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Michelle Carstensen
Minnesota Department of Natural Resources, michelle.carstensen@state.mn.us

John H. Giudice
Minnesota Department of Natural Resources

Erik C. Hildebrand
Minnesota Department of Natural Resources

J. P. Dubey
USDA-ARS, Jitender.dubey@ars.usda.gov

John Erb
Minnesota Department of Natural Resources

See next page for additional authors

Authors
Michelle Carstensen, John H. Giudice, Erik C. Hildebrand, J. P. Dubey, John Erb, Dan Stark, John Hart, Shannon M. Barber-Meyer, L. David Mech, Steve K. Windels, and Andrew J. Edwards

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A SEROSURVEY OF DISEASES OF FREE-RANGING GRAY WOLVES (*CANIS LUPUS*) IN MINNESOTA, USA

Michelle Carstensen,[1,8] John H. Giudice,[1] Erik C. Hildebrand,[1] J. P. Dubey,[2] John Erb,[3] Dan Stark,[3] John Hart,[4] Shannon Barber-Meyer,[5] L. David Mech,[5] Steve K. Windels,[6] and Andrew J. Edwards[7]

[1] Minnesota Department of Natural Resources, 5463 W Broadway, Forest Lake, Minnesota 55025, USA
[2] US Department of Agriculture, Agricultural Research Service, Animal Parasitic Disease Laboratory, Building 1001, Beltsville, Maryland 20705, USA
[3] Minnesota Department of Natural Resources, 1201 E Hwy. 2, Grand Rapids, Minnesota 55744, USA
[4] US Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, 34912 US Hwy. 2, Grand Rapids, Minnesota 55744, USA
[5] US Geological Survey, Northern Prairie Wildlife Research Center, 8711 37th St. SE, Jamestown, North Dakota 58401-7317, USA
[6] Voyageurs National Park, National Park Service, 360 Hwy. 11 E, International Falls, Minnesota 56649, USA
[7] 1854 Treaty Authority, Resource Management Division, 4428 Haines Rd., Duluth, Minnesota 55811, USA
[8] Corresponding author (email: michelle.carstensen@state.mn.us)

ABSTRACT: We tested serum samples from 387 free-ranging wolves (*Canis lupus*) from 2007 to 2013 for exposure to eight canid pathogens to establish baseline data on disease prevalence and spatial distribution in Minnesota's wolf population. We found high exposure to canine adenoviruses 1 and 2 (88% adults, 45% pups), canine parvovirus (82% adults, 24% pups), and Lyme disease (76% adults, 39% pups). Sixty-six percent of adults and 36% of pups exhibited exposure to the protozoan parasite *Neospora caninum*. Exposure to arboviruses was confirmed, including West Nile virus (37% adults, 18% pups) and eastern equine encephalitis (3% adults). Exposure rates were lower for canine distemper (19% adults, 5% pups) and heartworm (7% adults, 3% pups). Significant spatial trends were observed in wolves exposed to canine parvovirus and Lyme disease. Serologic data do not confirm clinical disease, but better understanding of disease ecology of wolves can provide valuable insight into wildlife population dynamics and improve management of these species.

Key words: Antibody titer, canine distemper, canine parvovirus, *Canis lupus*, eastern equine encephalitis, Lyme disease, *Neospora caninum*.

INTRODUCTION

Wolf (*Canis lupus*) management in Minnesota, US has repeatedly changed since the wolf population was estimated at 750 animals in the 1950s. Protected under the 1973 Endangered Species Act, Minnesota wolves soon reached the federal delisting goal of 1,250–1,400 wolves and were reclassified as threatened in 1978. Although legal battles delayed wolf delisting, wolf range in Minnesota expanded to approximately 95,000 km^2 and the population rose to 3,020 wolves by 2003 (Erb and Sampson 2013). In preparation for delisting, the Minnesota Department of Natural Resources (MNDNR) developed a plan for managing wolves under state control (MNDNR 2001). This plan was designed to protect wolves and monitor the population, while giving owners of domestic animals more flexibility in addressing wolf depredation. A primary component of monitoring the wolf population is to understand the diseases and parasites that impact the species.

Several diseases and parasites can have population-level effects on wolves. Most notably, relatively high prevalence of canine parvovirus (CPV) may reduce pup survival and limit population growth (Mech et al. 2008). Other diseases, including canine distemper virus (CDV), canine adenovirus (CAV), and parasites may kill infected wolves and impact population performance (Stephenson et al. 1982; Gese et al. 1997; Almberg et al. 2009). Some parasites of wolves, such as *Neospora caninum*, can cause high abortion rates in cattle (Gondim 2006). Understanding the prevalence and distribution of this disease may improve wolf management strategies at the wildlife–livestock interface. Our objective

was to estimate seroprevalence and spatial distribution of eight important canid diseases in Minnesota's wolves, including CPV, CDV, CAV, neosporosis (NEO), Lyme disease (LYM), West Nile virus (WNV), eastern equine encephalitis (EEE), and heartworm. Serosurveys can provide useful baseline data on disease exposure rates and improve understanding of disease ecology. Further, improved understanding of temporal and spatial patterns of disease can provide valuable insight into changes in population size or structure related to epizootic disease outbreaks.

