Source and seasonality of epizootic mycoplasmosis in free-ranging pronghorn (*Antilocapra americana*)

Marguerite Johnson[1], Christopher MacGlover[1], Erika Peckham[2], Halcyon Killion[3], Samantha E. Allen[4], Terry Creekmore[5], William H. Edwards[5], Madison Blaeser[1], McKenzi Davison[1], Erin Schwalbe[1], Amy K. Wray[1], Thomas K. Bragg[6], Kerry S. Sondgeroth[1,3], Jennifer L. Malmberg[1,3]*

[1]University of Wyoming, Department of Veterinary Sciences, 1174 Snowy Range Road, Laramie, WY 82070, USA

[2]Wyoming Game and Fish Department, Gillette, WY 82716, USA

[3]Wyoming State Veterinary Laboratory, 1174 Snowy Range Road, Laramie, WY 82070, USA

[4]Wyoming Game and Fish Department, 1212 S. Adams St, Laramie, WY 82070, USA

[5]Wyoming Game and Fish Department, 1174 Snowy Range Road, Laramie, WY 82070, USA

[6]Turner Institute of Ecoagriculture, 901 Technology Blvd, Bozeman, MT 59718, USA

*Phone 307-766-9977, Email: Jennifer.Malmberg@uwyo.edu, Fax: (307) 721-2051

Word Count: 3,941

ABSTRACT

Mycoplasma bovis is an economically important bacterial pathogen of cattle and bison that most commonly causes pneumonia, polyarthritis and mastitis. *M. bovis* is prevalent in cattle and commercial bison; however, infections in other host species are rare. In early 2019, we identified the first known cases of *M. bovis* in free-ranging pronghorn (*Antilocapra americana*). Here we report on additional pronghorn mortalities caused by *M. bovis* occurring in the same geographic region of northeastern Wyoming one year later. Genetic analysis by multilocus sequence typing (MLST) revealed that the mortalities were caused by the same *M. bovis* sequence type, which is unique among all sequence types documented in North America. To determine if pronghorn maintain chronic infections and to assess *M. bovis* status in other sympatric species, we performed surveillance in free-ranging ungulates. We found no evidence of subclinical infections in pronghorn (*n*=231) or mule deer (*Odocoileus hemionus*) (*n*=231) based on PCR testing of nasal swabs. To assess the likelihood of environmental transmission from livestock to pronghorn, we examined persistence of *M. bovis* in various substrates and conditions, revealing that *M. bovis* remains viable for 6 hours following inoculation of shaded water, and up to 3 hours in direct sunlight substrates. Our results indicate that environmental transmission of *M. bovis* from livestock to pronghorn is possible, and seasonality of infection could be due to shared resources during the late winter. This study also highlights the importance of further investigations to better understand transmission dynamics, to assess population level impacts to pronghorn, and to determine disease risks among other ungulate taxa.

KEYWORDS

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—human and animal movement, grazing of livestock, and human land use are 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 (580–1840 Kb), 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 *M. ovipneumoniae* in bighorn sheep (*Ovis canadensis*) (Besser et al. 2008), *M. gallisepticum* in house finches (*Carpodacus mexicanus*) (Ley et al. 1996), and *M. agassizii* in wild tortoises (*Gopherus* spp.) (Brown et al. 1994). The most recent example of mycoplasma emergence in wildlife is *M. bovis* in pronghorn *(Antilocapra americana*) (Malmberg et al. 2020).

Mycoplasma bovis is a globally distributed bacterial pathogen of economic importance in cattle and commercial 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) as well as in free-ranging mule deer (*O. 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. Here we use a combination of genetic analysis, multihost surveillance, and environmental assessment to investigate the source and seasonality of pronghorn *M. bovis* infections. We hypothesize that pronghorn may be attracted to livestock resources during the late winter and that shared environmental resources may be an important consideration for transmission.

