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SOURCE AND SEASONALITY OF EPIZOOTIC MYCOPLASMOSIS IN FREE-RANGING PRONGHORN (*ANTILOCAPRA AMERICANA*)

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ABSTRACT: *Mycoplasma bovis* is an economically important bacterial pathogen of cattle (*Bos taurus*) and bison (*Bison bison*) that most commonly causes pneumonia, polyarthritis, and mastitis. It is prevalent in cattle and ranched bison; however, infections in other species are rare. In early 2019, we identified *M. bovis* in free-ranging pronghorn (*Antilocapra americana*) in northeastern Wyoming. Here, we report on additional pronghorn mortalities caused by *M. bovis,* in the same approximately 120-km^2 geographic region 1 yr later. Genetic analysis by multilocus sequence typing revealed that the mortalities were caused by the same *M. bovis* sequence type, which is unique among all sequence types documented thus far in North America. To explore whether pronghorn maintain chronic infections and begin assessing *M. bovis* status in other sympatric species, we used PCR testing of nasal swabs to opportunistically survey select free-ranging ungulates. We found no evidence of subclinical infections in 13 pronghorn sampled from the outbreak area (upper 95% binomial confidence limit [bCL], ~24.7%) or among 217 additional pronghorn (upper 95% bCL, ~1.7%) sampled from eight additional counties in Wyoming and 10 in Montana. All mule deer (*Odocoileus hemionus*; *n*=231; upper 95% bCL, ~1.6%) sampled from 11 counties in Wyoming also were PCR negative. To assess the potential for environmental transmission, we examined persistence of *M. bovis* in various substrates and conditions. Controlled experiments revealed that *M. bovis* can remain viable for 6 h in shaded water and 2 h in direct sunlight. Our results indicate that environmental transmission of *M. bovis* from livestock to pronghorn is possible and that seasonality of infection could be due to shared resources during late winter. Further investigations to better understand transmission dynamics, to assess population level impacts to pronghorn, and to determine disease risks among pronghorn and other ungulate taxa appear warranted.

Key words: *Mycoplasma bovis*, pneumonia, wildlife-livestock interface.

INTRODUCTION

Understanding the dynamics of disease transmission at the wildlife-livestock interface is critical for both conservation of wildlife and preservation of ranching practices. Spillover events are of great concern, with human and animal movement, grazing of livestock, and human land use being primary determinants in the exchange of infectious pathogens between domestic animals and wildlife (Rhyan and Spraker 2010). However, a critical gap remains in our understanding of disease processes at the wildlife-livestock interface, particularly for emerging diseases in which ecological and economic impacts are not fully characterized (Miller et al. 2013).

Mycoplasmas are the smallest self-replicating organisms, comprising a ubiquitous family of bacteria with over 100 described species (Razin et al. 1998). Despite the small genome size (580–1,380 kilobases), many *Mycoplasma* spp. pose major threats to human and animal health (Rosengarten et al. 2000). Spillover of *Mycoplasma* spp. from domestic to free-ranging animals has impacted wildlife conservation in multiple instances. Notable examples include *Mycoplasma ovipneumoniae* in bighorn sheep (*Ovis canadensis*; Besser et al.

2008), *Mycoplasma gallisepticum* in house finches (*Carpodacus mexicanus*; Ley et al. 1996), and *Mycoplasma agassizii* in wild tortoises (*Gopherus* spp.; Brown et al. 1994). The most recent example of mycoplasma emergence in wildlife is *Mycoplasma bovis* in pronghorn (*Antilocapra americana*; Malmberg et al. 2020).

Mycoplasma bovis is a globally distributed bacterial pathogen of economic importance in cattle (*Bos taurus*) and ranched bison (*Bison bison*). Other species are rarely infected, but cases have been documented in captive and free-ranging white-tailed deer (*Odocoileus virginianus*; Dyer et al. 2004; Register et al. 2019) and in free-ranging mule deer (*Odocoileus hemionus*; Register et al. 2019). We previously identified *M. bovis* as the cause of epizootic pneumonia in pronghorn in northeastern Wyoming during early 2019 (Malmberg et al. 2020) and herein document additional mortalities occurring in early 2020. Documented cases have been limited in geographic distribution; however, population impacts remain unclear. Further, the drivers of emergence of this disease in a new host are unknown. We used a combination of genetic analysis, multihost surveillance, and environmental assessment to investigate the source and seasonality of pronghorn *M. bovis* infections. We hypothesized that pronghorn might be attracted to livestock resources during the late winter and that shared environmental resources might be an important consideration for transmission.

MATERIALS AND METHODS

Study site

Epizootics of *M. bovis* were documented within an approximately 120-km^2 area in Campbell County, Wyoming, predominantly on privately owned pasture and range land (Fig. 1). Elevations ranged from 1,250–1,455 m above sea level. Habitat in the area consists of mixed grassland and sagebrush steppe. Industrial mining and cattle ranching are primary land uses in this region.

Diagnostic workup

Field sample collection and evaluation: Wyoming Game and Fish Department and Wyoming State Veterinary Laboratory (WSVL) personnel obtained samples from pronghorn carcasses. When intact carcasses were available, a detailed postmortem examination was performed by a board-certified pathologist. Diagnostic evaluation included histopathology, bacteriology (aerobic and anaerobic culture, *Mycoplasma* spp. culture, and *M. bovis* PCR, all described soon), parasitology (Baermann technique for lungworm), and virology (PCR for bovine herpesvirus-1, parainfluenza virus-3, bovine viral diarrhea virus, bovine respiratory syncytial virus, epizootic hemorrhagic disease virus, bluetongue virus, and cervid adenovirus) as described (Malmberg et al. 2020; Supplementary Material Table S1). When only minimal tissues or nasal swabs were available, we performed PCR for *M. bovis* detection, followed by culture on a subset (n=13) of positive samples.

Diagnostic M. bovis real-time PCR: We extracted DNA from fresh lung tissue or nasal swab by using the DNeasy Blood & Tissue Kit (Qiagen, Germantown, Maryland, USA). Real-time PCR targeting the *oppD* gene was performed by using primers and probes described in Loy et al. (2018). Each reaction contained 1.0 µL of primer probe mix (primers diluted to 10 µM and probe to 2.5 µM in nuclease-free water), 5.0 µL (20–100 ng) of template DNA, 12.5 µL of master mix (BioRad, Hercules, California, USA), 0.5 µL of Xeno Internal Control Positive LIZ Assay (Applied Biosystems, Waltham, Massachusetts, USA), and nuclease-free water to 25 µL. Cycling conditions were as follows: 95 C for 2 min, 40 cycles of 95 C for 15 s, and 56 C for 60 s.

