in these opossums. For example, a study explicitly designed to address this question would reject their statistical independence at $P < 0.04$ if these infection frequencies were observed in a sample twice the size as the one available here.

Opossums are generalist predators and feed on a variety of foods, including, but not limited to, carrion, insects, and nestling birds. In the United States, *D. virginiana* can achieve a population density that averages 0.26/ha (0.02–1.16 per hectare), a home range averaged about 20 ha (4.7–254.0 ha), and a nightly foraging distance of 1.6–2.4 km.[10] Their prolonged and profuse excretion of sporocysts of *Sarcocystis* spp.[12] and their 2- to 3-year life expectancy may elevate the risk they pose as a reservoir for parasites that can be transmitted to wildlife, domestic animals, and humans. Thus, opossums can contribute significantly to environmental sporocysts abundance and may play an important role in the dissemination of a diverse array of parasitic infections.

Interactions between protozoa are poorly understood, despite examples such as the present report on frequent natural coinfection. Analysis of the diversity of their interactions will contribute to the development of ecological models of parasite transmission. Such knowledge will be necessary to better understand in what ways laboratory observations of single experimental infections can be extrapolated to natural host populations.

An integrated approach, combining field and laboratory data on tissue cyst–forming coccidia in wildlife, would provide needed information to promote effective prevention and control measures. As the diversity of coccidia parasitizing New World marsupials becomes more completely known, it will be interesting to use parasite phylogenies as a means to determine whether these parasites are distinct from, or rather members of, coccidia of the canine and feline carnivores that predominate in the New World.

Sources and manufacturers

a. Laser Scanning Microscope (LSM Zeiss 210), Carl Zeiss, Thornwood, NY.
b. CR/CT4.12 centrifuge with a M4 swinging bucket rotor, Forma Scientific, Marietta, OH.
c. BT cells, ATCC no. CRL-1390, American Type Culture Collection, Manassas, VA.
d. Antibiotic–antimycotic mixture, GIBCO, Grand Island, NY.
e. Permount, Fisher Scientific, Fair Lawn, NJ.
f. Reichert-Jung, Pegasus Scientific, Frederick, MD.
g. DNeasy Tissue kit, Qiagen Inc., Valencia, CA.

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Abdominal fat necrosis in a pygmy goat associated with fescue toxicosis

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Abstract. Abdominal fat necrosis was diagnosed in an 11-year-old female pygmy goat with a 10-day history of lethargy, anorexia, and progressive abdominal distension. Gross necropsy findings revealed multiple firm, dark yellow, nodular masses of fat throughout the abdominal cavity, which compressed several abdominal organs including the rumen, small intestine, spiral colon, and gall bladder. Histologically, multiple to coalescing adipocyte necrosis, saponification, and infiltration with variable numbers of macrophages, lymphocytes, and plasma cells was observed. Fat necrosis in this case was attributed to tall fescue toxicity based on the presence of high levels of endophyte (*Neotyphodium coenophialum*)–infected fescue identified in the goat's pasture. This is the first known report of abdominal fat necrosis in a goat and demonstrates the fat necrosis syndrome of fescue toxicosis in ruminants.

Abdominal fat necrosis is a condition that has been reported in several species including cattle,[8,10,19,22] pigs,[19] horses,[21] and Eld's deer.[23] The disease is characterized by the formation of diffuse masses of necrotic fat of various shapes and sizes located in the mesentery of the abdominal cavity. These masses eventually compress the gastrointestinal and urinary tracts, potentially leading to obstruction of viscous organs. Although the etiology of fat necrosis is unknown, it may be related to dietary factors such as the consumption of feeds high in long-chain, saturated fatty acids.[4] The condition has also been associated with ingestion of endophyte-infected fescue and is considered one of the 4 ''syndromes'' commonly associated with fescue toxicosis in ruminants.[11,13,18,22,23] The purpose of this report is to describe a case of abdominal fat necrosis in a goat.

The Food Animal Field Service of the North Carolina State University College of Veterinary Medicine (Raleigh, NC) was called to euthanatize an 11-year-old female pygmy goat with a progressive history of anorexia and abdominal distension. The goat had been housed all its life on a 3-acre pasture with 9–10 other goats of various breeds. It was vaccinated annually for *Clostridium perfringens* types C and D, tetanus, and rabies. It was also dewormed 3–4 times per year with either ivermectin or fenbendazole and dusted every

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