MATERIALS AND METHODS

Study Site

Epizootics of *M. bovis* were documented north of Gillette, Wyoming, USA predominantly on privately owned pasture and range land (Figure 1). Elevations ranged from 1250 meters to 1455 meters 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 (WGFD) and Wyoming State Veterinary Laboratory (WSVL) personnel obtained samples from pronghorn carcasses. When intact carcasses were available, a detailed post-mortem examination was performed by a board-certified pathologist. Diagnostic evaluation included histopathology, bacteriology (aerobic and anaerobic culture, *Mycoplasma* culture, and *M. bovis* PCR (all respectively described below); 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 in Malmberg et al. 2020

(Supplemental Table 1). 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 Mycoplasma bovis real-time PCR

DNA was extracted from fresh lung tissue or nasal swab using the DNeasy blood and tissue kit (Qiagen, Germantown, MD, USA). Real-time PCR targeting the *oppD* gene was performed using primers and probes described in Loy et al. 2018. Each reaction contained 1 µL of primer probe mix (primers diluted to 10 µM and probe to 2.5 µM in nuclease-free water), 5 µL (20-100 ng) of template DNA, 12.5 µL of master mix (BioRad, Hercules, CA, USA), 0.5 µL of Xeno Internal Control Positive LIZ Assay (Applied Biosystems, Waltham MA, 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 (Hardy Diagnostics Mycoplasma broth, Hardy Diagnostics, Santa Maria, CA, USA) and incubated with a loose lid at 37°C in 10% CO_2 for 72 hours. Subsequently, we inoculated 100 µl of broth onto commercial *Mycoplasma* spp. agar (Hardy Diagnostics, Mycoplasma Agar with Cefoperazone) and spread evenly over the entire plate with a sterile swab. Plates were incubated at 37°C in 10% CO_2 for 24–72 hours. Incubation period was complete after a confluent layer of colonies was present throughout 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/swabs were aseptically inoculated onto Columbia Blood Agar plates (CBA) with 5 % sheep blood and MacConkey Agar plates (Hardy Diagnostics, Santa Maria, CA, USA). The plates were struck to form three zones for isolation and incubated at 37 C in 10% CO_2. A CBA plate was also incubated in anaerobic conditions in a Baker-Ruskinn Bugbox (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 using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) (Bruker Daltonics GmbH & Co. KG, Bremen, Germany).

Surveillance

Nasal swabs (polyester fiber-tipped applicators, Cardinal Health, Waukegan IL, USA) were collected from free-ranging pronghorn throughout Wyoming and Montana and mule deer throughout Wyoming and frozen in media comprised of tryptic soy broth (TSB) with 15% glycerol for transport to the laboratory. Sources of samples included cases submitted to WSVL for routine post-mortem examination, swabs collected at hunter check stations, and swabs obtained from live animal captures in conjunction with other wildlife research (Table 1). All live animals were handled in accordance with IACUC 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=231) and mule deer (n=231) collected from 2019 to 2021 (Table 2). Samples included both post-mortem (n=346) and ante-mortem (n=116) nasal swabs. A total of 45 post-mortem swabs were collected from pronghorn in 2020 at WGFD hunter check stations

(Antelope Hunt Area 73 near Casper, Wyoming (*n*=34) and Antelope Hunt Areas 24 and 17 near Gillette, Wyoming (*n*=11)). Thirty-one swabs were collected from pronghorn during routine diagnostic cases at WSVL. Samples from Montana consisted of 150 swabs collected during live captures, and 5 aditional samples collected post-mortem. For mule deer, samples included 196 swabs from live captures and 35 post-mortem swabs from WSVL cases. To confirm that nasal swabs were suitable for *M. bovis* detection, we performed paired diagnostic *M. bovis* PCR as described above on lung tissue and nasal swabs from infected pronghorn (*n*=23).