Mycoplasma *culture:* Swab or lung tissue (approximately 20 mg) was placed in a 4-mL *Mycoplasma* enrichment broth (mycoplasma broth, Hardy Diagnostics, Santa Maria, California, USA) and incubated with a loose lid at 37 C in 10% CO_2 for 72 h. Subsequently, we inoculated 100 µL of broth onto commercial *Mycoplasma* spp. agar (mycoplasma agar with cefoperazone, Hardy Diagnostics), spreading this evenly over the entire plate with a sterile swab. Plates were incubated at 37 C in 10% CO_2 for 24–72 h. Incubation was considered complete after a confluent layer of colonies was present throughout the entire plate. In addition to 13 pronghorn isolates from 2020, we cultured *M. bovis* from cattle (n=6) and bison (n=5) samples submitted to WSVL for routine diagnostic testing (Table 1).

Aerobic and anaerobic culture: Tissues or swabs were aseptically inoculated onto Columbia blood agar plates with 5% sheep blood and MacConkey agar plates (Hardy Diagnostics). The plates were struck to form three zones for isolation and incubated at 37 C in 10% CO_2. A Columbia blood agar plate was also incubated in anaerobic conditions in a Baker-Ruskinn Bugbox

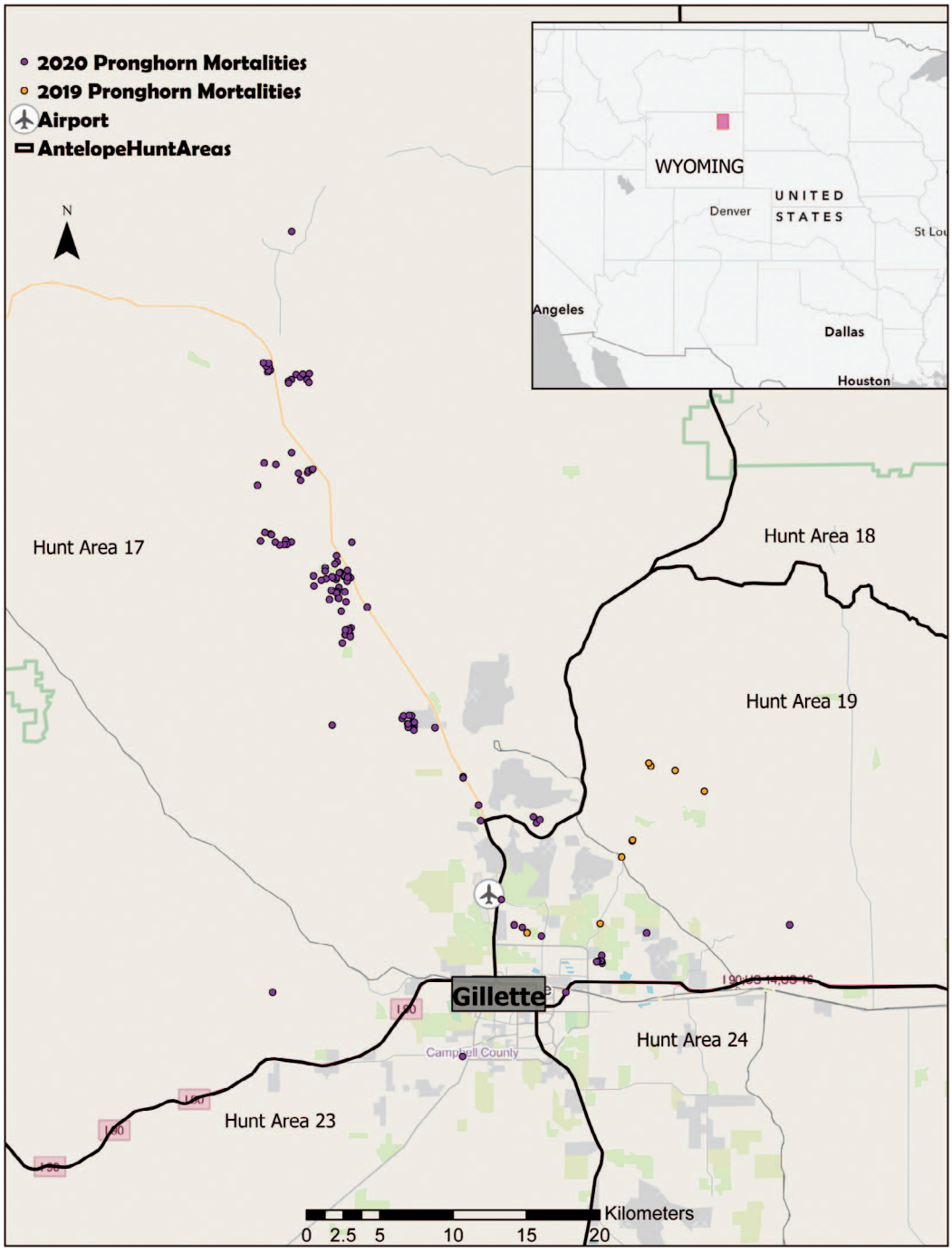

FIGURE 1. Pronghorn (*Antilocapra americana)* mortalities associated with *Mycoplasma bovis* infections in northeastern Wyoming, USA. Orange circles represent confirmed cases in 2019 (n=9), while purple circles indicate confirmed cases from 2020 (n=37) and sites of additional carcasses (presumptive positives; n=120) identified during the outbreak period (February–April). Gray shaded areas represent industrial mining, and green areas represents grassy parks. Black lines represent borders of antelope hunt areas.

TABLE 1. Summary of *Mycoplasma bovis* isolates collected from pronghorn (*Antilocapra americana*), bison (*Bison bison*), and cattle in various USA areas, 2017–21, and used for phylogenetic analysis.