MATERIALS AND METHODS

Study area

The range of wolves in Minnesota encompasses approximately 95,000 km^2 of the northern portion of the state (Fig. 1). During our study, from 2007–13, the state's wolf population was estimated in 2008 (n=2,921 wolves) and 2013 (n=2,211 wolves) where average pack and territory size was 4.9 wolves and 142 km^2, and 4.3 wolves and 161 km^2, respectively (Erb and Sampson 2013). Wolves were federally protected during the first 5 yr of this study, then they were delisted in 2012, after which 413 and 238 wolves were harvested in 2012 and 2013, respectively (Stark and Erb 2013, 2014). Approximately 200 depredating wolves were removed annually by federal agents. Long-term studies of wolves, which included their capture and release, were conducted by state, federal, and tribal agencies throughout the wolf range in Minnesota.

Sample collection

The MNDNR contracted with the US Department of Agriculture–Animal and Plant Health Inspection Service–Wildlife Services to collect biological samples from depredating wolves. Researchers within the MNDNR, the US Geological Survey, Voyageurs National Park, Minnesota National Guard–Camp Ripley Training Center and 1854 Treaty Authority obtained samples from wolves that were live-captured, radiocollared, and released. Conservation officers and MNDNR wildlife staff collected samples from other wolves found dead (e.g., vehicle kills) to improve our spatial coverage. We recorded date and method of collection, geographic location (Universal Transverse Mercator coordinates), age (adult [≥1 yr] or pup [<1 yr], based on morphological characteristics and date of collection), and gender for all samples.

Field personnel collected blood from a jugular, cephalic, or saphenous vein. For euthanized wolves, blood was collected from the site of a bullet wound, heart puncture, or from the chest cavity immediately after death. Whole blood was centrifuged and serum decanted into cryovials and stored at –20 C.

Serological screening

Serum was submitted for antibody screening for eight diseases to the Veterinary Diagnostic Laboratory at the University of Minnesota (St. Paul, Minnesota, USA) and then outsourced as needed. Titers were expressed as the reciprocal of the final dilution. Canine parvovirus was confirmed via a hemagglutination inhibition test; titers ≥256 were considered positive (Colorado State University, Fort Collins, Colorado, USA; Carmichael et al. 1980, 1983). Exposure to canine adenovirus type-1 (CAV-1) and type-2 (CAV-2) was confirmed via a serum neutralization test; titers ≥8 were considered positive (Cornell University, Ithaca, New York, USA; Carmichael et al. 1963). Canine distemper virus was also detected via a serum neutralization test; titers ≥25 were considered positive (Colorado State University; Carbrey et al. 1971). A plaque reduction neutralization test was used to confirm exposure to EEE and WNV, and titers ≥10 were considered positive (National Veterinary Services Laboratory, Ames, Iowa, USA; Ostlund 2004). Heartworm, *Dirofilaria immitis*, was detected by a Dirochek heartworm antigen test (Synbiotics©; Veterinary Diagnostic Laboratory at the University of Minnesota). An immunofluorescence assay was used for LYM (*Borrelia burgdorferi*); titers ≥160 were considered positive (Veterinary Diagnostic Laboratory at the University of Minnesota; Artsob et al. 1993). *Neospora caninum* was detected with the use of immunofluorescence assay from slides and reagents provided by Veterinary Medical Research and Development (Pullman, Washington, USA); titers ≥50 were considered positive (Veterinary Diagnostic Laboratory, University of Minnesota). Antibody titers were categorically grouped as "low", "medium", and "high" based on the percentile distribution of the observed positive titer values using quantiles of 0.33 and 0.66 as cut-off values to create three bins.

Wolf serum was also sent to the US Department of Agriculture–Agriculture Research Service (Beltsville, Maryland, USA) for a collaborative research project on *Neospora caninum* and *Toxoplasma gondii*. Serum was screened for NEO via a *Neospora* agglutination test and for *T. gondii* via a modified agglutination test; titers

