2010

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Journal of Wildlife Diseases, 46(3), 2010, pp. 988–993

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ABSTRACT: Lungworm infections are common among bighorn sheep (*Ovis canadensis*) in North America, and the predominant species reported are *Protostrongylus stilesi* and *P. rushi*. The only records of another lungworm species, *Muellerius capillaris*, infecting bighorns come from South Dakota, USA. At the National Bison Range (NBR), Montana, USA we found that across six sampling periods, 100% of wild bighorn sheep surveyed were passing first-stage dorsal-spined larvae (DSL) which appeared to be consistent with *M. capillaris*. By contrast, only 39% or fewer sheep were passing *Protostrongylus* larvae. Using molecular techniques, we positively identified the DSL from the NBR bighorns as *M. capillaris*. This is the first definitive record of *M. capillaris* infection in a free-ranging bighorn sheep population outside of South Dakota.

Key words: Bighorn sheep, lungworms, *Muellerius capillaris*, *Ovis canadensis*, *Protostrongylus*.

Lungworms have been a major focus of health-related research in bighorn sheep (*Ovis canadensis*) because of their possible role in pneumonia-associated die-offs in this species (Forrester, 1971). In North America, lungworm infections are widely reported in bighorns, and the most common species known to infect these sheep are *Protostrongylus stilesi* and *P. rushi* (Forrester and Senger, 1964; Kistner et al., 1977; Festa-Bianchet 1991); a third species, *P. frosti*, has apparently not been reported beyond the original description. Although not as widespread as *Protostrongylus*, there have been limited reports of another lungworm species, *Muellerius capillaris*, infecting wild bighorn sheep (Demartini and Davies, 1977; Pybus and Shave, 1984; Goldstein et al., 2005). *Muellerius capillaris* typically infects domestic sheep and goats, but a recent study of captive bighorn sheep demonstrated their competence as suitable maintenance hosts in the absence of a domestic reservoir (Foreyt et al., 2009). Most of the reported negative effects of lungworms on bighorn sheep have been linked to species of *Protostrongylus*. However, Demartini and Davies (1977) found that *Muellerius* infections were associated with an epizootic of translocation-related pneumonia in a population from South Dakota. This suggests that, much like *Protostrongylus*, *Muellerius* may have important negative effects on bighorn sheep, particularly during periods of high stress.

Despite the possible importance of *M. capillaris* infections in bighorn sheep from North America, definitive records of this species are presently restricted to South Dakota (Pybus and Shave, 1984). Although dorsal-spined larvae (DSL), thought to be characteristic of *M. capillaries*, have been reported in bighorn from Alberta and British Columbia, Canada and from Montana, North Dakota, and Washington, USA, these records remain unsubstantiated (Pybus and Shave, 1984; Foreyt et al., 2009). Dorsal-spined larvae of several genera and species of protostrongylid nematodes cannot be distinguished morphologically; consequently, the presence of such larvae is not sufficient evidence of *M. capillaris* infection. Only recently, and based on comparative molecular techniques (Jenkins et al., 2005; Kutz et al., 2007), has it become possible to provide unequivocal identification of

DSL, including all seven species known to occur in free-ranging or domestic ungulates from North America. Application of these techniques, and related methods, have demonstrated that DSL in bighorn may not always represent *M. capillaris*. For example, analyses using single strand conformation polymorphism of DSL isolated from bighorn sheep feces in Washington State identified these larvae as the muscle-dwelling nematode *Parelaphostrongylus odocoilei* (Huby-Chilton et al., 2006). As such, it has been difficult to assess the degree to which *M. capillaris* represents an important new or emerging component of the lungworm community of North American bighorn sheep. Here, we report on the occurrence of DSL in fecal samples collected from bighorn sheep and five other native ungulates at the National Bison Range (NBR), Montana, USA. We used molecular techniques for positive identification of *M. capillaris* in this population of wild sheep and compared the relative prevalence of *Protostrongylus* spp. and DSL in bighorn sheep.