ID	Year	Host	Site	Region	Sequence type	GenBank accession numbers						
						dnaA	*gltX*	*gspA*	*gyrB*	*pta2*	*tdk*	*tkt*
WSVL1	2019	Bovine	Joint	Colorado	191	OL744082	OL780044	OL780056	OL780068	OL780080	OL780092	OL780104
WSVL2	2020	Bovine	Lung	Colorado	192	OL744083	OL780045	OL780057	OL780069	OL780081	OL780093	OL780105
WSVL3	2018	Bovine	Joint	Nebraska	193	OL744091	OL780053	OL780065	OL780077	OL780089	OL780101	OL780113
WSVL4	2021	Bison	Lung	South Dakota	194	OL744087	OL780049	OL780061	OL780073	OL780085	OL780097	OL780109
WSVL5	2021	Bison	Lung	South Dakota	194	OL744088	OL780050	OL780062	OL780074	OL780086	OL780098	OL780110
WSVL6	2021	Bison	Lung	Nebraska	25	OL744086	OL780048	OL780060	OL780072	OL780084	OL780096	OL780108
WSVL7	2020	Bison	Lung	Wyoming	62	OL744084	OL780046	OL780058	OL780070	OL780082	OL780094	OL780106
WSVL8	2020	Bison	Lung	Wyoming	62	OL744085	OL780047	OL780059	OL780071	OL780083	OL780095	OL780107
WSVL9	2019	Bovine	Lung	Montana	60	OL744081	OL780043	OL780055	OL780067	OL780079	OL780091	OL780103
WSVL10	2017	Bovine	Nasal	Nebraska	23	OL744089	OL780051	OL780063	OL780075	OL780087	OL780099	OL780111
WSVL11	2017	Bovine	Lung	Nebraska	23	OL744090	OL780052	OL780064	OL780076	OL780088	OL780100	OL780112
WSVL12	2020	Pronghorn	Lung	Wyoming	111	OL780115	OL780054	OL780066	OL780078	OL780090	OL780102	OL780114

TABLE 2. Summary of *Mycoplasma bovis* surveillance samples (nasal swabs) collected from pronghorn (*Antilocapra americana*) and mule deer (*Odocoileus hemonius*) in Wyoming and Montana, USA. *Mycoplasma bovis* was not detected in the surveillance samples.

	Mule deer		Pronghorn		
Year	Antemortem	Postmortem	Antemortem	Postmortem	Total
2019	0	2	0	2	4
2020	168	26	0	60	254
2021	28	7	150	18	203
Total	196	35	150	80	461

(The Baker Company, Sanford, Maine, USA). Culture plates were read and documented once at ~18–24 h, and again at ~36–48 h. Bacterial colonies were identified by using matrix-assisted laser desorption–ionization time of flight (Bruker Daltonics GmbH & Co. KG, Bremen, Germany).

Surveillance

Nasal swabs (polyester fiber–tipped applicators, Cardinal Health, Waukegan, Illinois, USA) were collected from free-ranging pronghorn throughout Wyoming and Montana and mule deer throughout Wyoming and frozen in media that included tryptic soy broth with 15% glycerol for transport to the laboratory. Sources of samples included cases submitted to WSVL for routine postmortem examination, swabs collected at hunter check stations, and swabs obtained from live animal captures in conjunction with other wildlife research (Table 2). All live animals were handled in accordance with institutional animal care and use committee policies, following the general guidelines for handling wild mammals (Sikes 2016).

We performed the previously described diagnostic *M. bovis* PCR on DNA extracted from nasal swabs from pronghorn (n=230) and mule deer (n=231) collected 2019–21 (Table 2) across 23 counties in Wyoming and Montana (Fig. 2 and Table 3). Samples included both antemortem (n=346) and postmortem (n=115) nasal swabs. A total of 47 postmortem swabs were collected from pronghorn in 2020 at Wyoming Game and Fish Department hunter check stations (Antelope

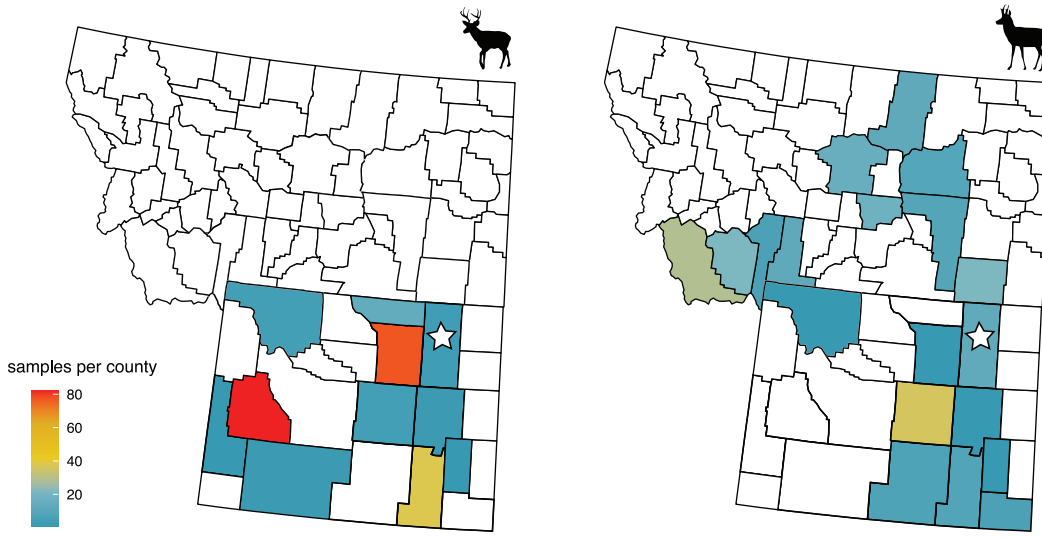

FIGURE 2. Map of Wyoming and Montana, USA, with county boundaries and corresponding surveillance sample (nasal swab) numbers represented for (left) mule deer (*Odocoileus hemionus*) and (right) pronghorn (*Antilocapra americana*). The star indicates Campbell County, Wyoming, within which pronghorn mortalities caused by *Mycoplasma bovis* were identified in 2019 and 2020. *Mycoplasma bovis* was not detected in surveillance samples.

TABLE 3. Number of pronghorn (*Antilocapra americana*) and mule deer (*Odocoileus hemonius*) nasal swabs tested for *Mycoplasma bovis*, by state (USA) name and county.

State, county	Species		Total
	Pronghorn	Mule deer	
Montana			
Beaverhead	29	0	29
Fergus	16	0	16
Gallatin	6	0	6
Garfield	10	0	10
Madison	22	0	22
Musselshell	18	0	18
Park	12	0	12
Phillips	12	0	12
Powder River	22	0	22
Rosebud	9	0	9
Wyoming			
Albany	8	38	46
Campbell	13	4	17
Carbon	7	0	7
Converse	1	2	3
Johnson	1	77	78
Laramie	6	0	6
Lincoln	0	1	1
Natrona	36	5	41
Park	1	5	6
Platte	1	1	2
Sheridan	0	14	14
Sublette	0	82	82
Sweetwater	0	2	2

Hunt Area 73 in Converse County, Wyoming, n=34; and Antelope Hunt Areas 24 and 17 in Campbell County, Wyoming, n=13). Another 28 swabs were collected from pronghorn during routine diagnostic cases at WSVL. Samples from Montana included 150 swabs collected during live captures and five samples collected postmortem. Mule deer samples included 196 swabs from live captures and 35 postmortem swabs from WSVL cases. To confirm that nasal swabs were suitable for *M. bovis* detection, we performed paired diagnostic *M. bovis* PCR as described earlier on lung tissue and nasal swabs from infected pronghorn (n=23).