Whole Genome Sequencing

DNA extractions of pure isolates subcultured from broth onto solid media were performed using the DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA) on an automated nucleic acid extraction platform (QIAcube, Qiagen). DNA extract was assessed for purity (260/280 nm) (NanoDrop™ 2000, Thermo Scientific, Grand Island, NY, USA) and concentration (Qubit 3.0 fluorometer, Thermo Scientific). A DNA sequencing library was prepared using the Nextera DNA Flex library Prep Kit (Illumina, San Diego, CA, USA). The library quantity was measured using the Qubit 3.0 fluorometer (Thermo Scientific). Lengths of DNA library fragments were assessed using the TapeStation 4200 (Agilent, Santa Clara, CA, USA), and after normalization, DNA libraries were sequenced on a MiSeq using V2 2 x 250 base pair (bp) cycle chemistry with ~8.0 Gb of output (Illumina). Post-sequencing statistics were evaluated 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) using Cutadapt (Naikare et al. 2015); (2) mapping of trimmed reads to the genome of *M. bovis* international reference strain PG45 (GenBank accession number NC_014760) using Bowtie2 (Ziegler et al. 2014); (3) conversion of .sam files

to .bam files using Samtools (Martin 2011); and (4) viewing of sorted .bam files in Geneious Prime 2019.1.3. Consensus sequences were generated from mapped reads using the highest quality parameter in Geneious Prime as a threshold (sum of residue quality exceeds 60%). 'N' was assigned to sites with coverage <three reads to represent missing data. Consensus sequences were trimmed to seven 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 numbers 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 to other publicly available North American sequence types from the *M. bovis* PubMLST database (Jolley et al. 2018 Wellcome Open Res 3:124). Unique sequence types were retained and were aligned using Muscle (Li et al. 2009). Bayesian phylogenetic inference was performed 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 MCMC 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 quarts) (MilliQ), and loose mineral supplement (2.0 kg) (Agland 12:12 Mineral, Agfinity Inc). Each substrate was prepared in duplicate and placed in a small

primary container. All contents were autoclaved, then inoculated with approximately 2.14 x10^5 CFUs of *M. bovis* recovered from pronghorn in 2020. Final concentrations were determined using colony counts on *Mycoplasma* spp. agar (Hardy Diagnostics, Mycoplasma Agar with Cefoperazone). One set of substrates was placed in direct sunlight, and one set in a shaded area.

Substrate Sampling

On day one, sampling was performed every hour for 8 hours. Substrates were further sampled at hours 24, 48, and 72, and days 5, 7, 14, 21, and 28 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 ºC for 72 hours at 5% CO_2 for culture. Mineral, hay, and topsoil substrates were swabbed and individually placed in 1 mL of PBS 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-hour incubation, samples in broth were plated on *Mycoplasma* spp. agar (Hardy Diagnostics, Mycoplasma Agar with Cefoperazone), then incubated for an additional 72 hours prior to assessment. Colony growth was confirmed as *M. bovis* using real-time PCR as described below.

Environmental Real-time PCR

Real-time PCR was performed in triplicate on all environmental samples using a protocol adapted from Rossetti et al. 2010. Briefly, PCR reactions consisted of 5µL 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 Path-ID™ qPCR Master Mix (428864, Applied Biosystems), 0.5 µL of Xeno Internal Control Positive LIZ Assay (Applied Biosystems, Waltham MA, USA) and nuclease-free water to 25 µL.

Cycling conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 56°C for 60s.

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 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 (Supplemental Figure 1). Infections were identified in both sexes and all age classes. Cattle with respiratory disease were not identified in the region of the outbreak. While 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 located approximately 64 kilometers to the southeast of the study site. An outbreak of *M. bovis* was not reported in these bison during the study period.

Diagnostic Evaluation

Thirty-seven cases of *M. bovis* were confirmed in pronghorn in 2020 via the previously described diagnostic *M. bovis* real-time PCR (Supplemental Table 1). *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 15/20 (75%).

Surveillance

Mycoplasma bovis was not detected in any pronghorn or mule deer nasal swabs collected for surveillance. Using lung tissue harvested post-mortem 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 post-mortem interval of ~1 week (*n*=3).

