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EVIDENCE OF *LEPTOSPIRA* SEROVARS IN WILDLIFE AND LEPTOSPIRAL DNA IN WATER SOURCES IN A NATURAL AREA IN EAST-CENTRAL ILLINOIS, USA

Kourtney Grimm,[1,5] Nelda A. Rivera,[2,5] Shannon Fredebaugh-Siller,[3] Hsin-Yi Weng,[4] Richard E. Warner,[3] Carol W. Maddox,[1,6] and Nohra E. Mateus-Pinilla[2,6]

[1] College of Veterinary Medicine, University of Illinois Urbana-Champaign, Department of Pathobiology, Veterinary Medicine Basic Sciences Building, 2001 S Lincoln Avenue, Urbana, Illinois 61802, USA
[2] Illinois Natural History Survey, Prairie Research Institute, University of Illinois Urbana-Champaign, 1816 S Oak Street, Champaign, Illinois 61820, USA
[3] Department of Natural Resources and Environmental Sciences, University of Illinois Urbana-Champaign, 1102 S Goodwin Avenue, Urbana, Illinois 61801, USA
[4] Department of Comparative Pathobiology, Purdue University, 725 Harrison Street, West Lafayette, Illinois 47907, USA
[5] These authors contributed equally to this study.
[6] Corresponding authors (emails: maddox@illinois.edu; nohram@illinois.edu)

ABSTRACT: We identified seven *Leptospira* serovars in wildlife and the presence of leptospiral DNA in water sources at a natural area within a fragmented habitat in Illinois, US. These serovars have been implicated in domestic animal and human leptospirosis, a reemerging zoonotic disease, whose reservoirs include wildlife and domestic animals. We live trapped medium-sized mammals (n=351) near building (H-sites) or forest sites (F-sites). Using serology, we evaluated exposure to *Leptospira* (*L. interrogans* serovars Autumnalis, Bratislava, Canicola, Icterohaemorrhagiae, Pomona; *L. kirschneri* serovar Grippotyphosa; *L. borgpetersenii* serovar Hardjo). Using PCR, we tested for the presence of leptospires in eight water samples (ponds, creeks, and rainwater runoff) collected near trapping sites. We identified antibody titers in raccoons (*Procyon lotor*; 121/221) and Virginia opossums (*Didelphis virginiana*; 60/112), but not in feral cats (*Felis catus*; 0/18). We found significant differences in overall *Leptospira* seroprevalence between years (P=0.043) and animal's age in 2008 (P=0.005) and 2009 (P=0.003). Serovars Autumnalis, Bratislava, and Grippotyphosa showed significant differences among age groups with the highest seroprevalence in adults. Females had a higher seroprevalence for Icterohaemorragiae in 2008 (P=0.003) and Hardjo in 2009 (P=0.041). Risk of exposure to *Leptospira* was higher at F-sites compared to H-sites (odds ratio 2.3, 95% confidence interval 1.3–3.9, P=0.002). We captured more animals with titers >1:800 at H-sites, but there was no association between titer levels and capture site. Six of eight water sources were *Leptospira*-positive; however, there was no correlation between trapping locations of seropositive animals and positive water sources. Natural areas create opportunities for interspecies interactions, favoring leptospires transmission across species. Understanding that *Leptospira* serovars are present in natural areas is an integral part of the safe human and pet recreational use of these areas. Our study should raise awareness and build on public education designed to prevent disease transmission between species.

Key words: *Didelphis virginiana*, *Felis catus*, feral cat, leptospirosis, *Procyon lotor*, raccoon, Virginia opossum, zoonosis.

INTRODUCTION

Leptospirosis is a zoonosis caused by spirochetes of the genus *Leptospira* (Hartskeerl et al. 2011), which include species pathogenic for mammals (Adler and de la Peña-Moctezuma 2010). Leptospires survive in fresh water (Andre-Fontaine et al. 2015) and in warm, moist areas for weeks to months, contributing to the risk of animal exposure (Bolin 2000). Infection is acquired via exposure of mucus membranes or skin lesions to urine of an infected animal, or ingestion of contaminated water (Levett 2015). Clinical symptoms can include dysuria, abortion, and meningitis, among others (Wohl 1996; Bolin 2000). Wildlife and domestic animals serve as reservoirs. Asymptomatic reservoirs can shed leptospires in urine for months to years (Adler and de la Peña-Moctezuma 2010).