Whole-genome sequencing

From pure isolates subcultured from broth onto solid media, DNA extractions were performed by using the DNeasy Blood & Tissue Kit on an automated nucleic acid extraction platform (QIAcube, Qiagen). Each DNA extract was assessed for purity by using a spectrophotometer at 260/280 nm (NanoDrop™ 2000, Thermo Scientific, Grand Island, New York, USA) and for concentration by using a Qubit 3.0 fluorometer (Thermo Scientific). A DNA sequencing library was prepared with the Nextera DNA Flex library Prep Kit (Illumina, San Diego, California, USA). The library quantity was measured by using the Qubit 3.0 fluorometer. Lengths of DNA library fragments were assessed by using the TapeStation 4200 (Agilent, Santa Clara, California, USA), and after normalization, DNA libraries were sequenced on a MiSeq by using V2 2×250 base pair (bp) cycle chemistry with ~8.0 Gb of output (Illumina). Postsequencing statistics were evaluated by using FastQC (Andrews 2010).

Multilocus sequence typing and phylogenetics

Paired end fastq reads of ~250 bp were analyzed as follows: 1) trimming of indexes, primers, low quality (phred <20), and short reads (<50 bp) by using Cutadapt (Martin 2011); 2) mapping of trimmed reads to the genome of *M. bovis* international reference strain PG45 (GenBank accession no. NC_014760) by using Bowtie2 (Langmead and Salzberg 2012); 3) conversion of .sam files to .bam files by using Samtools (Li et al. 2009); and 4) viewing of sorted .bam files in Geneious Prime 2019.1.3 (Biomatters Ltd., Auckland, New Zealand). Consensus sequences were generated from mapped reads with the highest quality parameter in Geneious Prime as a threshold (sum of residue quality exceeds 60%). "N" was assigned to sites with coverage of less than three reads to represent missing data. Consensus sequences were trimmed to seven multilocus sequence typing (MLST) loci as described (Register et al. 2020) and concatenated in frame, yielding a final length of 3,015 bp. Consensus sequences for each target loci were submitted to GenBank (accession nos. OL744081-OL744091 and OL780043-OL780115; Table 1).

Concatenated sequences were generated for the following: pronghorn isolates recovered from lung in 2020 (n=10) and 2019 (n=4); bison isolates recovered from lung (n=5); and cattle isolates (n=6) recovered from lung, nasal swabs, or joint swabs (Table 1). These sequences were then compared with other publicly available North American sequence types (STs) from the *M. bovis* PubMLST database (Jolley et al. 2018). Unique STs were retained and were aligned by using Muscle (Li et al. 2009). Bayesian phylogenetic inference was performed by using MrBayes version 3.2.7a (Huelsenbeck and Ronquist 2001) with a mixed nucleotide substitution model. Specifically, we performed two independent runs

for four million generations each, with four simultaneous Markov chain Monte Carlo chains subsampled every 1,000 generations. The first 25% of the results were discarded as analytical burn-in, and we used an average standard deviation of split frequencies below 0.01 as the convergence diagnostic.

Environmental persistence

Environmental substrates: Four substrates were selected for assessment, including grass hay (0.30 kg), topsoil (3.0 kg; Laramie, Wyoming), water (6 qt [5.67 L]; MilliQ IQ, MilliporeSigma, Burlington, Massachusetts, USA), and loose mineral supplement (2.0 kg; Agland 12:12 Mineral, Agfinity Inc., Loveland, Colorado, USA). Each substrate was prepared in duplicate and placed in a small primary container. All contents were autoclaved and inoculated with approximately 2.14×10^5 colony-forming units of *M. bovis* recovered from pronghorn in 2020. Final concentrations were determined by using colony counts on *Mycoplasma* spp. agar (mycoplasma agar with cefoperazone, Hardy Diagnostics). One set of substrates was placed in direct sunlight, and one set in a shaded area. Temperature and relative humidity were measured at all sampling points by using digital thermometers and hygrometers (ThermoPro TP60S and TP450, ThermoPro, Toronto, Ontario, Canada).

Substrate sampling: On d 1, sampling was performed every hour for 8 h. Substrates were further sampled at 24, 48, and 72 h, and 5, 7, 14, 21, and 28 d for both the shade and sunlight treatments. For water sampling, 1 mL was collected and placed in a microcentrifuge tube for PCR. An additional 1 mL was placed in 3 mL of *Mycoplasma* broth (Hardy Diagnostics) and incubated at 37 C for 72 h in 5% CO_2 for culture. Mineral, hay, and topsoil substrates were swabbed and individually placed in 1 mL of phosphate-buffered saline for PCR and 3 mL *Mycoplasma* broth for culture. Swabs were collected by dabbing the entire substrate, including the surface and deep aspects. Following a 72-h incubation, samples in broth were plated on *Mycoplasma* spp. agar (mycoplasma agar with cefoperazone, Hardy Diagnostics) and incubated for an additional 72 h prior to assessment. Colony growth was confirmed as *M. bovis* by using real-time PCR (described soon).

Environmental real-time PCR: Real-time PCR was performed in triplicate on all environmental samples by using a protocol adapted from Rossetti et al. (2010). Briefly, PCR reactions consisted of 5 µL of sample (20–100 ng of template DNA), 1 µL of 20 µM forward and reverse primers, 1 µL of 8 µM *urvC* probe, 12.5 µL of Path-ID™ qPCR Master Mix (428864, Applied Biosystems), 0.5 µL of Xeno Internal Control Positive LIZ Assay (Applied Biosystems), and nuclease-free water to 25 µL. Cycling conditions were 95 C for 10 min, 40 cycles of 95 C for 15 s, and 56 C for 60 s.

RESULTS

Field investigation

An estimated 400 pronghorn died of pulmonary mycoplasmosis in northeastern Wyoming in 2020. This represents a sizeable increase compared to 2019 (n=60 estimated mortalities). Mortality estimates are based on a combination of ground observations, confirmed landowner reports, and aerial carcass counts. No mortalities due to pulmonary mycoplasmosis were identified in 2021. In both 2019 and 2020, the first mortalities were identified in late February, and the last mortalities were documented in late April (Supplementary Material Fig. S1). Infections were identified in both sexes and all age classes. Cattle with respiratory disease were not identified in the region of the outbreak. Although observations of cattle on pasture were limited to several ranches that granted access, no livestock in the vicinity of carcasses exhibited obvious clinical signs of respiratory disease. The closest commercial bison operation is approximately 64 km southeast of the study site. No outbreaks of *M. bovis* were reported in these bison during the study period.

