Mycoplasma ovipneumoniae Strains Associated with Pneumonia Outbreaks in North American Bighorn Sheep

LOGAN K. WEYAND, *Washington State University, College of Veterinary Medicine, PO Box 647010, Pullman, WA 99164-7010, USA; weyandl@vetmed.wsu.edu*
E. FRANCES CASSIRER, *Idaho Department of Fish and Game, 3316 16th St., Lewiston, ID, 83501, USA*
GRETCHEN E. KAUFMAN, *Paul G. Allen School for Global Animal Health, PO Box 647090, Washington State University, Pullman, WA, 99164-647090, USA*
THOMAS E. BESSER, *Washington State University, College of Veterinary Medicine, Department of Veterinary Microbiology and Pathology, PO Box 647010, Pullman, WA 99164-7010, USA*

ABSTRACT Epizootic all-age pneumonia outbreaks (and subsequent recurrent lamb pneumonia outbreaks) significantly limit many North American bighorn sheep (*Ovis canadensis*) populations but vary widely in severity and duration. We conducted a pilot study to determine whether *Mycoplasma ovipneumoniae* genetic strain types were associated with outbreak severity, using 1) *M. ovipneumoniae p113,* the gene encoding a putative *M. ovipneumoniae* adhesin, and 2) multi-locus sequence typing (MLST) based on partial DNA sequences of four loci (the small ribosomal subunit, the 16S-23S intergenic spacer, and *rpoB* and *gyrB* housekeeping genes). For both of these comparisons, we examined whether clusters of genetically similar *M. ovipneumoniae* strains shared similar outbreak severity, using data from the Wild Sheep Working Group of the Western Association of Fish and Wildlife. The *p113* locus data and the MLST data each produced several well-supported clusters, but these clusters were not detectably associated with outbreak severity. Our study may have been limited by several factors, including detection bias towards severe outbreaks, insufficient sequence data, and perhaps the impact of selection pressure on *p113,* which is a cell surface protein. Future research may provide better assessment of the role of *M. ovipneumoniae* genetic strain types in contributing to outbreak severity.

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When developing solutions for complex and persistent wildlife disease, it is important to understand the factors that influence the pathology, transmission dynamics, and severity of the disease. Since the introduction of pneumonia causing pathogens to bighorn sheep in North America in the mid-nineteenth century, bighorn sheep *(Ovis canadensis*) have been limited by all-age epizootic pneumonia and annual recurrent lamb pneumonia events that combine to create significant population limiting effects (Cassirer et al. 2013, Manlove et al. 2016). Our understanding of bighorn sheep pneumonia is still developing and there are many aspects of the disease that require investigation, including the observed variation in disease severity and persistence. Here we report the results of a pilot study designed to analyze potential relationships between the genetics of *Mycoplasma ovipneumoniae* (*M. ovipneumoniae*) and disease severity (Besser et al. 2013).

In the all-age epizootic form of

bighorn sheep pneumonia, there is considerable variation in outbreak mortality (Cassirer et al. 2013). Because there is typically only a single *M. ovipneumoniae* genetic strain associated with each outbreak, we hypothesized that variation in outbreak severity may be attributable, in part, to differences in strain-specific genetic repertoires that are potentially associated with virulence (Besser et al. 2012). To evaluate strain-specific *M. ovipneumoniae* genetics, we utilized two approaches. First, we analyzed the partial sequence of *p113,* which encodes the protein P113, a putative *M. ovipneumoniae* adhesin thought to be involved in binding to host respiratory epithelial cilia. The sequenced region included bases 375-659 of the 3,240 bp gene, predicted to encode amino acids 92-186 of the extracellular domain (NCBI 2015, Yang et al. 2014). Second, we analyzed the overall. *ovipneumoniae* phylogeny as assessed by multi-locus sequence typing (MLST) developed for a separate study (Cassirer et al. 2017). The MLST included sequence data from four loci: the small ribosomal subunit (343 bp), the 16S-23S intergenic region (390– 420 bp), *rpoB* (562 bp), and *gyrB* (400 bp). In both of these approaches, we looked for association between *M. ovipneumoniae* genetic strain clusters and the reported severity of the associated outbreaks from which they were detected.