We collected fecal samples from bighorn sheep at the NBR, Moiese, Montana (47°20′N, 114°15′W) to assess the prevalence and composition of lungworm infections. Fecal samples were collected from individually identifiable sheep in July–August 2007 ($n=18$), October 2007 ($n=24$), November 2007 ($n=19$), October 2008 ($n=25$), November 2008 ($n=26$), and December 2008 ($n=20$). We also collected fresh fecal samples from five other native ungulate species at NBR: bison (*Bison bison*, $n=18$), elk (*Cervus elpahus*, $n=20$), pronghorn (*Antilocapra americana*, $n=19$), mule deer (*Odocoileus hemionus*, $n=14$), and white-tailed deer (*Odocoileus virginianus*, $n=15$). These samples were collected in June–July 2007 during driving transects conducted on all major and minor NBR roads. Once a group of animals was located, we stopped and recorded the species, time of day, size and composition of the group, and the GPS coordinates. We waited until at least one animal in the group defecated and then collected all fresh fecal samples found. Samples from all species were immediately placed in a cooler and, upon returning from the field, were kept at 4 C until processing. Lungworm larvae were isolated from 10 g of feces using a beaker-modified Baermann technique (Forrester and Lankester, 1997). Larvae were identified and quantified using a binocular microscope at 100–400× magnification. We identified larvae as *Protostrongylus* spp. and dorsal-spined larvae (DSL) based on morphologic features described by Bowman (1999) and Foreyt (2001).

Individual larvae from a subset of fecal samples were collected for molecular identification. Ten DSL each were collected from two bighorn sheep and two white-tailed deer samples. For species in which DSL were rare (bison and mule deer), we collected 10–30 individual larvae from cultures containing mixed pools of gastrointestinal nematode and lungworm larvae. In this case, larvae were collected from 16 individual bison and 19 mule deer. We also collected pooled larvae from 13 additional bighorn sheep. DNA was extracted from individual larvae in 0.2-ml tubes containing 5 μl of deionized water. To each tube we added 15 μl of lysis buffer containing 2 μl 10× polymerase chain reaction (PCR) buffer (Buffer I with 15 mM $MgCl_2$, Applied Biosystems, Foster City, California, USA), 0.8 μl 1.5 mM $MgCl_2$, 2 μl 4.5% Nonidet P-40, 2 μl 4.5% Tween® 20 (Fisher Scientific, Pittsburgh, Pennsylvania, USA), 2 μl of a 2 mg/ml solution of proteinase K, and 6.2 μl H_2O (Redman et al., 2008). DNA was extracted by incubating each larva in buffer at 60 C for 98 min, then at 94 C for 20 min. DNA extracts were diluted 1:5 with purified (Milli-Q) water and stored at −80 C. To identify DSL to species, we amplified and sequenced a 425-base pair (bp) region of the ITS-2 rRNA (Gasser et al., 1993). PCR amplification was performed in 12-μl reactions containing 1 μl of DNA extract,

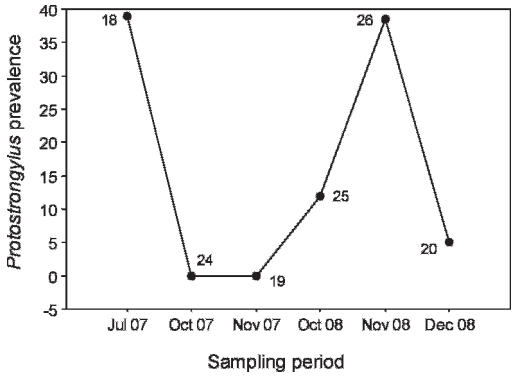

FIGURE 1. Prevalence of *Protostrongylus* spp. larvae in bighorn sheep fecal samples collected at the National Bison Range, Montana, USA in 2007–2008. Samples sizes are shown next to sample points. Prevalence of dorsal-spined larvae was 100% across all sampling periods.

0.6 μl of each 10 μM primer (NC1-ACGTCTGGTTCAGGGTTGTT; NC2-TTAGTTTCTTTTCCTCCGCT), 1.2 μl of 2 mM dNTP (Invitrogen, Carlsbad, California, USA), 1.2 μl 10× PCR buffer without $MgCl_2$, 0.96 μl of 1.5 mM $MgCl_2$, 0.16 μl of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 5.08 μl of water. All reactions included a negative control wherein the DNA extract was replaced with purified water. Amplification was preceded by a 10-min denaturation and polymerase activation step at 95 C, followed by 40 cycles of 45 sec each at 55 C annealing, 72 C extension, and 95 C denaturation. These cycles were followed by a 5-min extension step at 72 C. Positive reactions were identified by the presence of a 425-bp band visualized by agarose gel electrophoresis. Positive PCRs were cleaned using ExoSAP and sequenced using Dye Terminator Cycle Sequencing (Applied Biosystems). BLAST searches were used to compare the resulting sequences to ITS-2 rRNA sequences available in GenBank, including sequences from the seven species of protostrongylids from North America known to produce DSL (Kutz et al., 2007; Asmundsson et al., 2008).