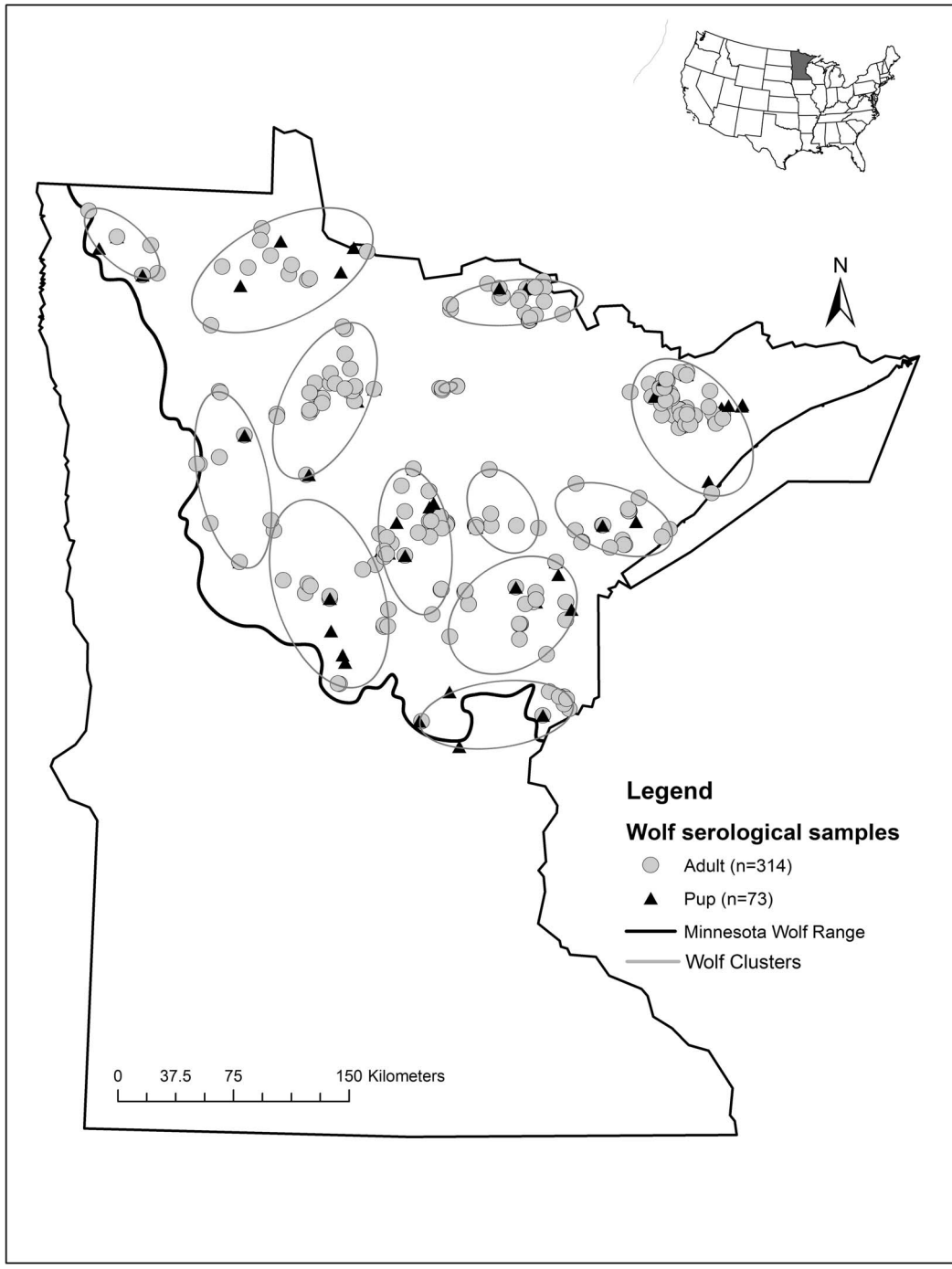

FIGURE 1. Spatial distribution of serum samples taken from wolves (*Canis lupus*) in northern Minnesota, USA, 2007–13, for evidence of exposure to infectious diseases. Gray lines denote spatial clusters of points based on Euclidian distance and the function 'pam' in the R package 'cluster' (Maechler et al. 2015).

≥25 were considered positive. Results for toxoplasmosis infection in wolves were published elsewhere (Dubey et al. 2013a).

Analytical methods

We used a simple binomial estimator to compute overall prevalence rates $\hat{p}=k/n$, where k=number of positive events and n=number of independent Bernoulli trials (wolves with serological results). We computed separate estimates for adults and pups but pooled data over gender and year for overall prevalence estimates. We used the score interval (Agresti and Coull 1998) to compute approximate confidence intervals (CI) for $\hat{p}$'s, except where $k=0$ (no positive events). In the latter case we used the "rule of three" (Jovanovic and Levy 1997) to compute an upper 95% CI for $\hat{p}|k=0$. We did not adjust seroprevalence for test sensitivity or specificity because this information is lacking for wolves and the validity of applying estimates from tests on dogs (*Canis lupus familiaris*) is questionable. Thus, overall seroprevalence served as an index of exposure of adults and pups to the eight pathogens during 2007–13. We also used overall prevalence to identify which pathogens to investigate more closely for spatiotemporal variation and gender differences in exposure rates. We used $\min(k, n-k)>30$ and a degrees-of-freedom (df) spending approach (Harrell 2001; Giudice et al. 2012) to determine which pathogen data sets had sufficient information to warrant fitting binary logistic models to investigate demographic, spatial, and temporal patterns of pathogen exposure. General guidelines for avoiding model overfitting recommend limiting the df associated with predictors (including complex terms such as interactions and nonlinear terms) to $m/10$ or $m/20$, where m is the limiting sample size (Harrell 2001). In the case of a binary response variable, $m=\min(k, n-k)$. The limiting sample size m in our seroprevalence data sets ranged from 0 to 95 (median=23.5). We used $m\geq30$ as a cutoff because at a minimum we wanted to include categorical predictors for gender and year, which would require at least 4 df. We did not consider models that included both adults and pups because sample sizes were highly unbalanced (314 vs. 73) and these models would require 1–4 additional df for the categorical predictor age and potential interactions involving age and year. Likewise, we did not consider a gender:year interaction in our df-spending approach because it was not biologically intuitive. Based on this criterion, we fit models to adult serological data for CPV (m=45), CDV (m=47), NEO (m=56), WNV (m=95), CAV-1 (m=32), and LYM (m=62).