Leptospirosis in human and canines has increased in North America. From 1997 through 2001, the average number of cases

of leptospirosis in humans—serovars representative of all serogroups—increased from 2.8% to 6.8% annually (Meites et al. 2004). Although *Leptospira interrogans* serovars Canicola and Icterohemorrhagiae are commonly associated with canine leptospirosis, and *L. interrogans* serovar Bratislava is maintained in dogs (*Canis lupus familiaris*) worldwide, clinical cases in dogs have emerged associated with *L. interrogans* serovars Pomona and *L. kirchneri* serovar Grippotyphosa in the US (Ellis 2015). Predictive models used to analyze 14 yr of canine leptospirosis in the US identified the Midwest, East, and Southwest as areas of higher prevalence (White et al. 2017). In west-central Illinois, 48% (222/459) of raccoons (*Procyon lotor*) tested seropositive; 220 raccoons had antibody titers for *L. interrogans* serovar Grippotyphosa, and two for *L. interrogans* serovars Canicola and Icterohemorrhagiae (Mitchell et al. 1999). Blanding's turtles (*Emydoidea blandingii*) in an urban setting in northeast Illinois showed antibody titers, suggesting exposure to *L. kirschneri* serovar Grippotyphosa, and *L. interrogans* serovars Brataslava and Icterohemorrhagiae (Grimm et al. 2015).

Many studies have reported on the seroprevalence of *Leptospira* in mammals and reptiles, and on the presence of leptospiral DNA in water sources across Illinois, yet little work has been done to identify local *Leptospira* serovars in a single natural habitat. There are concerns that feral cats (*Felis catus*) and wildlife in natural areas serve as reservoirs of pathogens that affect humans, other wildlife, and domestic animals visiting the area (Pedersen et al. 2018). Our objectives were to: 1) determine seroprevalence of *Leptospira* serovars in medium-sized mammals in relation to capture sites (building or forest sites); 2) compare seroprevalence and antibody titers over two sampling periods; and 3) evaluate the presence of leptospires in water sources. We hypothesized that *Leptospira* seroprevalence would differ between trapping sites and sampling periods.

MATERIALS AND METHODS

Study area and site selection

We evaluated the seroprevalence of *Leptospira* among medium-sized mammals in Robert Allerton Park, the largest natural area in a predominantly agricultural landscape, located along 4 km of the Sangamon River and 7 km southwest of Monticello, in Piatt County, Illinois (39°59′37″N, 88°39′5″W). It encompasses 607 ha of river corridor, meadows, prairies, and upland and bottomland forests surrounded by agricultural lands and dispersed buildings on the edge of the park (Robert Allerton Park 2018). The park supports Illinois endangered and threatened species (Szafoni et al. 2012). Its predominant recreational use and relevance as a natural area makes it a valuable resource for the ecoepidemiology research of zoonotic diseases. Site selection criteria followed study findings by Fredebaugh et al. (2011), reporting a high occurrence of raccoons, Virginia opossums (*Didelphis virginiana*), and feral cats. Trapping sites (Fig. 1) included four sites within 300 m of a building (H-sites), and four sites within the forest (F-sites) more than 300 m away from a building.

Mammal trapping

We live trapped mammals from June–October of 2008 and April–September of 2009 at eight sites within Robert Allerton Park (Fig. 1). Each trapping event consisted of forty tomahawk traps (model 108, Tomahawk Live Trap, Tomahawk, Wisconsin, USA) baited with sardines (*Clupea pilchardus*) for two overnight live trappings per site. We conducted 44 trap nights in 2008 and 64 in 2009, with equal trap nights per site (54 at each site). We sedated captured animals using a combination of ketamine (Butler Schein Animal Health, Dublin, Ohio, USA) and xylazine (Akorn Inc., Decatur, Illinois, USA; Nielsen 1999; Kreeger et al. 2002), and recorded species, sex, and age. Blood was drawn from the cephalic, ventral coccygeal (opossums only), or saphenous veins. Opossums and raccoons were tagged with a passive integrated transponder (Biomark, Inc., Boise, Idaho, USA) for future identification. All animals reached full recovery from sedation prior to their release at their original trapping site. We identified feral cats based on photographs. We allowed at least 2 wk prior to retesting an animal. The University of Illinois Veterinary Diagnostic Laboratory (Urbana, Illinois, USA) conducted the microscopic agglutination test (MAT) using a seven serovar *Leptospira* panel and following standard protocols (Center for Veterinary Biologics and National Veterinary Services Laboratories [NVSL], Ames, Iowa, USA). The study was conducted under approved University of Illinois