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 sequence type among all previously published isolates from North America. Based on phylogenetic analysis of concatenated MLST loci, the closest relatives of the pronghorn sequence type originated from cattle and/or bison from central Canada (ST 2 and ST 40), as well as the Pacific Northwest region (ST 3) (Figure 2). 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 comprised previously unreported sequence types, while six were 100% homologous to published sequence types (Table 1). Our results are consistent with previous studies suggesting that there is minimal geographic structuring of *M. bovis* sequence types within North America based on MLST analyses, and sequence types found in bison do not form a monophyletic group (Register et al. 2019). When compared globally, the pronghorn sequence type 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 employed 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-day study period in both sunlight and shade. In water, DNA was detectable for 5-7 days (Supplemental Figure 3). Culture revealed *M. bovis* growth in substrates placed in direct sunlight for 1 hour (water and topsoil) and 2 hours (hay). In shaded substrates, *M. bovis* remained viable for 3 hours (hay and topsoil) and 6 hours (water). Over an 8-hour span, average temperature was 19.09 ºC (±12.47 ºC) in the sunlight and 15.17 ºC (±14.26 ºC) in the shade, with an average relative humidity of 39.78% (±13.02%) and 50.78% (±11.51%), respectively (Supplemental Table 2). 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 sequence type was implicated in mortalities occurring in 2019 and 2020. It is possible that this sequence types represents a unique strain that is more 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 in 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 sequence type 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, co-infections, or a combination of such variables (Maunsell et al. 2011). In contrast, *M. bovis* is more virulent in bison, and disease can occur in absence of co-infecting 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. 2021b). These differences may be partially attributed to host-pathogen coevolution in cattle, in which the disease was initially identified approximately 60 years ago (Hale et al. 1962). Mycoplasmosis is a more recently identified disease in bison, emerging in the early 20th century (USDA 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 using nasal swabs tested by PCR. Given that pronghorn have high connectivity in Wyoming (LaCava et al. 2020) and that little is known about movement of pronghorn in northeastern Wyoming, we reasoned that broad, opportunistic surveillance of pronghorn in Wyoming and adjacent regions could be useful to elucidate persistent or subclinical infections. Although we did not identify any subclinical infections, only eight samples tested by PCR were obtained from hunt units within or adjacent to

the outbreak region. Surveillance 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 close 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 that remains unquantified in most systems (Yang et al. 2021). While 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 months (Justice-Allen et al. 2010). We therefore hypothesized that if spillover from cattle resulted in pronghorn infection(s), environmental transmission may have played an important role. To assess the potential for environmental transmission from livestock to pronghorn, 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 hours and in grass hay and topsoil for up to 3 hours. We found that shade, humidity, and cool temperatures prolong the persistence of viable *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 persistence of the bacteria 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 (i.e., nasopharyngeal swab, bronchoalveolar lavage, transtracheal wash) could improve sensitivity as compared to standard nasal swabs, and systematic sampling of regional livestock could aid in elucidating transmission pathways. Further, while PCR is adequate to detect active infections and shedding of bacteria, a serologic assay for pronghorn would be ideal to assess exposure. *M. bovis* serology, however, has proven challenging in bison, and cross-reactivity with other mycoplasmas is possible (Register et al. 2013, 2021a; Bras et al. 2017).

Lastly, 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. Snow depths are relatively low in this region as compared to other parts of Wyoming, 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 currently tracked using GPS collars or otherwise closely monitored.

Our collective findings comprise 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). While such high connectivity can 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 ecologic and economic impacts at the wildlife-livestock interface and inform future studies to better understand host range and risk of interspecific transmission.

ACKNOWLEDGEMENTS

We are thankful for the contributions and collaboration from the Wyoming Game and Fish Department and faculty and staff at the Wyoming State Veterinary Laboratory, specifically Marce Vasquez for his knowledge and assistance with qPCR. We would also like to thank members of the Wyoming Cooperative Fish and Wildlife Research Unit, specifically members of the Monteith

Shop who collected swabs from their respective wildlife captures. Additionally, we would like to thank Dr. Jennifer Ramsey and other members of Montana Fish, Parks and Wildlife for contributing nasal swabs from pronghorn in Montana. Authors would like to thank all landowners who allowed access to their land for carcass sampling and all hunters who allowed sample collection at check stations. This work was supported by the Y Cross Ranch Scholarship, The Riverbend Ranch Endowment in Wildlife-Livestock Health, and numerous supporters of the WDA Crowd Funding Campaign.

LITERATURE CITED

Andrews S. 2010. Babraham Bioinformatics - 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.