Across each of six sampling periods, spanning 2007–2008, the prevalence of DSL in bighorn sheep fecal samples was 100%. By contrast, the prevalence of *Protostrongylus* larvae ranged from 0–39% (Fig. 1). There was no evidence of *Protostrongylus* infection in any other ungulate species examined in summer 2007. However, DSL were recovered from three other species: bison, white-tailed deer, and mule deer (Table 1). Of 20 individual DSL sequenced from two bighorn sheep, all were identified as *M. capillaris*. The DSL specimens sequenced from 13 additional sheep were also positively identified as *M. capillaris*. All sequences were identical to two *M. capillaris* ITS-2 sequences available on GenBank (accession numbers AY679529 and AY679527). We also detected *M. capillaris* from larvae sequenced from a bison, whereas DSL in white-tailed deer and mule deer were identified as *Parelaphostrongylus andersoni* and *Pa. odocoilei*, respectively, based on comparisons of the ITS-2 sequences. Voucher specimens of DSL representing species of *Muellerius* or *Parelaphostrongylus* were not retained, as all larvae were destructively sampled.

TABLE 1. Prevalence of dorsal-spined larvae (DSL) and *Protostrongylus* larvae in six wild ungulate species at the National Bison Range, Montana, USA, June–August 2007.

Host species	*n*	DSL % infected	*Protostrongylus* spp. % infected
Bighorn sheep	18	100	38.9
Bison	18	5.6	0
Elk	20	0	0
Pronghorn	19	0	0
Mule deer	14	21.4	0
White-tailed deer	15	86.7	0

Our findings indicate that lungworm infections in bighorn sheep at NBR are dominated by *M. capillaris*. Larvae from this species were detected at high prevalence in sheep across all sampling periods, while larvae of the more commonly described bighorn lungworms, *Protostrongylus* spp., occurred at varying, but

consistently low, frequencies. Forrester and Senger (1964) sampled bighorn sheep across 10 sites in Montana between 1958 and 1963 and found evidence of *P. stilesi*, but not of *P. rushi*, infection in the lungs of sheep at NBR. However, overall infection prevalence, measured from feces, was extremely low at NBR (19%) compared to all other sites (83–100%). Similarly, a study of a related lungworm reported to infect pronghorn (*Orthostrongylus macrotis*) failed to find any evidence of infection in the NBR pronghorn population, despite high prevalence (97%) in Yellowstone National Park (Greiner et al., 1974). Greiner hypothesized that the low prevalence of protostrongylids at NBR was a consequence of a lack of appropriate intermediate hosts in the area, an idea supported by Forrester (1962) who, in a survey of bighorn at 10 sites across Montana, found potential gastropod hosts at all localities except the NBR.

Pybus and Shave (1984) examined nine bighorn sheep from South Dakota and found that all individuals were infected with *M. capillaris* and none were infected with *Protostrongylus*. Our results show a similar pattern, with dominance of *M. capillaris* and limited occurrence of *Protostrongylus*. In combination with the findings of Pybus and Shave (1984), our results suggest that co-occurrence of these two parasites may be rare, or that in cases where they co-infect bighorn, *M. capillaris* tends to dominate. Indeed, studies of related host species, including domestic sheep (*Ovis aries*) and wild mouflon (*Ovis musimon*), have shown *M. capillaris* to be dominant when it co-occurs with *Protostrongylus rufescens* and other protostrogylids (Meana et al., 1996; Reguera-Feo et al., 1996). This may be a consequence of habitat segregation between the intermediate hosts for the different parasites or possible competitive interactions between the parasites themselves (Reguera-Feo et al., 1996).