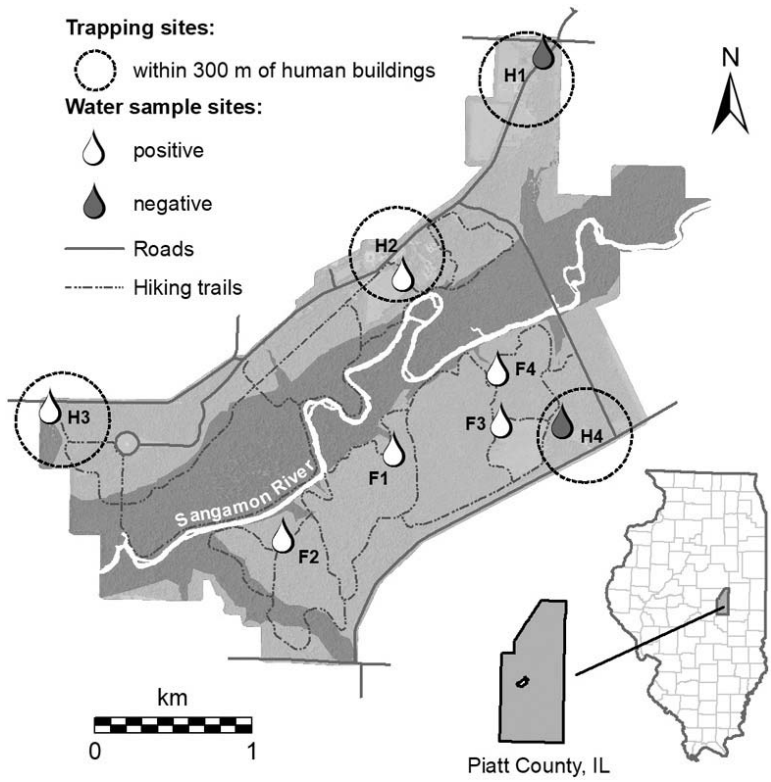

FIGURE 1. Map of Robert Allerton Park (Piatt County, Illinois, USA) including trapping sites and water sample locations. Blood samples from raccoons (*Procyon lotor*), Virginia opossums (*Didelphis virginiana*), and feral cats (*Felis catus*) were collected from June to October of 2008 and April to September of 2009. Human (H) sites (dashed circles) are within 300 m of human dwellings and include H1, H2, H3, and H4. Forest (F) sites are far from human dwellings and encompass a greater area than 300 m. Forest sites include F1, F2, F3, and F4. Water samples were collected near trapping sites and tested by quantitative PCR for leptospiral DNA (white drop=positive; black drop=negative).

at Urbana-Champaign Institutional Animal Care and Use Committee protocol (IACUC protocol no. 06110).

Microscopic agglutination test

The evaluation of serum included twofold serial dilutions from 1:25 to 1:800 against seven serovars (*Leptospira interrogans* serovars Autumnalis, Bratislava, Canicola, Icterohaemorrhagiae, Pomona; *Leptospira kirschneri* serovar Grippotyphosa; *Leptospira borgpetersenii* serovar Hardjo). The antigen was prepared from cultures grown in Probumin media (Millipore, Billeria, Massachusetts, USA) and centrifuged at 349 × G for 10 min at room temperature to remove dead bacteria. The supernatant was diluted 1:6 with sterile phosphate buffered saline.

Serum samples were centrifuged at 349 × G for 1 min at room temperature to remove red blood cells and lipids, pipetted into a 96-well flat-bottom plate, and diluted 1:25 to 1:800 with phosphate buffered saline. We added 50 μL of antigen to the 50 μL of diluted sera. Plates were examined under dark-field microscopy following incubation for 2 h at room temperature. The endpoint was determined by the last positive (>50% agglutination) dilution. We considered a titer of ≥1:25 as seropositive for exposure to *Leptospira*, and ≥1:800 as a potential indicator of recent or active infection (Veterinary Diagnostics Laboratory Standard Operating Procedure, Center for Veterinary Biologics, and NVSL). Samples with a titer of 1:800 were retitered using a serial dilution of 1:400–1:12,800 to determine the end point.