Bras AL, Suleman M, Woodbury M, Register K, Barkema HW, Perez-Casal J, Windeyer MC. 2017. A serologic survey of *Mycoplasma* spp. in farmed bison (*Bison bison*) herds in western Canada. *J Vet Diagnostic Investig* 29:513–521.

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* 65:91–109.

Cassirer EF, Manlove KR, Almberg ES, Kamath PL, Cox M, Wolff P, Roug A, Shannon J, Robinson R, Harris RB, 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. *Management of Disease in Wild Mammals*. 9–29.

Dhondt AA, Dhondt K V, 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.

Hale H, 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* 85:1–14.

Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Yang and Rannala. *Bioinforma Appl NOTE* 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.

Jones PF, Jakes AF, Eacker DR, Hebblewhite M. 2020. Annual Pronghorn Survival of a Partially Migratory Population. *J Wildl Manage* 84:1114–1126.

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 Mammal* 101:1061–1071.

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, Project G, et al. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinforma Appl NOTE* 25:2078–2079.

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

Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* 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.

Naikare H, Bruno D, Mahapatra D, Reinisch A, Raleigh R, Sprowls R, Ferguson DC, Hoenig M. 2015. Development and evaluation of a novel taqman real-time pcr assay for rapid detection of *Mycoplasma bovis*: Comparison of assay performance with a conventional PCR assay and another taqman real-time PCR assay. *Vet Sci* 2:32–42.

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. 1094–1156.

Register KB, Jelinski MD, Waldner M, Boatwright WD, Anderson TK, Hunter DL, Hamilton RG, Burrage P, Shury T, Bildfell R, 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 Diagnostic Investig* 31:899–904.

Register KB, Jones LC, Boatwright WD, Shury TK, Woodbury M, Hamilton RG, Treanor J, Dyer N, Nol P. 2021a. Prevalence of *Mycoplasma* spp. in the respiratory tract of healthy North American Bison (*Bison bison*) and comparison with serum antibody status. *J Wildl Dis* 57:683–688.

Register KB, Jones LC, Boatwright WD, Shury TK, Woodbury M, Hamilton RG, Treanor J, Dyer N, Nol P. 2021b. 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.

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, Sacco RE, Olsen SC. 2013. Evaluation of enzyme-linked immunosorbent assays for detection of *Mycoplasma bovis*-specific antibody in bison sera. *Clin Vaccine Immunol* 20:1405–1409.

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. *U.S. Bureau of Land Management Papers.* 5.

Sawyer H, Lindzey F, WcWhirter D. 2005. Mule deer and pronghorn migration in western Wyoming. *Wildlife Society Bulletin* 33(4):1266–1273.

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

USDA (United States Department of Agriculture). 2013. *Mycoplasma bovi*s- An emerging pathogen in ranched Bison. 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.

Ziegler JC, Lahmers KK, Barrington GM, Parish SM, Kilzer K, Baker K, Besser TE. 2014. Safety and immunogenicity of a *Mycoplasma ovipneumoniae* bacterin for domestic sheep (*Ovis aries*). *PLoS One* 9.

Table 1. Summary of *Mycoplasma bovis* isolates from this study used in phylogenetic analysis.

						GenBank accession numbers						
ID	Year	Host	Site	Region	Sequence type	*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 ungulates in Wyoming and Montana, USA. *Mycoplasma bovis* was not detected in surveillance samples.

Year	Mule Deer		Pronghorn		Total
	ante-mortem	post-mortem	ante-mortem	post-mortem	
2019	0	2	0	2	4
2020	168	26	0	62	256
2021	28	7	150	17	202
Total	196	35	150	81	**462**