METHODS

Study areas

We included bighorn pneumonia outbreaks where both mortality estimates and information on *M. ovipneumoniae* DNA were available (n = 40; Fig. 1). Geographically these outbreaks ranged from North Dakota to southern Nevada, and from Washington to Nebraska. These outbreaks spanned a 29-year period from 1985 to 2014. Climate, topography, disturbance regimes, vegetation character, and other ecological factors vary widely amongst all populations (Fig. 1).

Experimental design

Epizootic all-age die offs commonly characterize the first observations of disease after initial introduction of an *M. ovipneumoniae* strain to a bighorn population (Plowright et al. 2013). Typically there is only one *M. ovipneumoniae* strain per epizootic, so we used outbreak data compiled by the Western Association of Fish and Wildlife Agencies' Wild Sheep Working Group to identify outbreaks from which banked *M. ovipneumoniae* DNA extracts were available (Besser et al. 2012). For each *M. ovipneumoniae* strain, we identified the year of the outbreak, the population involved and its location, and the estimate of percent mortality associated with the outbreak (Table 1).

Laboratory technique

Gene amplification: We amplified a 285 bp fragment of *p113* from each *M. ovipneumoniae* strain using the PCR technique reported by Yang et al. (2014). For each strain, the reaction mixture included 2.0 µL of genomic DNA, 12.5 µL Qiagen master mix, 1.0 µL each of *p113* forward and reverse primers (10 µM each), and 8.5 µL deionized water. Thermocycling conditions included initial denaturation (15 min at 95 C), 45 cycles of denaturation (30 sec, 95 C), annealing (30 sec, 56 C), and extension (30 sec, 72 C), and a final extension (7 min, 72 C). The positive control was *M. ovipneumoniae* strain 'Crony' and the negative control was a DNA extract of deionized water.

Gel electrophoresis: We separated *p113* PCR product amplicons by gel electrophoresis to confirm the appropriate size. Gels (80 mL TAE, 1 g. agarose, 4.0 µL EtBr) were electrophoresed (60 min, 100 v, 261 mAmps) with the 15 wells including size ladders (product, source) in lanes 1 and 15, and samples (5.0 µL) of positive controls (lane 13), negative controls (lane 14), and test samples (lanes 2-12). Following electrophoresis, gels were imaged (Alpha

Imager HP system, ProteinSimple, San Jose, CA) to confirm the presence of a DNA product equivalent in size to the positive control in each test sample lane.

Preparation of amplicon for sequencing: We treated *p113* PCR products (20.0 μL) with 0.4 μL ExoI (20 U/μL), 1.6 μL FastAP (1U/μL), 0.4 μL 10x FastAP RB buffer, and 1.6 μL deionized water, and incubated them for 20 min at 37 C, followed by 15 min at 80 C). Treated products (20.0 μL) were submitted for sequencing to Eurofins Genomics Company, Louisville, KY.

Sequence analysis: We used Sequencher 5.1 software (Gene Codes Corporation, Ann Arbor, MI) to assemble forward and reverse DNA sequence reads, and to trim the assembled sequences. Sequencing chromatograms were manually examined and edited to remove unsupported insertions or deletions, and to correct ambiguous base calls due to artifacts.

Sequences were aligned in Clustal Omega (Clustal Omega 1.2.1, www.ebi.ac.uk/Tools/msa/clustalo/, accessed 1 November 2015).