Before fitting any models, we considered data limitations associated with the opportunistic sampling design, which resulted in clustered samples, unbalanced data, and potential confounding across space, time (years), and gender. Almberg et al. (2009) used pack affiliation as a random effect to account for their clustered sampling design, but we lacked pack-affiliation data. Therefore, we used function 'pam' in the R package 'cluster' (Maechler et al. 2015; R Core Team 2016) and a Euclidean distance matrix based on UTM coordinates to construct spatial clusters of data without viewing the relationship to seroprevalence, age, gender, or year. We used average silhouette width (Maechler et al. 2015) over 2–20 clusters to determine the optimal number of clusters. We constructed 13 clusters (Fig. 1) to account for the clustered sampling design and investigate spatial variation in exposure rates after accounting for gender and year differences. We also used the R package 'ape' version 3.4 (Paradis et al. 2004; R Core Team 2016) to compute Moran's I statistic for each pathogen, providing a measure of global spatial correlation for the binary response variable (−1=perfect dispersion, 0=random, 1=perfect correlation or clustering).

We investigated modeling spatial autocorrelation and first-order spatial trends directly via geostatistical models (Berke 1999; Christensen and Ribeiro 2002; Dormann et al. 2007), but the data were insufficient to fit such models (e.g., estimated effects were imprecise and inconsistent in direction and magnitude). Therefore, we chose to use a simpler approach, by modeling spatial autocorrelation and first-order spatial trends of pathogen exposure among wolves in Minnesota during 2010–13. This approach is described in the Supplementary Materials.

RESULTS

We collected serum from 387 wolves: 247 depredation animals (2010–13), 118 live-caught animals (2007–13), 17 animals found dead (2010–12), three road-kills (2010–11), and two with unrecorded collection method (2010–11). Our final sample consisted of 314 adult wolves (48% females, 52% males) and 73 wolf pups (39% females, 61% males), as shown in Figure 2. Seventy-nine percent of pup samples were collected during August–September (range: July–December). Thus, most wolf pups were at least 5 mo old. Adult wolves were sampled year-round, although most (94%) were collected during May–October each year.

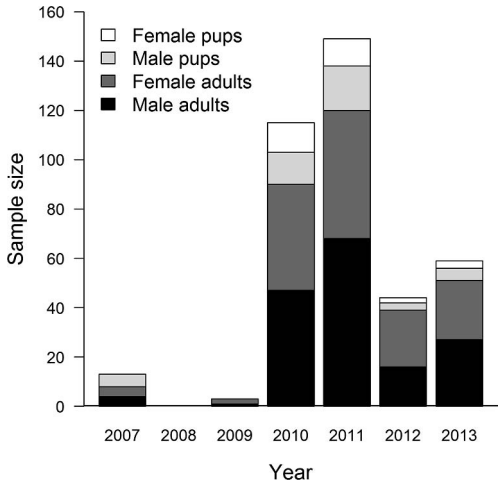

FIGURE 2. Wolf (*Canis lupus*) serum samples taken from northern Minnesota, USA for evidence of exposure to infectious diseases, arranged by gender, age class, and year, 2007–13 (does not include three adults and one pup with missing gender data).

Adult wolves had higher pathogen exposure than pups (Table 1). Pathogens CAV-1, CAV-2, CPV, and LYM seroprevalence rates exceeded 75% in adults (Table 1). The highest seroprevalence in pups was for CAV-2 (45%) and CAV-1 (44%). Exposure to EEE and heartworm was low (≤7%) in both adults and pups. High antibody titers for CAV-1 and CPV were observed for 30% and 25% of seropositive adult wolves, respectively (Table 2). Thirty-four percent of adults and 67% of pups seropositive for CDV had high antibody titers for this pathogen (Table 2).

Gender and year did not explain significant variation in the exposure of adult wolves to CDV, CAV-1, or NEO (Supplementary Material Table S1). We found no evidence of spatial variation or correlation in CAV-1 or NEO exposure rates, whereas CDV exposure was slightly clustered in space (Moran's I statistic=0.138, SD=0.047) and the predicted probability of exposure varied among sampling clusters (range: 9–37%; lowest in central part of range). However, overall seroprevalence (Table 1) was our best estimate of population-level exposure rates for CDV, CAV-1, and NEO.