Water source collection

One-liter water samples were collected in July 2009 near each trapping site and within 6 m of marked hiking trails (Fig. 1). We sampled ponds, creeks, and rainwater runoff; avoided rapidly

moving water; and collected samples far from the bank to avoid contamination by algae, plants, and sediment.

DNA preparation of water samples

To pellet the bacteria for DNA extraction we: 1) centrifuged each water sample (1 L) for 20 min at 4 C and 6164 × G; 2) removed supernatant and further centrifuged the pelleted bacteria at 6800 × G for 10 min to tighten up the pellet; 3) resuspended the pellet in 200 µL of tissue lysis buffer (Buffer ATL®, QIAGEN Inc., Valencia, California, USA) and 20 µL of proteinase K to begin DNA extraction. We isolated genomic DNA with the QIAamp DNA mini kit (QIAGEN Inc., Valencia, California, USA). A culture of the *L. interrogans* serovar Autumnalis obtained from the NVSL served as a positive control.

Primer selection and PCR conditions

We screened water samples using the quantitative (q)PCR (Smart Cycler system, Cepheid, Sunnyvale, California, USA) with Omnimix® (Cepheid) master mix and primers designed for pathogenic *Leptospira*, which amplified an 87-base pair fragment of the 16S rRNA gene between positions 171 to 258, and a fluorescent dual-labeled probe with fluorescent reporter dye (FAM) and quencher (TAMRA) as described in Table 1 (Smythe et al. 2002). To validate the real-time PCR assay, we amplified the template DNA with longer 16S rRNA *Leptospira* spp. primers (Merien et al. 1992), performing conventional gel electrophoresis, extracting the amplicon, and performing Sanger sequencing. Thirty of 30 qPCR-positive templates yielded *Leptospira* 16S rRNA gene sequences. We included positive and negative controls with each sample set. We followed the parameters of the Veterinary Diagnostic Laboratory where a sample with a cycle threshold (Ct) <38 was positive, a Ct>40 was negative, and samples with Ct results in between 38 and 40 were considered suspect.

Statistical analyses

We used IBM SPSS version 23 (IBM Corp., Armonk, New York) for the statistical analyses. Statistically significant covariates (e.g., host species, sex, or age) from the univariate analysis of the association between individual variables and *Leptospira* seroprevalence using Pearson chi-square and Fisher's exact tests entered the logistic regression models for comparing differences in seroprevalence and antibody titers (previous vs. recent infection) between years, sampling sites (H-sites vs. F-sites), and water sources (positive vs. negative sources). We calculated adjusted odds ratios (OR) and 95% confidence intervals (CI).

We used Poisson regression models to compare the mean number of positive serovars per host between host species, sexes, and ages, separately by year, and to compare differences between years in the mean number of positive serovars per host, adjusting for host species, sex, and age. For animals recaptured more than twice in a year, and in both 2008 and 2009, we only used the first and last sample within a year in the analysis. We considered $P \leq 0.05$ significant.

RESULTS

We captured 351 medium-sized mammals and collected 448 samples including from recaptures (n=202 in 2008 and n=246 in 2009). Feral cats (n=9 in 2008 and n=9 in 2009) had no measurable antibody titers ($<$1:25) and were removed from the analysis. Most raccoons and opossums were captured near H-sites (244/333; 173 raccoons and 71 opossums), compared to 48 raccoons and 41 opossums captured at F-sites. We captured adults (171/333; 120 raccoons and 51 opossums), juveniles (99/333; 66 raccoons and 33 opossums), and subadults (63/333; 35 raccoons and 28 opossums); females (162/333; 109 raccoons and 53 opossums), and males (171/333; 112 raccoons and 59 opossums).

Leptospira seroprevalence and titers in raccoons and opossums

Overall, 54.8% of raccoons and 53.6% of opossums exhibited antibody titers to at least one of the seven *Leptospira* serovars (Table 2). The serovar Autumnalis was the most prevalent (38.7%), with Bratislava (28.5%) and Grippotyphosa (21.3%) as the next most common serovars in raccoons and opossums. Raccoons and opossums showed antibody titers for the seven serovars evaluated in this study at the three lowest cutoffs. However, serovars Bratislava and Icterohaemorragiae were not detected in opossums at $\geq$1:100 levels (Table 3).