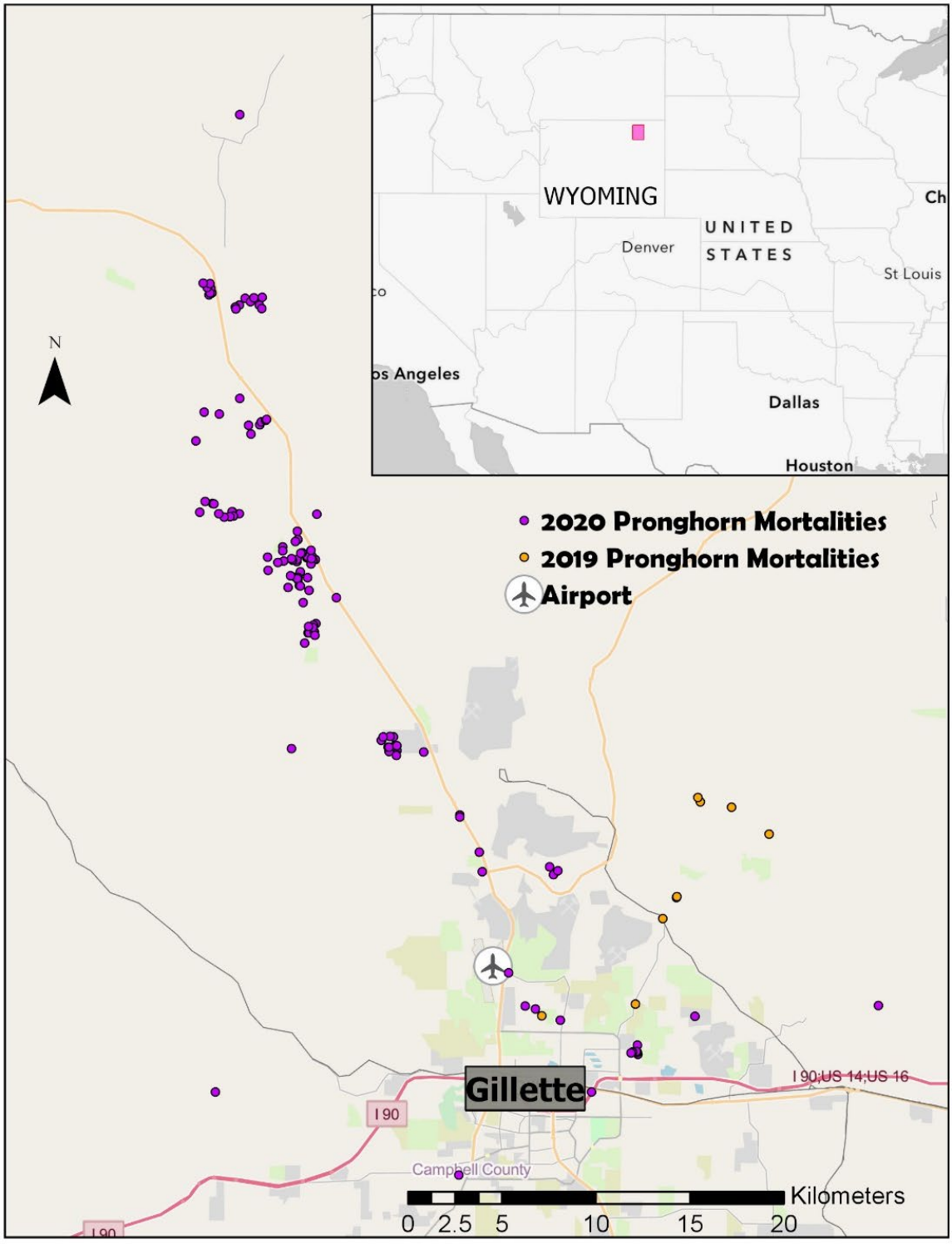

Figure 1. Pronghorn (*Antilocapra americana)* mortalities associated with *Mycoplasma bovis* infections in northeastern Wyoming, USA. Orange circles represent confirmed cases in 2019 (*n*=9), while purple circles indicate confirmed cases from 2020 (*n*=37) and sites of additional carcasses (presumptive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