Diagnostic evaluation

During 2020, 37 cases of *M. bovis* were confirmed in pronghorn by *M. bovis* real-time PCR (Supplementary Material Table S1). *Mycoplasma* culture was performed on a subset (n=13) of these cases. No coinfecting respiratory pathogens were consistently detected in pronghorn that died of mycoplasmosis; *Trueperella pyogenes* was detected by aerobic culture in two cases, while *Mannheimia* sp. was detected in two other cases. No respiratory viruses were detected by PCR. Lungworm (*Protostrongylus* sp.) were identified in 15 of 20 (75%).

Surveillance

Using lung tissue harvested postmortem from infected pronghorn as the gold standard, we demonstrated 87% sensitivity and 100% specificity of nasal swabs (n=23) for *M. bovis* detection by PCR. Sensitivity improved to 100% by excluding samples collected beyond a postmortem interval of ~1 wk (n=3). We found no evidence of subclinical infections in nasal swabs from 13 pronghorn that were collected within the outbreak area (upper 95% binomial confidence limit [bCL], 24.7%) or among nasal swabs from 217 additional pronghorn (upper 95% bCL, 1.7%) sampled from eight additional counties in Wyoming and 10 in Montana. All nasal swabs from mule deer (n=231; upper 95% bCL, ~1.6%), collected from 11 counties in Wyoming, also were PCR negative.

Multilocus sequence typing and phylogenetics

Fourteen *M. bovis* isolates recovered from pronghorn in 2019 (n=4) and 2020 (n=10) shared 100% sequence identity across loci used for MLST, and comprise a unique ST among all previously published isolates from North America. Based on phylogenetic analysis of concatenated MLST loci, the closest relatives of the pronghorn ST originated from cattle or bison from central Canada (ST 2 and ST 40), as well as the Pacific Northwest region (ST 3; Fig. 3). Isolates from pronghorn fall within a clade that includes the majority of bison isolates, as well as single isolates from mule deer and white-tailed deer. Of the *M. bovis* isolates recovered from regional bison and cattle for this study, five were previously unreported STs, while six were 100% homologous to published STs (Table 1). Our results are consistent with previous studies suggesting, based on MLST analyses, there is minimal geographic structuring of *M. bovis* STs within North America (Register et al. 2019). When compared globally, the pronghorn ST is identical to a single isolate recovered from a bovine with mastitis in Japan (ST 111; Hata et al. 2019). These findings build on previous characterization of pronghorn isolates from 2019 (Malmberg et al. 2020), which was limited due to a gene deletion in one of seven target loci used in a previous MLST scheme (Register et al. 2015). The 2020 assemblies from pronghorn contained the same deletion of the *adh-1* gene that was documented in isolates from 2019.

Environmental persistence

Mycoplasma bovis DNA was detectable by real-time PCR in hay and topsoil substrates for the entire 28-d study period in both sunlight and shade. In water, DNA was detectable for 5–7 d (Supplementary Material Fig. S2). Culture revealed *M. bovis* growth in substrates placed in direct sunlight for 1 h (water and topsoil) and 2 h (hay). In shaded substrates, *M. bovis* remained viable for 3 h (hay and topsoil) and 6 h (water). Over an 8-h span, the average temperature was 19.09 C (SD±12.47 C) in the sunlight and 15.17±14.26 C in the shade, with an average relative humidity of 39.78±13.02% and 50.78±11.51%, respectively (Supplementary Material Table S2). In mineral substrates, we did not detect *M. bovis* DNA or growth in culture at any point.

DISCUSSION

Disease emergence in a new host involves either novel exposure or pathogen mutation to surmount species barriers of infection (Plowright et al. 2017). Following a spillover event, infection and transmission dynamics can follow several courses. Many spillover events are "silent" and do not progress to detectable intensities, while others result in epidemics with variable adaption to and persistence in the new host (Parrish et al. 2008). Understanding such dynamics is critical to management of emerging diseases at the wildlife-livestock interface, including mycoplasmosis.

Multilocus sequence typing of *M. bovis* isolates from pronghorn revealed that a single ST was implicated in mortalities occurring in 2019 and 2020. It is possible that this ST represents a unique strain that is more