There have been no historic reports of *M. capillaris* in NBR bighorns, so our results suggest a fairly recent invasion (within the last 40 yr) of this species into the system. Since *M. capillaris* occurred in only one other ungulate species (bison) at the NBR, and at very low frequency, it is unlikely that these infections in bighorn were driven by spillover from other free-ranging bovids or cervids. Cross-transmission of *M. capillaris* between bighorn sheep and domestic goats can occur (Foreyt et al., 2009), and although NBR is a fenced refuge, bighorn, particularly rams, can easily jump or otherwise pass through the fence and have frequently been observed to do so. Thus, a contact event between NBR bighorns and surrounding domestic livestock may account for the initial introduction of *M. capillaris*, with subsequent maintenance of the parasite by bighorns. Alternatively, *M. capillaris* may have been introduced into the NBR by migrant or translocated individuals. Two natural immigrants to the population have been documented since 2001 (both rams), and 23 individuals (11 rams and 12 ewes) were introduced experimentally during 1985–1994 (Hogg et al., 2006). The introduced sheep originated from herds in Thompson Falls, Wildhorse Island, and Rock Creek, Montana and Whiskey Basin, Wyoming. However, at two of these sites, Wildhorse Island and Rock Creek, Forrester and Senger (1964) found no evidence of *M. capillaris* infection in resident sheep. Regardless of the route of entry of *M. capillaris* into the NBR, we have documented a previously undetected invasion of a new parasite into the NBR bighorn population. Given that both species of *Protostrongylus* and *M. capillaris* have been implicated in die-offs of bighorns, a more-detailed understanding of the community structure of lungworms and their intermediate hosts may have important implications for understanding the role these parasites play in the health of both individuals and populations of these free-ranging sheep.

Our results further suggest that circulation of multiple genera and species of

protostrongylids among the sympatric assemblage of ungulate hosts at the NBR is limited. For example, *M. capillaris* and species of *Protostrongylus* seem to be restricted to bighorn sheep, while species of *Parelaphostrongylus* were only found among white-tailed and mule deer. *Parelaphostrongylus andersoni* had not been demonstrated previously in white-tailed deer from Montana (e.g., Lankester, 2001; Asmundsson et al., 2008). This appears to be the second observation of sympatry for *Pa. andersoni* in white-tailed deer and *Pa. odocoilei* in mule deer, where these muscle worms appear restricted to their respective hosts (Asmundsson et al., 2008). Additionally, mixed infections involving *Protostrongylus*, *Parelaphostrongylus*, and *Muellerius* were not apparent in bighorn sheep or in any other bovid or cervid host at the NBR. This is despite the fact that bighorns have been shown to be competent hosts for *Pa. odocoilei*.

We thank the National Bison Range for permission to conduct research, and Stefan Ekernas, Bree Hogg, Mike Dacey, Morgan Anderson, Lila Tauzer, Kei Yasuda, Zea Walton, Charlie Henderson, Carson Lindbeck, Vicky Zero, and Wesley Sarmento for assistance collecting and processing samples. This work was funded by the Division of Biological Sciences, University of Montana, and stimulated in part by collaborations generated through a University of Montana Project PACE Award to V.O.E. A.M.H was supported by an HHMI MILES Undergraduate Fellowship and a Davidson's Honors College Watkin's Research Award.

LITERATURE CITED

ASMUNDSSON, I. M., J. A. MORTENSON, AND E. P. HOBERG. 2008. Muscleworms, *Parelaphostrongylus andersoni* (Nematoda: Protostrongylidae), discovered in Columbia white-tailed deer from Oregon and Washington: Implications for biogeography and host associations. Journal of Wildlife Diseases 44: 16–27.

BOWMAN, D. D. 1999. Georgis' parasitology for veterinarians, 7th Edition. W. B. Saunders, Philadelphia, Pennsylvania, 414 pp.

DEMARTINI, J. C., AND R. B. DAVIES. 1977. Epizootic of pneumonia in captive bighorn sheep infected with *Muellerius* sp. Journal of Wildlife Diseases 13: 117–124.

FESTA-BIANCHET, M. 1991. Numbers of lungworm larvae in feces of bighorn sheep—Yearly changes, influence of host sex, and effects on host survival. Canadian Journal of Zoology 69: 547–554.

FOREYT, W. J. 2001. Veterinary parasitology reference manual, 5th Edition. Iowa State University Press, Ames, Iowa, 235 pp.

———, E. J. JENKINS, AND G. D. APPLEYARD. 2009. Transmission of lungworms (*Muellerius capillaris*) from domestic goats to bighorn sheep on common pasture. Journal of Wildlife Diseases 45: 272–278.

FORRESTER, D. J. 1962. Land molluscs as possible intermediate hosts of *Protostrongylus stilesi*, a lungworm of bighorn sheep in Western Montana. Proceedings of the Montana Academy of Sciences 22: 82–92.

———. 1971. Bighorn sheep lungworm-pneumonia complex. *In* Parasitic diseases of wild mammals, J. W. Davis and R. C. Anderson (eds.). Iowa State University Press, Ames, Iowa, pp. 158–173.