Phylogenetic analysis

Phylogeny construction: We formatted aligned *p113* and MLST sequences to NEXUS file format and used these for analyses in Bayesian phylogenetic analytical software (MrBayes http://mrbayes.sourceforge.net/, accessed 4 March 2016). The number of generations in the analyses was 6 million for *p113* and 8 million for MLST, and burn-in was 25% of generations. The evolutionary model is a general time reversible model with a proportion of invariable sites and a gamma shaped distribution of substitution rates across sites. We used Dirichlet (non-informative) priors. Convergence was assessed by standard deviation of split frequencies (*p113*: 0.005; MLST: 0.008). For cluster identification, clusters were selected by clade probability>90%, lumped to ensure at least 3 strains per clade. Where multiple well-supported nodes existed within a clade, we attempted to maximize the number of well-supported clusters each containing at least 3 strains. The topologies of the phylogenetic trees for *p113* and MLST were compared to subjectively identify concordant clusters.

Statistical analysis: We conducted a simple analysis of variance of estimated outbreak percent mortality by cluster to detect possible association between outbreak mortality rates and genetic clusters detected within either *p113* or MLST sequence phylogenies (RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/). Our null hypothesis predicted that average mortality estimates would not vary significantly among genetic clusters, and our research hypothesis predicted that average mortality estimates would vary significantly among genetic clusters. We used the median outbreak severity (estimated 45% mortality) to define the cut-point between severe and moderate/mild outbreaks.

Selection analysis

To evaluate differences in selection pressure on *p113* relative to two *M. ovipneumoniae* housekeeping genes, *rpoB* and *gyrB*, we determined the average dN/dS ratios for each set of sequences. The dN/dS ratio is the ratio of non-synonymous mutations (mutations that change the amino acid sequence) to synonymous mutations (mutations that do not change the amino acid sequence), a measure that is possible because of the degeneracy in the genetic code: multiple codon sequences may encode a single amino acid residue. dN/dS greater than 1.0 denotes positive selection (when non-synonymous changes are favored), equal to 1.0 denotes neutral selection or drift, and less than 1.0 denotes constraining selection against altered amino acid sequence (Graur and Li 2000).

Table 1. Population outbreak strain samples and their corresponding year of outbreak, state in which outbreak was first reported, population, estimated outbreak percent mortality, and membership in *M. ovipneumoniae p113* and/or *M. ovipneumoniae* MLST phylogenetic clusters.

Year	State	Population	Population Abbreviation	Outbreak Mortality (%)	*p113* Cluster	MLST Cluster
1985	NV	Desert Sheep Range	DSR	75	3	1
1991	MT	Anaconda	AN	75	2	4
1994	MT	Tendoys	TE	87		2
1995	OR	Wenaha	WE	54		1
1995	WA	Black Butte404**	BB404	55		
1997	MT	Taylor Hilgard***	TH	75		
1997	ND	Ice Box Canyon	IBC	87		2
2000	ID/OR	Sheep Mtn***	SM	51		
2003	SD	Custer State Park	CU	75*		3
2004	NE	Fort Robinson	FR	54		1
2006	WA	Black Butte415***	BB415	35		
2007	CO	Fossil Ridge	FO	74	1	3
2008	MT	Elkhorns	EL	83	1	3
2009	CA	White Mountains	WH	Mild	3	3
2009	MT	Bonner	BO	71*		4
2009	MT	East Fork Bitterroot***	EFB	52*		
2009	MT	Lower Rock Creek	LRC	54	2	4
2009	WA	Yakima Canyon	YC	34*		2
2010	MT	Sun River389	SR389	34		4
2010	MT	Sun River398	SR398	34	3	3
2010	NE	Barrel Butte	BA	17	3	3
2010	SD	Spring Creek	SC	20*	3	3
2010	UT/NV	Pilot Leppy405	PL405	44		1
2010	UT/NV	Pilot Leppy398	PL398	44	3	3
2011	NV	Pancakes	PA	14	2	4
2011	NV	Snowstorms	SN	50		4
2012	NV	Grant Range	GR	45	2	3
2012	NV	McCullough	MC	27	3	1
2012	NV	Slate Mountain	SL	28		3
2012	WA	Asotin Creek	AS	36		1
2013	MT	Cinnabar	CI	21	1	3
2013	MT	Gardiner	GA	21	1	3
2013	MT	Mt. Everts	ME	21		3
2013	NV	Spring Range401	SP401	19		4
2013	NV	Spring Range406	SP406	19	3	
2013	WA	Tieton	TI	Severe		4
2014	ND	Sheep Creek	SC	30		3
2014	NE	Sowbelly	SO	40	3	3
2014	NV	Santa Rosa	SA	40		3
2014	WA	Black Butte393	BB393***	33		