Exposure to CPV varied by gender, year, and cluster (Fig. 3A and Supplementary Material Table S1). Exposure was twofold higher for males than females, and exposure in 2010 and 2012 was approximately threefold higher than in 2011 and 2013. The random cluster effect for CPV was significant (estimated SD=0.998, 95% CI: 0.326 to 2.18) and Moran's I statistic indicated a weak spatial clustering of CPV exposures (Moran's I=0.107, SD=0.048). Positive random effects (increasing log odds of exposure) were generally associated with clusters from western and northern portions of Minnesota's wolf range. Likewise, all 15 CPV-positive results for wolf pups occurred in the northwest and central portion of the range.

We found no evidence of spatial variation (cluster effect) in WNV (Supplementary Material Table S1), although Moran's I statistic (0.120, SD=0.047) indicated a weak clustering of WNV exposures. We found stronger evidence of temporal variation in WNV exposure rates (Fig. 3B). Most notably, exposure was fivefold higher in 2011 than 2010. Gender was not an important predictor of variation in WNV exposure ($\hat{\beta}$=0.167, 95% CI: –0.381 to 0.717; also see Fig. 3B).

Lastly, we found spatial and temporal variation in LYM exposure (Supplementary Material Table S1). The average predicted exposure declined from 90% in 2010 to 64% in 2013 (Fig. 3C). Predicted exposure also varied by cluster (Fig. 3C) and LYM cases were weakly clustered in space (Moran's I statistic=0.240, SD=0.047). The log odds of LYM exposure were lower in clusters from the northern part of Minnesota's wolf range. Gender was not an important predictor of variation in LYM exposure rates ($\hat{\beta}$=0.036, 95% CI: –0.752 to 0.668; Fig. 3C).

DISCUSSION

Serosurveys are useful to determine apparent disease prevalence and improve understanding of disease ecology. There are important caveats to consider, however, including 1) only "survivors" are available for sampling, 2) a positive antibody titer only indicates exposure and not clinical disease,

TABLE 1. Overall seroprevalence of nine pathogens among wolves in Minnesota, USA, 2007–13. Titers are given as the reciprocal of the final dilution.

Age class Pathogen[a]	No. of years	No. of wolves	No. of positive samples	$\hat{p}$[b]	95% Confidence interval		Titer range for positive tests
					Lower	Upper	
Adults							
CAV-1	6	259	227	0.88	0.83	0.91	12–12,288
CAV-2	5	197	174	0.88	0.83	0.92	8–6,144
CPV	6	253	208	0.82	0.77	0.87	256–16,384
CDV	5	255	47	0.19	0.14	0.24	32–4,096
LYM	6	260	198	0.76	0.71	0.81	160–1,280
NEO	5	232	154	0.66	0.60	0.72	50–51,200[c]
WNV	5	257	95	0.37	0.31	0.43	10–100
EEE	5	256	8	0.03	0.02	0.06	10–10
HWM	6	261	18	0.07	0.04	0.11	—[e]
Pups							
CAV-1	5	57	25	0.44	0.31	0.57	8–12,288
CAV-2	5	40	18	0.45	0.30	0.61	128–2,048
CPV	5	62	15	0.24	0.15	0.37	256–16,384
CDV	5	62	3	0.05	0.01	0.14	1,024–4,096
LYM	5	62	24	0.39	0.27	0.52	160–1,280
NEO	4	53	19	0.36	0.24	0.50	50–1,600[d]
WNV	5	62	11	0.18	0.10	0.30	10–100
EEE	5	62	0	0.00	0.00	0.05	—[e]
HWM	5	62	2	0.03	<0.01	0.12	—[e]

[a] CAV-1 and CAV-2 = canine adenovirus type-1 and type-2; CPV = canine parvovirus; CDV = canine distemper virus; LYM = Lyme disease; NEO = *Neospora caninum*; WNV = West Nile virus; EEE = equine encephalitis virus; HWM = heartworm.

[b] $\hat{p}$ = apparent prevalence.

[c] Titer range for immunofluorescence assay test. Titer range for positive *Neospora* agglutination test was 25–1,600.

[d] Titer range for immunofluorescence assay test. Titer range for positive *Neospora* agglutination test was 25–200.

[e] — = not applicable.

and 3) antibodies to certain pathogens can persist at a maintenance level, so the titer magnitude can reflect historic/past exposure, recent initial exposure, re-exposure, current infection, or clinical disease; thus, it may not be possible to discern health impacts.

We obtained serological data on nearly 400 wolves across their range in Minnesota from 2007 to 2013; however, nearly 70% of samples were collected during 2010–11. Gender of adult wolves was nearly equal, but males were favored in the pup cohort. Our sample may be biased by the collection method, as approximately two thirds of wolves were euthanized for depredation. Spatial distribution of these wolves may not be random; as depredating wolves disproportionately occurred near high proportions of pasture (an index of cattle [*Bos taurus*] abundance) and high white-tailed deer (*Odocoileus virginianus*) densities (Treves et al. 2004).