———, AND C. M. SENGER. 1964. A survey of lungworm infection in bighorn sheep of Montana. Journal of Wildlife Management 28: 481–491.

FORRESTER, S. G., AND M. W. LANKESTER. 1997. Extracting protostrongylid nematode larvae from ungulate feces. Journal of Wildlife Diseases 33: 511–516.

GASSER, R. B., N. B. CHILTON, H. HOSTE, AND I. BEVERIDGE. 1993. Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. Nucleic Acids Research 21: 2525–2526.

GOLDSTEIN, E. J., J. J. MILLSPAUGH, B. E. WASHBURN, G. C. BRUNDIGE, AND K. J. RAEDEKE. 2005. Relationships among fecal lungworm loads, fecal glucocorticoid metabolites, and lamb recruitment in free-ranging Rocky Mountain bighorn sheep. Journal of Wildlife Diseases 41: 416–425.

GREINER, E. C., D. WORLEY, AND B. W. O'GARA. 1974. *Protostrongylus macrotis* (Nematoda: Metastrongyloidea) in pronghorn antelope from Montana and Wyoming. Journal of Wildlife Diseases 10: 70–74.

HOGG, J. T., S. H. FORBES, B. M. STEELE, AND G. LUIKART. 2006. Genetic rescue of an insular population of large mammals. Proceedings of the Royal Society B–Biological Sciences 273: 1491–1499.

HUBY-CHILTON, F., A. A. GAJADHAR, K. MANSFIELD, W. J. FOREYT, AND N. B. CHILTON. 2006. Bighorn sheep, a new host record for *Parelaphostrongylus odocoilei* (Nematoda: Protostrongylidae). Journal of Wildlife Diseases 42: 877–882.

JENKINS, E. J., G. D. APPLEYARD, E. P. HOBERG, B. M.

Rosenthal, S. J. Kutz, A. M. Veitch, H. M. Schwantje, B. T. Elkin, and L. Polley. 2005. Geographic distribution of the muscle dwelling nematode *Parelaphostrongylus odocoilei* in North America, using molecular identification of first stage larvae. Journal of Parasitology 91: 574–584.

Kistner, T. P., S. M. Matlock, D. Wyse, and G. E. Mason. 1977. Helminth parasites of bighorn sheep in Oregon. Journal of Wildlife Diseases 13: 125–130.

Kutz, S. J., I. M. Asmundsson, E. P. Hoberg, G. D. Appleyard, E. J. Jenkins, K. Beckmen, M. Branigan, L. Butler, N. B. Chilton, D. Cooley, B. Elkin, F. Huby-Chilton, D. Johnson, A. Kuchboev, J. Nagy, M. Oakley, L. Polley, R. Popko, A. Scheer, M. Simard, and A. Veitch. 2007. Serendipitous discovery of a novel protostrongylid (Nematoda: Metastrongyloidea) in caribou, muskoxen and moose from high latitudes of North America based on DNA sequence comparisons. Canadian Journal of Zoology 85: 1143–1156.

Lankester, M. J. 2001. Extrapulmonary lungworms of cervids. *In* Parasitic diseases of wild mammals, W. D. Samuel, M. J. Pybus and A. A. Kocan (eds.). Iowa State University Press, Ames, Iowa, pp. 228–278.

Meana, A., M. Luzon-Pena, J. Santiago-Moreno, A. De Bulnes, and M. Gomez-Bautista. 1996. Natural infection by gastrointestinal and bronchopulmonary nematodes in mouflons (*Ovis musimon*) and their responses to Netobimin treatment. Journal of Wildlife Diseases 32: 39–43.

Pybus, M. J., and H. Shave. 1984. *Muellerius capillaris* (Mueller, 1889) (Nematoda: Protostrongylidae): An unusual finding in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis* Shaw) in South Dakota. Journal of Wildlife Diseases 4: 284–288.

Redman, E., E. Packard, P. Grillo, J. Smith, F. Jackson, and J. S. Gilleard. 2008. Microsatellites analysis reveals marked genetic differentiation between *Haemonchus contortus* laboratory isolates and provides a rapid system of genetic fingerprinting. International Journal for Parasitology 38: 111–122.

Reguera-Feo, A., L. Castanon-Ordonez, and M. Corridero Del Campillo. 1996. Ecological relations among first-stage larvae of four species of Protostrongylidae (Nematoda) within their ovine host. Applied Parasitology 37: 66–73.

Submitted for publication 23 October 2009.
Accepted 29 January 2010.