Notes to Table 1: Strain clusters identified in both the *M. ovipneumoniae p113* and *M. ovipneumoniae* MLST phylogenies are denoted numerically (1, 2, 3, 4) and strain membership in a certain phylogenetic cluster is listed in columns 6 and 7; *culled (percent mortality value may have been higher or lower if symptomatic individuals had not been culled); **outbreak occurred at Sheep Mountain ID/OR; ***strain did not resolve into any phylogenetic cluster identified in our analysis.

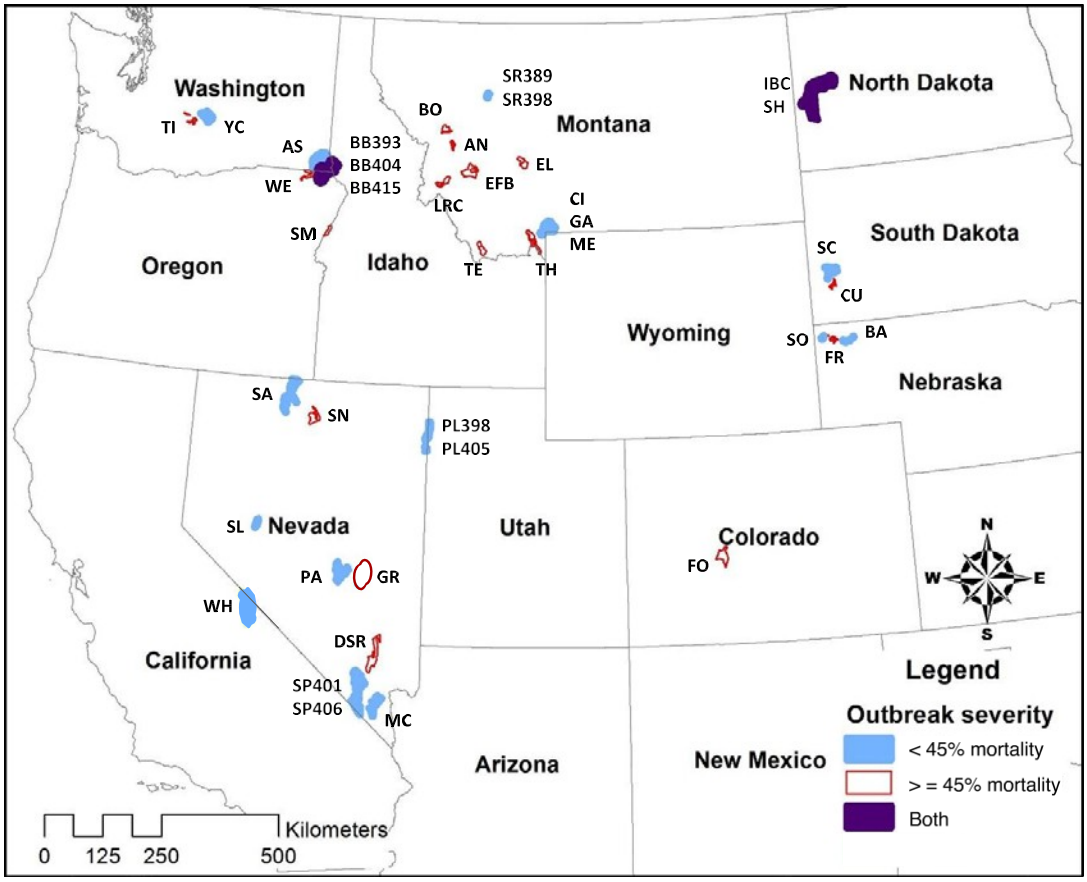

Figure 1. Geographic distribution of the 40 bighorn sheep populations in North America in which epizootic pneumonia outbreaks occurred with severity documented by the Western Association of Fish and Wildlife Agencies (WAFWA) Wild Sheep Working Group (WSWG), and for which *M. ovipneumoniae* DNA extracts were available. See Table 1 for population identification codes.