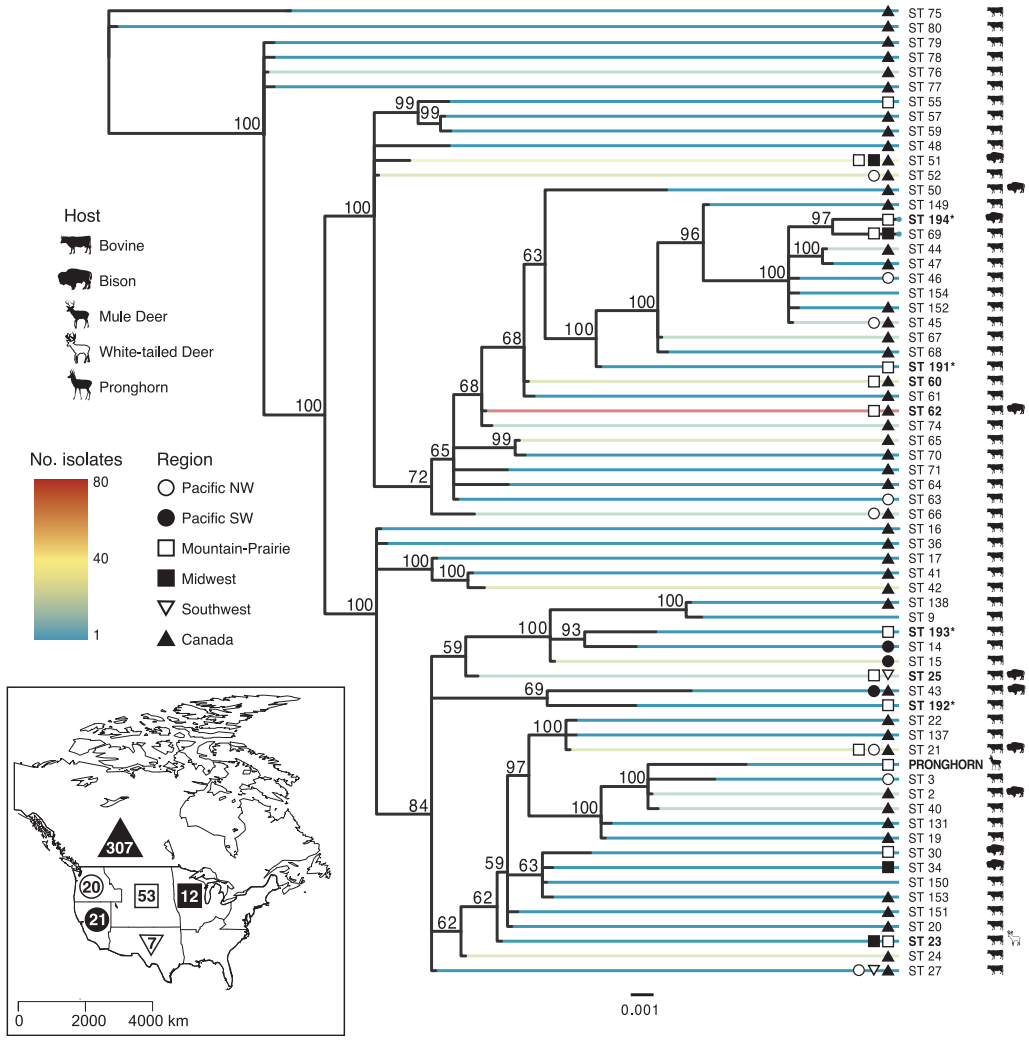

FIGURE 3. Bayesian phylogenetic consensus tree of *Mycoplasma bovis* multilocus sequence types found in North America (n=66). Numbers at nodes represent posterior probabilities. Branch length (shown in black) corresponds with the expected number of substitutions per site, while branch extension colors indicate the number of isolates of each sequence type (warmer colors indicate more isolates, and cooler colors indicate fewer isolates). Animal silhouettes represent hosts in which each sequence type has been identified). Shapes on the branches or branch extensions represent the region in which each sequence type has been found. Names beginning with "ST" correspond with PubMLST profiles (Jolley et al. 2018). Sequence types with names in bold text were detected in this study and described in Table 1. Stars indicate previously unreported sequence types. Map inset shows the number of isolates found in each respective geographic region for the contiguous USA and Canada, including isolates from PubMLST (Jolley et al. 2018) and from our study. Silhouettes from open source (MIT license), copyright T. Micheal Keesey (http://phylopic.org), used under Creative Commons License (Attribution Noncommercial 3.0 Unported, *Bison bison* silhouette) and Public Domain Dedication 1.0 (all other silhouettes).

transmissible across host species or specifically more infectious in pronghorn. However, it is also possible that pronghorn are susceptible to all strains of *M. bovis* and that spillover to pronghorn is due to novel exposure rather than evolution of the pathogen. Further, the sequence homology across 2019 and 2020 suggests that either infection of one ST was

maintained in pronghorn following a single spillover event or that recurrent, seasonally associated spillover events arose from a common source.

Persistent mycoplasma infections are well documented, though infection dynamics are variable across hosts. In cattle, subclinical infections are common, and clinical disease is often triggered by stress, immunosuppression, coinfections, or a combination of such variables (Maunsell et al. 2011). In contrast, *M. bovis* is more virulent in bison, and disease can occur in the absence of coinfecting pathogens (Register et al. 2018). Although little is known about chronic carrier status, prevalence in asymptomatic bison has been estimated at 3% (Register et al. 2021). These differences may be partially attributed to host-pathogen coevolution in cattle, in which the disease was initially identified approximately 60 yr ago (Hale et al. 1962). Mycoplasmosis is a more recently identified disease in bison, emerging in the early 2000s (Sweeney et al. 2013). Similar host-pathogen dynamics are well documented for *M. ovipneumoniae* in caprids; ancient infections in domestic sheep and goats have evolved toward low virulence, while more recent spillover to bighorn sheep is associated with high virulence (Cassirer et al. 2018).

We aimed to investigate the possibility of persistent infections in pronghorn by using nasal swabs tested by PCR. Given that pronghorn have high connectivity in Wyoming (LaCava et al. 2020), we reasoned that broad, opportunistic surveillance in Wyoming and adjacent regions could be useful to elucidate persistent or subclinical infections. Although we did not identify any subclinical infections, only 13 samples tested by PCR were obtained from hunt units within or adjacent to the outbreak region, yielding low confidence (upper 95% bCL, ~24.7%) for detection within the region of interest. Further, pronghorn in Wyoming are managed as 50 distinct herds that are delineated by natural or anthropogenic barriers (Emmerich et al. 2007). Despite permeability to gene flow (LaCava et al. 2020), such barriers could facilitate localized pathogen persistence that is not detectable through broad, opportunistic sampling. A systematic surveillance approach targeting the outbreak region would, thus, be useful to further assess for persistence of infection in pronghorn.

We frequently observed mule deer in close proximity to live pronghorn and pronghorn carcasses during field investigations in 2019 and 2020, yet we did not detect any *M. bovis* infections in mule deer. The absence of detectable infections suggests that mule deer were not sufficiently exposed despite proximity to infected pronghorn. Negative findings also help to exclude mule deer as a potential source of interspecific transmission.

Indirect contact via shared resources is thought to be an important driver of disease transmission at the wildlife-livestock interface, which remains unquantified in most systems (Yang et al. 2021). Although spatiotemporal overlap of pronghorn and cattle on range is perceptibly common, direct contact (i.e., nose to nose) is not frequently observed. Although mycoplasmas lack a cell wall and are therefore highly susceptible to desiccation and heat (McAuliffe et al. 2006), some conditions (i.e., humidity, shade) have been documented to permit environmental persistence of *M. bovis* for up to 8 m (Justice-Allen et al. 2010). We, therefore, hypothesized that if spillover from cattle resulted in pronghorn infection, environmental transmission may play an important role. To assess the potential for environmental transmission, we examined persistence of *M. bovis* in a variety of substrates commonly found on cattle range. Our results indicate that viable *M. bovis* can persist in water for 6 h and in grass hay and topsoil for up to 3 h. We found that shade, humidity, and cool temperatures prolong the viability of *M. bovis* in the environment, consistent with previous work by Justice-Allen et al. (2010). These findings suggest that the observed seasonality of pronghorn mortalities caused by *M. bovis* may be related to environmental conditions that permit bacterial persistence outside the host. The months of February and March often have cooler temperatures with areas of snow cover that could provide optimal

conditions for persistence. Further, natural resources may be scarce at this time, and pronghorn may be attracted to anthropogenic resources intended for livestock. Birdfeeders have been implicated as an important fomite for transmission of *M. gallisepticum* in house finches (Dhondt et al. 2007), suggesting that despite the fragility of mycoplasmas outside the host, transmission via the environment is an important pathway to consider, particularly if congregation around resources occurs.