Adenoviruses may cause significant mortality during periods of increased stress, such as food scarcity, high density, or parasitism; pups <4 mo old are most vulnerable (Trainer and Knowlton 1968). Adenoviruses are transmitted through urine and are very resistant to chemical and physical degradation, allowing for high exposure in canid populations (Stephenson et al. 1982). The high prevalence of CAV-1 and 2 in our adult wolves (88%) and pups (45%), was similar to adult wolves in Yellowstone National Park (91–96%, Almberg et al. 2009) and Alaska (81–84%, Zarnke et al. 1987, 2004; 95%, Stephenson et al. 1982). Our results showing high exposure supports the hypothesis that these adenoviruses are en-

TABLE 2. The magnitude of antibody titers and the seroprevalence of eight pathogens in wolves (*Canis lupus*) sampled in Minnesota, USA (2007–13) pathogens. Antibody titers were categorically grouped as "low," "medium," and "high," based on the percentile distribution of the observed positive titer values using quantiles of 0.33 and 0.66 as cut-off values to create three bins.

Pathogen[a]	No. positive	Low titer samples		Medium titer samples		High titer samples	
		%	Range	%	Range	%	Range
CAV-1							
Adult	227	8	8–256	62	384–3,072	30	4,096–12,288
Pup	25	60		32		8	
CAV-2							
Adult	174	12	8–256	83	384-3,072	5	4,096–12,288
Pup	18	11		89		0	
CPV							
Adult	208	21	256–512	54	1,084–4,096	25	8,192–16,384
Pup	15	27		60		13	
CDV							
Adult	47	32	32–128	34	256–1,024	34	2,048–4,096
Pup	3	0		33		67	
LYM							
Adult	198	12	160	25	320–640	63	1,280
Pup	24	21		54		25	
NEO[b]							
Adult	154	77	25–400	18	800–3,200	5	6,400–51,200
Pup	19	84		16		0	
WNV							
Adult	95	54	10	46	100	0	—[c]
Pup	11	64		36		0	
EEE							
Adult	8	100	10	0	—[c]	0	—[c]
Pup	0	0		0		0	

[a] CAV-1 and CAV-2 = canine adenovirus type-1 and type-2; CPV = canine parvovirus; CDV = canine distemper virus; LYM = Lyme disease; NEO = *Neospora caninum*; WNV = West Nile virus; EEE = equine encephalitis virus.

[b] Titer range for immunofluorescence assay and *Neospora* agglutination tests combined.

[c] — = no data.

demic. Most adult wolves in our study had medium (62%, 384–3,072) or high (30%, 4,096–12,288) titers to CAV-1, which further indicates the pathogen was well established and opportunity for re-exposure was readily available.

The earliest evidence of CPV infection in wild canids came from serum of live-captured wolves from northeastern Minnesota in 1973 (Mech and Goyal 1995). Mortality of wolves from CPV has been confirmed (Mech et al. 1997) and CPV was implicated as impacting wild populations through high pup mortality (Wydeven et al. 1995; Gese et al. 1997; Mech et al. 2008). These authors correlated CPV prevalence in adults with increased mortality in 2–4-mo-old pups, when maternal antibodies are waning and pups become the most vulnerable to the virus (Barker and Parrish 2001). Limitations in the ability to monitor neonate survival made it difficult to confirm CPV as cause of death in wolves <5 mo old, but correlations between CPV exposure in adults and pup survival estimates were highly suggestive (Mech et al. 2008). Recently, Mech and Goyal (2011) concluded that the disease became endemic after 1994, and the population has since developed enough immunity to withstand infections. Our high CPV prevalence in adults supports the hypothesis that