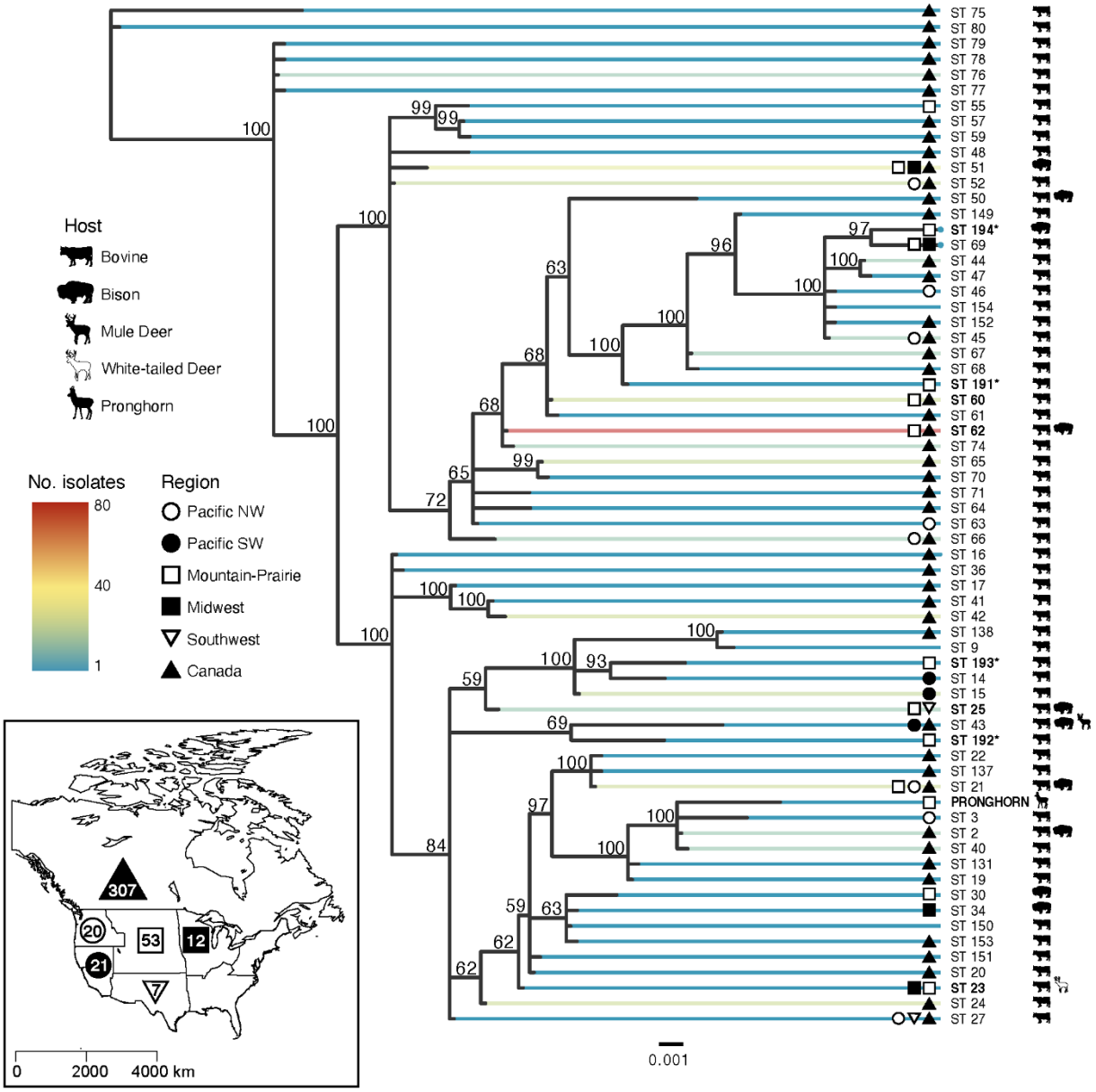

Figure 2. Bayesian phylogenetic consensus tree of *Mycoplasma bovis* multilocus sequence types found in North America (n=66). Numbers at nodes represent posterior probabilities. Branch length (shown in black) corresponds with the expected number of substitutions per site, while branch extension colors indicate the number of isolates of each sequence type (warmer colors indicate more isolates, while cooler colors indicate fewer isolates). Animal silhouettes represent hosts in which each sequence type has been identified (obtained from phylopic.org). Shapes on the branches or branch extensions represent the region where each sequence type has been found. Names beginning with "ST" correspond with pubMLST profiles. Sequence types with names in bolded text were detected in this study and described in Table 2. Stars indicate previously unreported sequence types. Map inset shows the number of isolates found in each respective

geographic region for the contiguous U.S. and Canada, including isolates from pubMLST and from this study.