RESULTS

We produced 285 bp DNA fragment sequences of *M. ovipneumoniae p113* for each population, and they were highly polymorphic. The *M. ovipneumoniae p113* phylogeny produced only a few well-supported clades, and the mean mortality estimates in each cluster were not significantly different [F = 0.628; df = 2,14; P = 0.548] (Fig. 2). *M. ovipneumoniae* MLST sequences produced slightly more well-supported clades; however, these were also not significantly associated with outbreak severity [F = 1.54; df = 3,27; P = 0.228] (Fig. 3).

Our dN/dS analysis indicates that the three *M. ovipneumoniae* genes we investigated (*p113, rpoB,* and *gyrB)* are under strong constraining selection, but that the two housekeeping genes *rpoB* and *gyrB* exhibit stronger constraining selection than *p113* (Table 2). Of the three genes, *p113* exhibits the highest frequency of positive selection occurring, with 2.43% of sites under strong positive selection (ω (+) = 1.54). For *rpoB,* 0.89% of sites were under positive selection (ω (+) = 2.04) and for *gyrB,* 0.91% of sites were under positive selection (ω (+) = 2.15).

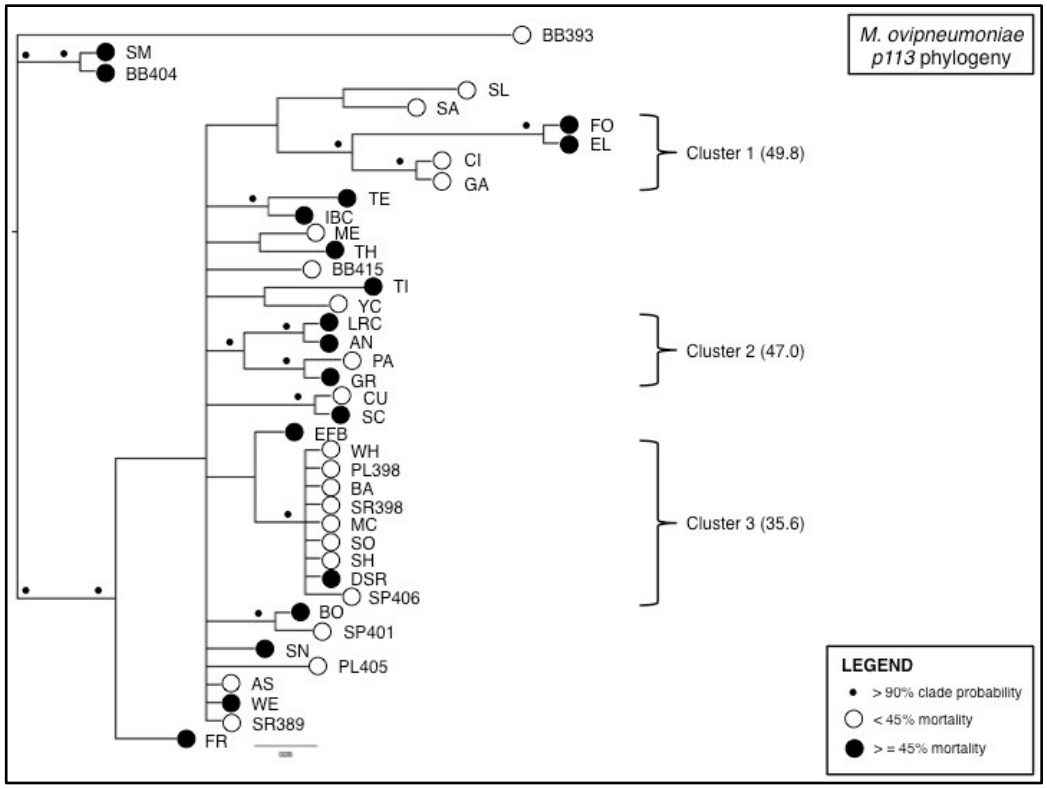

Figure 2. Bayesian *M. ovipneumoniae p113* phylogeny with strain clusters 1-3 identified and supported by >90% clade probability values. Average estimated mortalities for each cluster are listed parenthetically after the cluster number. See Table 1 for population identification codes.

Table 2. Summary data for selection at three *M. ovipneumoniae* loci (*p113,* and housekeeping genes *rpoB* and *gyrB*) using Bayesian analysis to determine dN/dS ratios.

	p113		rpoB		gyrB	
Parameter	**Mean**	**Variance**	**Mean**	**Variance**	**Mean**	**Variance**
p113						
ω (-)[1]	0.0441	0.000242	0.00221	0.00000248	0.0131	0.0000303
ω (N)	1.00	0.00	1.00	0.00	1.00	0.00
ω (+)	1.54	0.445	2.04	1.21	2.16	0.879
π (-)[2]	0.929	0.000893	0.962	0.000184	0.970	0.000372
π (N)	0.0470	0.000756	0.0290	0.000167	0.0211	0.000363
π (+)	0.0243	0.0004.81	0.00893	0.0000877	0.00912	0.0000942

[1] ω denotes the dN/dS ratios for negatively (-), neutral (N), and positively (+) selected sites; [2]π denotes the frequencies of the site categories (negatively (-), neutral (N), and positively (+) selected sites).