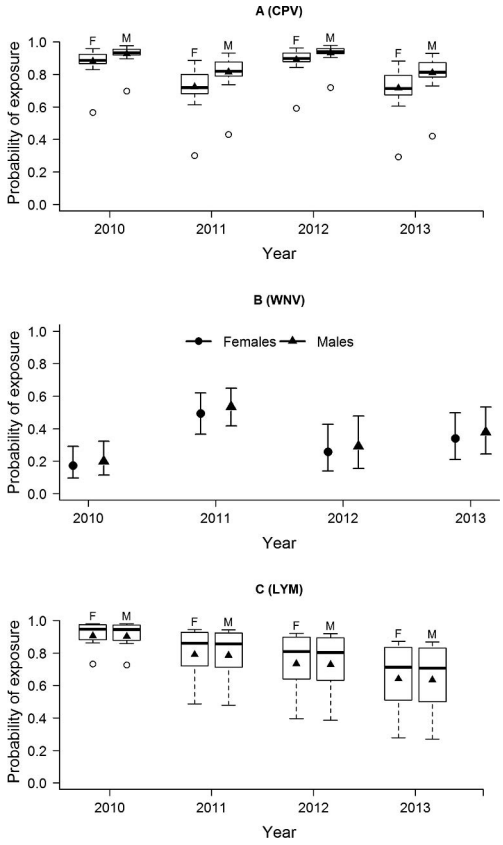

FIGURE 3. The probabilities that wolves (*Canis lupus*) in northern Minnesota, USA were exposed to infectious diseases, 2007–13. (A) Boxplots of predicted probabilities of exposure of adult wolves (F=females, M=males) to canine parvovirus (CPV), northeast Minnesota, 2010–13; based on a logistic mixed model with year and gender as additive fixed effects and location (spatial cluster) as a random effect. Predicted probabilities describe year and gender effects and the variation reflects uncertainty about the distribution of wolves among clusters. The arithmetic mean (black triangle) of the distribution of predicted probabilities denotes our best estimate of population-level prevalence given the sample distribution of wolves among clusters. (B) Model-based estimates of probability of exposure of adult wolves to West Nile virus (WNV), northern Minnesota, 2010–13. Error bars denote 95% confidence intervals. (C) Boxplots of predicted probabilities of exposure of adult wolves (F=females, M=males) to Lyme disease (LYM), northern Minnesota, 2010–13, based on a logistic mixed model with year and gender as additive fixed effects and location (spatial cluster) as a random effect. Predicted probabilities describe year and gender effects and the variation reflects uncertainty about the distribution of wolves among clusters. The arithmetic mean (black triangle) of the distribution of predicted probabilities ← denotes our best estimate of population-level prevalence given the sample distribution of wolves among clusters.

the disease is endemic across Minnesota's wolf range. We found males were twofold more likely than females to be exposed to CPV. Previous studies failed to find an effect of gender on CPV prevalence (Johnson et al. 1994; Mech et al. 2008), except Nelson et al. (2012) reported more males than females exposed in the Canadian Rockies.

The CPV titers we report are much higher than in previous studies. Gese et al. (1997) suggested a CPV titer >1,280 indicated recent infection, and this would decline with age; Arjo et al. (2003) reported a titer of >1,600 was indicative of recent exposure. Johnson et al. (1994) concluded that adult pack members with CPV titers >1,600 experienced pup mortalities in that year's litter. Mech et al. (2008) found <1% of their wolves positive at the highest titer (8,192). In our study, 63% of adult and 47% of pups had antibody titers >1,600. In fact, 25% and 13% of our adults and pups, respectively, had CPV titers between 8,192 and 16,384. These high CPV titers may indicate an epizootic had recently occurred across Minnesota's wolf range. Canine parvovirus prevalence in adults in this study consistently exceeded the 76% threshold, which Mech and Goyal (1995) predicted would result in wolf population declines. We did not assess overall health of euthanized or live-captured wolves, and some may have had clinical CPV infection. Alternatively, given the high CPV prevalence in adults and year-long persistence of the virus in feces (Muneer et al. 1988), wolves may be re-exposed often enough to increase maintenance antibody titers. We also found a significant spatial effect with CPV, with prevalences higher in the north and west portions of the wolf range, similar to the spatial effect found by Mech et al. (2008).

Unlike CAV and CPV, CDV is highly contagious and thought to spread quickly, but infected individuals shed virus for only a

short time, and the virus degrades rapidly (Almberg et al. 2009). Therefore, although we found a CDV prevalence of 19% in adult wolves and 5% in pups, an epizootic may have occurred outside our sampling frame. Our results are similar to the 7–12% CDV prevalence in coyotes (*Canis latrans*) in Utah, where the virus was presumed inactive over several years (Arjo et al. 2003). Gese et al. (1997) found CDV prevalence in coyotes in Yellowstone declined from 100% to 33% over 4 yr, and older animals had higher exposure.

Our CDV titers were higher than in previous studies. One third of our infected adult wolves and two of three infected pups had titers of 2,048–4,096, nearly sixfold higher than the 32–356 range described by Arjo et al. (2003). Johnson et al. (1994) suggested pup mortality increased in packs with adult wolves having CDV titers >1,250. Stephenson et al. (1982) reported that two collared adult wolves died of CDV in Alaska and Stronen et al. (2011) reported that one adult wolf died in Manitoba, but pups remain the highest risk cohort (Almberg et al. 2009).

Scant evidence exists about the occurrence and potential clinical effect of LYM in wild wolves. A captive wolf inoculated with *B. burgdorferi* produced a peak titer of 512; however, lymphadenopathy was the only observed clinical sign of disease (Kazmierczak et al. 1988). Wild wolves from Wisconsin and Minnesota from 1972–89 showed only 3% seroprevalence for LYM (Kazmierczak et al. 1988; Thieking et al. 1992). Johnson et al. (1994) failed to detect LYM disease in wild wolves in Montana from 1985–90; however, three of four wolves sampled on Isle Royale during 1988–94 were positive, as were 33 of 69 (48%) wolves in Wisconsin from 1991–96 (Peterson et al. 1998; Beheler-Amass et al. 1999).