There are several important limitations to this study. First, diagnosis of *M. bovis* is challenging due to complex shedding dynamics, confounding sampling approaches, and detection limitations (Sachse et al. 1993; Calcutt et al. 2018). Sampling by different methods (e.g., nasopharyngeal swab, bronchoalveolar lavage, transtracheal wash) might improve sensitivity compared with standard nasal swabs, and systematic sampling of regional livestock might aid in elucidating transmission pathways.

Second, it is important to consider that pronghorn range widely and exhibit variability in movement patterns. North to south seasonal migration has been well documented in some populations (Sawyer et al. 2005; Jakes et al. 2018), while year-round residency is an alternative strategy (Jones et al. 2020). Movement ecology and intraspecific contact dynamics of pronghorn in northeastern Wyoming have yet to be fully characterized. In northeastern Wyoming, snow depths are relatively low compared with other parts of the state, and pronghorn are observed year round. It is possible that unobserved mortalities associated with the outbreaks occurred following spring dispersal of pronghorn. Sporadic mortalities could also be difficult to detect, especially given that pronghorn in this area are not closely monitored or currently tracked using global positioning system collars.

Our collective findings are a foundational study of seasonally associated, virulent mycoplasmosis in a new species and suggest that livestock should be considered as potential reservoir hosts for *M. bovis* spillover into pronghorn and potentially other free-ranging ungulates. Though thus far localized to a single geographic region, the disease has shown potential for high mortality, seasonal occurrence, and rapid spread. Population-level assessment of pronghorn in Wyoming found no evidence of genetic subdivision and minimal evidence of isolation by distance in pronghorn (LaCava et al. 2020). Although such high connectivity may benefit the maintenance of genetic diversity, it may also increase opportunities for intraspecific transmission of an emerging infectious disease (Cross et al. 2009). Further, pronghorn are sensitive to anthropogenic habitat disturbances such as energy development (Sawyer et al. 2002; Beckmann et al. 2012; Christie et al. 2015), which could be exacerbated by additional population stressors. Finally, *Mycoplasma* spp. infections in other free-ranging hosts have significant conservation and management implications (Brown et al. 1994; Ley et al. 1996; Besser et al. 2008). Foundational study of mycoplasma spillover can help minimize ecological and economic impacts at the wildlife-livestock interface and inform future studies to better understand host range and risk of interspecific transmission.

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SUPPLEMENTARY MATERIAL

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LITERATURE CITED

Andrews S. 2010. *FastQC: A quality control tool for high throughput sequence data*. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/. Accessed September 2020.

Beckmann JP, Murray K, Seidler RG, Berger J. 2012. Human-mediated shifts in animal habitat use: Sequential changes in pronghorn use of a natural gas field in Greater Yellowstone. *Biol Conserv* 147:222–233.

Besser TE, Cassirer EF, Potter KA, VanderSchalie J, Fischer A, Knowles DP, Herndon DR, Rurangirwa FR, Weiser GC, Srikumaran S. 2008. Association of *Mycoplasma ovipneumoniae* infection with population-limiting respiratory disease in free-ranging Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*). *J Clin Microbiol* 46:423–430.

Brown MB, Schumacher IM, Klein PA, Harris K, Correll T, Jacobson ER. 1994. *Mycoplasma agassizii* causes upper respiratory tract disease in the desert tortoise. *Infect Immun* 62:4580–4586.

Calcutt MJ, Lysnyansky I, Sachse K, Fox LK, Nicholas RAJ, Ayling RD. 2018. Gap analysis of *Mycoplasma bovis* disease, diagnosis and control: An aid to identify future development requirements. *Transbound Emerg Dis* 65 (Suppl 1):91–109.

Cassirer EF, Manlove KR, Almberg ES, Kamath PL, Cox M, Wolff P, Roug A, Shannon J, Robinson R, et al. 2018. Pneumonia in bighorn sheep: Risk and resilience. *J Wildl Manage* 82:32–45.

Christie KS, Jensen WF, Schmidt JH, Boyce MS. 2015. Long-term changes in pronghorn abundance index linked to climate and oil development in North Dakota. *Biol Conserv* 192:445–453.

Cross PC, Drewe J, Patrek V, Pearce G, Samuel MD, Delahay RJ. 2009. Wildlife population structure and parasite transmission: Implications for disease management. In: *Management of disease in wild mammals*, Delahay RJ, Smith GC, Hutchings MR, editors. Springer, Tokyo, Japan, pp. 9–29.

Dhondt AA, Dhondt KV, Hawley DM, Jennelle CS. 2007. Experimental evidence for transmission of *Mycoplasma gallisepticum* in house finches by fomites. *Avian Pathol* 36:205–208.

Dyer NW, Krogh DF, Schaan LP. 2004. Pulmonary mycoplasmosis in farmed white-tailed deer (*Odocoileus virginianus*). *J Wildl Dis* 366–370.

Emmerich J, Guenzel R, Jahnke L, Kroger B, Nemick J, Rudd B, Woolley T. 2007. Pronghorn antelope (*Antilocapra americana*). In: *Handbook of biological techniques*, 3rd Ed., Tessmann SA, Bohne JR, editors. Wyoming Game and Fish Department, Cheyenne, Wyoming. pp. 1–59. https://wgfd.wyo.gov/WGFD/media/content/PDF/Wildlife/Handbook-BioTechniques/01-Pronghorn.pdf. Accessed February 2022.

Hale HH, Helboldt CF, Plastridge WN, Stula EF. 1962. Bovine mastitis caused by a *Mycoplasma* species. *Cornell Vet* 52:582–591.

Hata E, Harada T, Itoh M. 2019. Relationship between antimicrobial susceptibility and multilocus sequence type of *Mycoplasma bovis* Isolates and development of a method for rapid detection of point mutations involved in decreased susceptibility to macrolides, lincosamides, tetracyclines, and spectinomycin. *Appl Environ Microbiol* 85:e00575-19.

Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformtics* 17:754–755.

Jakes AF, Gates CC, DeCesare NJ, Jones PF, Goldberg JF, Kunkel KE, Hebblewhite M. 2018. Classifying the migration behaviors of pronghorn on their northern range. *J Wildl Manage* 82:1229–1242.

Jolley KA, Bray JE, Maiden MCJ. 2018. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Res* 3:124.