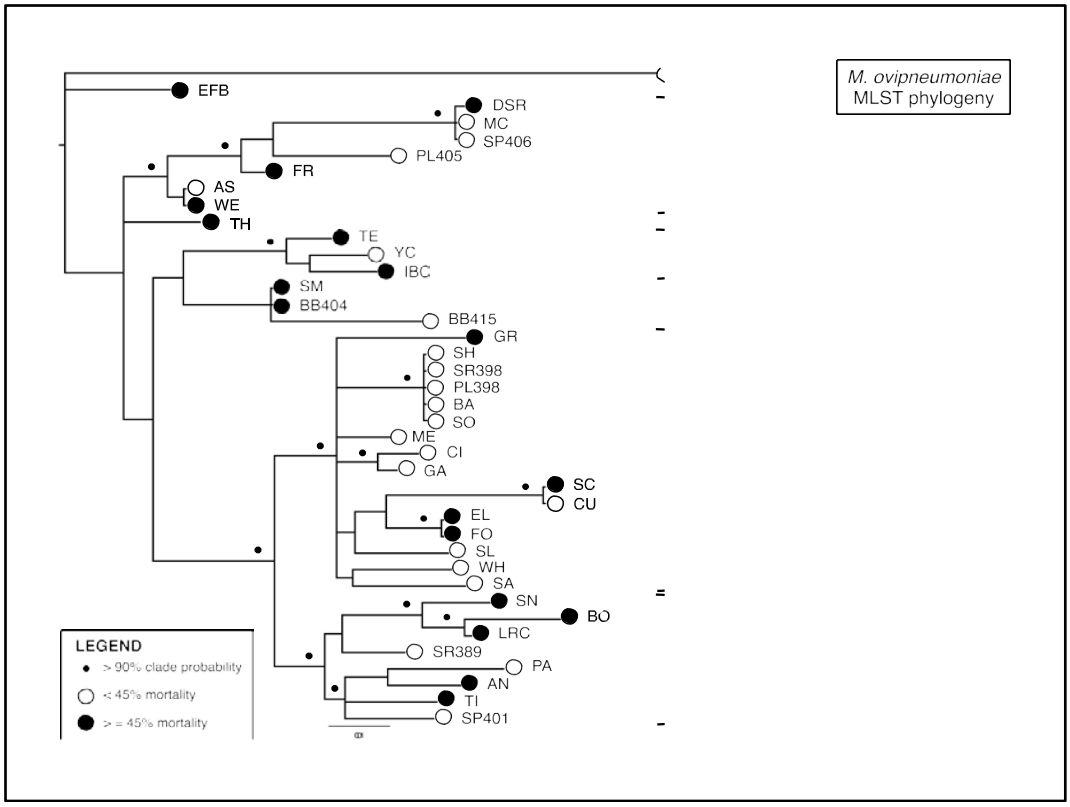

Figure 3. Bayesian *M. ovipneumoniae* MLST phylogeny with strain clusters 1-4 identified and supported by > 90% clade probability values. Average estimated mortalities for each cluster are listed parenthetically after the cluster number. See Table 1 for population identification codes.

DISCUSSION

We did not detect association of *M. ovipneumoniae* genetic clusters with outbreak severity, as least as we were able to characterize severity. We detected fewer well-supported clades based on *p113* sequences than MLST sequences. Neither *p113* nor MLST clusters were significantly associated with outbreak severity.

This was a pilot study, and as such, included several significant limitations that could be addressed by future, more extensive investigations; these include, a possible bias towards high severity in the outbreaks selected for inclusion in the study (under-representing *M. ovipneumoniae* genetic clusters that may be associated with less severe disease), the inclusion of insufficient *p113* sequence to detect genetic clustering, and the possibility that *p113*, if actually a cell surface gene associated with virulence, may undergo selection for immune escape that may obscure phylogenetic association with virulence. An important limitation of this study is likely bias towards severe outbreaks. Outbreak detection in the field and accurate disease-induced mortality estimates depend on the level of disease surveillance and sample recovery. It is likely that there was variation in the intensity of monitoring at the population and individual levels and that mild disease outbreaks would not have been recognized for inclusion in the WSWG data.