We found 76% of adult wolves and 39% of pups had been exposed to LYM. Antibody levels were >1,280 in 63% and 25% of exposed adults and pups, respectively. Titers >1,024 were considered indicative of active infection (Kazmierczak et al. 1988), suggesting that most adult wolves in our study were actively infected. Transplacental transmission of LYM has been confirmed in coyotes in Texas (Burgess and Windberg 1989), so it is possible that wolves also developed antibodies to LYM *in utero,* contributing to our high prevalence and persistently high antibody levels. Our data suggest LYM is endemic in Minnesota's wolves; adverse effects on the population are unknown.

Lyme infection in wolves occurred less frequently in the northern portion of Minnesota's wolf range. Similarly, most cases in humans and dogs occurred in the north-central counties, and they were absent in extreme north and western Minnesota (Companion Animal Parasite Council 2015; Minnesota Department of Health 2015).

Recently, the gray wolf was confirmed as a definitive host for *N. caninum* (Dubey et al. 2011, 2014). The sylvatic life cycle of this parasite includes definitive canid hosts (wolves, coyotes, or dogs) where sexual reproduction occurs, and intermediate hosts such as white-tailed deer (Dubey et al. 2009, 2013b). In northern Minnesota, approximately 80% of deer are infected with this parasite (Dubey et al. 2013b), and as the primary prey for wolves, deer provide continued opportunity for wolf re-exposure. Infected canids shed *N. caninum* oocysts in feces and, if ingested, NEO can cause abortion in cattle. Transplacental infection is common in cattle, and this vertical transmission can perpetuate the disease within a herd in the absence of a canid source (Trees and Williams 2005).

Our prevalence rates for NEO in adult wolves (66%) and pups (36%) suggest this parasite is endemic throughout wolf range; there was no spatial clustering of infected individuals. Most of our adult wolves and pups had low antibody titers to NEO. Almberg et al. (2009) reported 33% prevalence in adult wolves and 4% in pups in Yellowstone, and because *N. caninum* does not induce long-term immunity, these infections were presumed to be recent or active (Björkman and Uggla 1999). Gondim et al. (2004) found *N. caninum* antibodies in 64 (39%) of 164 wolves from Minnesota.

Eastern equine encephalitis outbreaks have been found primarily in the southeastern US, but EEE-infected horses have also occurred in Minnesota, Michigan, and Wisconsin, with mortality rates of 70–90% in Minnesota (Minnesota Department of Health 2016). Mosquitoes, particularly *Culiseta melanura*, are thought to be the primary source of exposure (Kinsley et al. 2016) in Minnesota. Little is known about EEE infection in wolves; however, the disease has been documented in domestic dogs (Farrar et al. 2005). Clinical signs in dogs were pyrexia, depression, nystagmus, and lateral recumbency. Farrar et al. (2005) concluded that young dogs are the most susceptible. To our knowledge, this is the first report of exposure to EEE in Minnesota wolves; it is unclear what effect it has on wolf survival.

West Nile virus is an avian virus that can be fatal in some species of mammals, reptiles, and birds. It is not clear what effect WNV has on the 37% of adult wolves and 18% of pups we found to be exposed to the disease, but neurological signs have been reported from rare clinical cases in dogs and wolves. A captive 4-mo-old Arctic wolf pup (*C. lupus arctos*; Lanthier et al. 2004) and a 3-mo-old wolf pup (Lichtensteiger et al. 2003) infected with WNV exhibited vomiting, anorexia, and ataxia prior to death, which occurred 24–48 h after the onset of neurological signs. Our prevalence rates are similar to a recent serosurvey of coyotes in Nebraska, where 48% were exposed to WNV (Bischof and Rogers 2005).

We found only 7% and 3% of adult wolves and pups, respectively, were exposed to canine heartworm (*Dirofilaria immitis*), which was documented in Minnesota wolves previously (Mech and Fritts 1987). Transmitted by mosquitos, this parasite is a significant pathogen in dogs and has been reported in other canids including coyotes, red fox (*Vulpes vulpes*), gray fox (*Urocyon cinereoargenteus*), and red wolf (*Canis rufus*) in the US (Anderson 2001). A single wolf in Wisconsin tested positive for heartworm in 1991 (Beheler-Amass et al. 1999). High infection rates (66%) were reported for coyotes in Arkansas, with 15–30 worms found in each of 50 hearts (King and Bohning 1984). This parasite could be a mortality factor in Minnesota wolves, especially during times of high stress.

Limitations in the temporal span of this study make it difficult to determine annual differences in exposure rates. Long-term data are needed to distinguish between epidemic and endemic patterns as well as effects of age of individuals. Although we elucidated several spatial trends in disease prevalence for CPV and LYM that appeared supported by previous study and current disease trends, our collection methods were biased toward depredating wolves, clustered in space and time, and may not be representative of wolves in Minnesota. We advise caution in interpretation of our results beyond our sampling frame. Future efforts to assess disease prevalence in Minnesota wolves should focus on interior areas within their range, which would provide a useful comparison to the results we reported in this study.

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SUPPLEMENTARY MATERIAL

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