Jones PF, Jakes AF, Eacker DR, Hebblewhite M. 2020. Annual pronghorn survival of a partially migratory population. *J Wildl Manage* 84:1114–1126.

Justice-Allen A, Trujillo J, Corbett R, Harding R, Goodell G, Wilson D. 2010. Survival and replication of *Mycoplasma* species in recycled bedding sand and association with mastitis on dairy farms in Utah. *J Dairy Sci* 93:192–202.

LaCava MEF, Gagne RB, Love Stowell SM, Gustafson KD, Buerkle CA, Knox L, Ernest HB. 2020. Pronghorn population genomics show connectivity in the core of their range. *J Mamm* 101:1061–1071.

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9:357–359.

Ley DH, Edward Berkhoff J, McLaren JM. 1996. *Mycoplasma gallisepticum* isolated from house finches (*Carpodacus mexicanus*) with conjunctivitis. *Avian Dis* 40:480–483.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25:2078–2079.

Loy JD, Leger L, Workman AM, Clawson ML, Bulut E, Wang B. 2018. Development of a multiplex real-time PCR assay using two thermocycling platforms for detection of major bacterial pathogens associated with bovine respiratory disease complex from clinical samples. *J Vet Diagn Invest* 30:837–847.

Malmberg JL, O'Toole D, Creekmore T, Peckham E, Killion H, Vance M, Ashley R, Johnson M, Anderson C, et al. 2020. *Mycoplasma bovis* infections in free-ranging pronghorn, Wyoming, USA. *Emerg Infect Dis* 26:2807–2814.

Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J* 17:10.

Maunsell FP, Woolums AR, Francoz D, Rosenbusch RF, Step DL, Wilson DJ, Janzen ED. 2011. *Mycoplasma bovis* infections in cattle. *J Vet Intern Med* 25:772–783.

McAuliffe L, Ellis RJ, Miles K, Ayling RD, Nicholas RAJ. 2006. Biofilm formation by mycoplasma species and its role in environmental persistence and survival. *Microbiology* 152:913–922.

Miller RS, Farnsworth ML, Malmberg JL. 2013. Diseases at the livestock-wildlife interface: Status, challenges, and opportunities in the United States. *Prev Vet Med* 110:119–132.

Parrish CR, Holmes EC, Morens DM, Park E-C, Burke DS, Calisher CH, Laughlin CA, Saif LJ, Daszak P. 2008. Cross-species virus transmission and the emergence of new epidemic diseases. *Microbiol Mol Biol Rev* 72:457–470.

Plowright RK, Parrish CR, McCallum H, Hudson PJ, Ko AI, Graham AL, Lloyd-Smith JO. 2017. Pathways to zoonotic spillover. *Nat Rev Microbiol* 15:502–510.

Razin S, Yogev D, Naot Y. 1998. Molecular biology and pathogenicity of mycoplasmas. *Microbiol Mol Biol Rev* 62:1094–1156.

Register KB, Jelinski MD, Waldner M, Boatwright WD, Anderson TK, Hunter DL, Hamilton RG, Burrage P, Shury T, et al. 2019. Comparison of multilocus sequence types found among North American isolates of *Mycoplasma bovis* from cattle, bison, and deer, 2007–2017. *J Vet Diagn Invest* 31:899–904.

Register KB, Jones LC, Boatwright WD, Shury TK, Woodbury M, Hamilton RG, Treanor J, Dyer N, Nol P. 2021. Prevalence of *Mycoplasma* spp. in the respiratory tract of healthy North American Bison (*Bison bison*) and comparison with serum antibody status. *J Wildl Dis* 57:683–388.

Register KB, Lysnyansky I, Jelinski MD, Boatwright WD, Waldner M, Bayles DO, Pilo P, Alt DP. 2020. Comparison of two multilocus sequence typing schemes for *Mycoplasma bovis* and revision of the PubMLST reference method. *J Clin Microbiol* 58: e00283–20.

Register KB, Olsen SC, Sacco RE, Ridpath J, Falkenberg S, Briggs R, Kanipe C, Madison R. 2018. Relative virulence in bison and cattle of bison-associated genotypes of *Mycoplasma bovis*. *Vet Microbiol* 222: 55–63.

Register KB, Thole L, Rosenbush RF, Minion FC. 2015. Multilocus sequence typing of *Mycoplasma bovis* reveals host-specific genotypes in cattle versus bison. *Vet Microbiol* 175:92–98.

Rhyan JC, Spraker TR. 2010. Emergence of diseases from wildlife reservoirs. *Vet Pathol* 47:34–39.

Rosengarten R, Citti C, Glew M, Lischewski A, Droeße M, Much P, Winner F, Brank M, Spergser J. 2000. Host-pathogen interactions in mycoplasma pathogenesis: Virulence and survival strategies of minimalist prokaryotes. *Int J Med Microbiol* 290:15–25.

Rossetti BC, Frey J, Pilo P. 2010. Direct detection of *Mycoplasma bovis* in milk and tissue samples by real-time PCR. *Mol Cell Probes* 24:321–323.

Sachse K, Pfützner H, Hotzel H, Demuth B, Heller M, Berthold E. 1993. Comparison of various diagnostic methods for the detection of *Mycoplasma bovis*. *Rev Sci Tech* 12:571–580.

Sawyer H, Lindzey F, McWhirter D, Andrews K. 2002. Potential effects of oil and gas development on mule deer and pronghorn populations in western Wyoming. In: *Transactions of the sixty-seventh North American wildlife and natural resources conference*, Wildlife Management Institute, Dallas, Texas, 3–7 April, pp. 350–365.

Sawyer H, Lindzey F, McWhirter D. 2005. Mule deer and pronghorn migration in western Wyoming. *Wildl Soc Bull* 33:1266–1273.

Sikes RS. 2016. 2016 Guidelines of the American Society of Mammalogists for the use of wild mammals in research and education. *J Mamm* 97:663–688.

Sweeney SS, Jones RJ, Patyk K, LoSapio C. 2013. *Mycoplasma bovis*—An emerging pathogen in ranched bison. *Emerging Disease Notice. Document 222.0913*. Animal and Plant Health Inspection Service, US Department of Agriculture, Washington, DC, pp. 1–6. https://www.aphis.usda.gov/animal_health/nahms/bison/downloads/bison14/Bison14_Mbovis_1.pdf. Accessed November 2021.

Yang A, Boughton RK, Miller RS, Wight B, Anderson WM, Beasley JC, VerCauteren KC, Pepin KM, Wittemyer G. 2021. Spatial variation in direct and indirect contact rates at the wildlife-livestock interface for informing disease management. *Prev Vet Med* 194:105423.

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