If P113 is a virulence determinant due to its hypothesized role in interaction with respiratory epithelial cilia receptors, association of its amino acid sequence with virulence may well be limited to the receptor-ligand region. Given that the ligand region is not currently identified, it is very possible that it is encoded outside the 285 bp regions we analyzed, since the complete *p113* gene sequence is 3,240 bp (Yang et al. 2014). Finally, if antibody responses to P113 reduce its ability to mediate interaction with cilia, one would expect strong genetic selection on *M. ovipneumoniae* to avoid these immune responses that could confound any association between *p113* phylogeny and virulence.

There are also other factors that affect outbreak severity that may blur an underlying association of genetic clusters with virulence. These include host genetics and nutrition, host social contact networks, and climatic conditions as confounding factors that, along with pathogen virulence, may contribute to outbreak percent mortality estimates (Hudson et al. 2002, Manlove et al. 2014, Plowright et al. 2013, Tompkins et al. 2011).

Progress in determining whether genetic variability of *M. ovipneumoniae* is associated with the severity of bighorn sheep pneumonia all-age outbreaks will require continued efforts to identify specific bighorn sheep populations in which the introduction of *M. ovipneumoniae* is associated with low all-age mortality. More complete sequencing of *p113*, other virulence-associated genes, or generation of whole genome sequences will provide a stronger basis for these comparisons. In addition to their potential to improve our understanding of the factors that contribute to the variation in severity of pneumonia outbreaks in bighorn sheep, such studies will also offer the potential to improve outbreak response, and may contribute to development of candidate *M. ovipneumoniae* vaccines (Hudson et al. 2002, Sells et al. 2015, Tompkins et al. 2011, Weiser et al. 2012, Zeigler et al. 2014).

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LITERATURE CITED

Besser, T. E., M. A. Highland, K. Baker, E. F. Cassirer, N. J. Anderson, J. M. Ramsey, K. Mansfield, D. L. Bruning, P. Wolff, J.

B. Smith, J. A. Jenks. 2012. Causes of pneumonia epizootics among bighorn sheep, western united states, 2008-2010. USDA National Wildlife Resource Center – Staff Pub, Paper 1101.

Besser, T. E., E. F. Cassirer, M. A. Highland, P. Wolff, A. Justice-Allen, K. Mansfield, M. A. Davis, W. Foreyt. 2013. Bighorn sheep pneumonia: sorting out the cause of a polymicrobial disease. Preventative Veterinary Medicine 108:85-93.

Cassirer, E. F., R. K. Plowright, K. R. Manlove, P. C. Cross, A. P. Dobson, K. A. Potter, P. J. Hudson. 2013. Spatio-temporal dynamics of pneumonia in bighorn sheep. Journal of Animal Ecology 82:518-528.

Cassirer, E. F., K. R. Manlove, R. K. Plowright, T. E. Besser. 2017. Evidence for strain specific immunity to pneumonia in bighorn sheep. Journal of Wildlife Management 81:133-143.

Graur, D., and Li, W. 2000. Fundamentals of Molecular Evolution. Sinauer. 2000.

Hudson, P. J., A. Rizzoli, B. T. Grenfell, H. Heesterbeek, A. P. Dobson. 2002. The Ecology of Wildlife Diseases.

Manlove, K. R., E. F. Cassirer, P.C. Cross, R. K. Plowright, P. J. Hudson. 2014. Costs and benefits of group living with disease: a case study of pneumonia in bighorn lambs (*Ovis canadensis).* Proceedings of the Royal Society of Biological Sciences 281:2014-2331.

Manlove, K. R., E. F. Cassirer, P.C. Cross, R. K. Plowright, P. J. Hudson. 2016. Disease introduction is associated with a phase transition in bighorn sheep demographics. Ecology 2016 0:1-10.

National Center for Biotechnology Information [NCBI]. 2015. NCBI Mycoplasma ovipneumoniae strain GZ-QXI p113 gene, complete cds>. Accessed 14 Oct 2015. <https://www.ncbi.nlm.nih.gov/nuccore/930435519

Plowright, R. K., K. R. Manlove, E. F. Cassirer, P. C. Cross, T. E. Besser, P. J. Hudson. 2013. Use of exposure history to identify patterns of immunity to pneumonia in bighorn sheep (*Ovis canadensis*). PLoS ONE 8, e61919.

Sells, S. N., M. S. Mitchell, J. J. Nowak, P. M. Lukacs, N. J. Anderson, J. M. Ramsey, J. A. Gude, P. R. Krausman. 2015. Modeling risk of pneumonia epizootics in bighorn sheep: pneumonia risk model for bighorn sheep. Journal of Wildlife Management 79:195-210.

Tompkins, D. M., A. M. Dunn, M. J. Smith, S. Telfer. 2011. Wildlife diseases: from individuals to ecosystems: ecology of wildlife diseases. Journal of Animal Ecology 80:19-38.

Weiser, G. C., M. L. Drew, E. F. Cassirer, A. C. S. Ward. 2012. Detection of *Mycoplasma ovipneumoniae* and *M. arginini* in bighorn sheep using enrichment culture coupled with genus- and species-specific polymerase chain reaction. Journal of Wildlife Disease 48:449-453.

Yang, F., X. F. Dao, A. Rodriguez-Palacios, X. F. Feng, C. Tang, X. N. Yang, and H. Yue.
2014. A real-time PCR for detection and quantification of *Mycoplasma ovipneumoniae.* The Journal of Veterinary Medical Science 76:1631.

Ziegler, J. C., K. K. Lahmers, G. M. Barrington, S. M. Parish, K. Kilzer, K. Baker, T. E. Besser. 2014. Safety and immunogenicity of a *Mycoplasma ovipneumoniae* bacteria for domestic sheep (*Ovis aries*). PLoS ONE 9, e95698.