In 2008, the serovar Autumnalis was higher in opossums compared to raccoons (P=0.002), whereas the serovars Bratislava, Grippotyphosa, and Icterohaemorragiae were higher in

TABLE 1. *Leptospira* primers and probe sequences used to screen water samples (rain runoff, creek, and pond) collected in 2008 and 2009 at Robert Allerton Park, Piatt County, Illinois, USA.

qPCR	Name	Position	Sequence (5′ to 3′)[a]
Primer[b]	Lepto F1	171	CCC GCG TCC GAT TAG
Primer[b]	Lepto R1	258	TCC ATT GTG GCC GRA/GA CAC
Probe[c]	FAM[d] (5′); TAMRA[e] (3′)	205; 228	CTC ACC AAG GCG ACG ATC GGT AGC

[a] The quantitative (q)PCR primers and probe are based upon those of Smyth et al. (2002). We programmed the Cepheid SmartCycler® for an initial denaturation step at 95 C for 120 s followed by 40 denaturation cycles at 95 C for 15 s and 64 C for 20 s of annealing and extension.

[b] The F1 and R1 primers result in an 88-base pair amplicon that displaces the FAM from the probe, leading to signal detection proportionate to the amount of *Leptospira* DNA target present in the sample.

[c] This is a dual-labeled probe with FAM at the 5′ end (position 205) and TAMRA at the 3′ end (position 228).

[d] FAM = dye (6-carboxy-fluorescein).

[e] TAMRA = quencher (6-carboxy-tetramethyl-rhodamine).

raccoons than opossums ($P<0.001$, $P=0.011$, and $P=0.049$, respectively). In 2009 raccoons presented higher seroprevalences for serovars Bratislava ($P<0.001$) and Grippotyphosa ($P=0.007$). Seroprevalence for all serovars decreased from 2008 to 2009, except for Hardjo (which increased in raccoons and opossums), and Canicola (which increased only in opossums).

We identified antibody titers suggestive of recent or active infection (≥1:800; Table 2) in 27 raccoons and four opossums, including serovars Grippotyphosa, Autumnalis, and Bratislava in raccoons, and Grippotyphosa, Autumnalis, and Pomona in opossums. A total of 35.7% raccoons and 23.2% opossums had titers for two or more serovars. In 2008 more raccoons showed antibody titers suggestive of recent infection compared to opossums ($P=0.035$). Six opossums captured in 2008 exhibited the same antibody titers to multiple serovars (Animal Health Diagnostic Center 2018). Raccoons, 17 in 2008 and 12 in 2009, were not serovar-specific.

Association with age and sex

We found significant differences in the overall seroprevalence of *Leptospira* antibodies by age ($P=0.005$ in 2008 and $P=0.003$ in 2009). Age-associated differences were significant for serovars Autumnalis ($P=0.011$ in 2008 and $P=0.005$ in 2009), Bratislava ($P<0.001$ in both years), and Gripotyphosa ($P=0.023$ in 2008 and $P=0.004$ in 2009), with a higher proportion of seropositive adults than subadults and juveniles in all cases. Only serovars Icterohaemorragiae ($P=0.003$ in 2008) and Hardjo ($P=0.041$ in 2009) showed significant differences with sex (females>males). However, titer levels—recent (≥1:800) or previous (1:25–1:800) infection—were not associated with age or sex.

Association with years, sampling sites, and water sample results

We found significant differences in the number of positive serovars detected per animal, which can range from zero to seven, between mammal hosts ($P=0.007$ in 2008 and $P=0.008$ in 2009), males and females ($P=0.005$ in 2008), adults and subadults ($P=0.001$ in 2008), and between adults and juveniles ($P<0.011$ in both years; Table 4). We found significant differences ($P<0.001$) in the number of positive serovar titers per animal between years after adjusting for age, sex, and host species.

Leptospira seroprevalence was higher at F-sites than H-sites (OR=2.3, 95% CI 1.3–3.9, $P=0.002$). However, we found more animals with antibody titers reflective of recent infection at H-sites than F-sites (17 at H-sites and one at F-sites in 2008; 12 at H-sites and four at F-sites in 2009). There was no significant association between the number of animals with antibody titers ≥1:800 or 1:25–1:800, and capture sites (H-sites or F-sites; OR=0.8, 95% CI 0.3–1.9, $P=0.551$).

TABLE 2. Within-year comparison of seroprevalence of seven leptospiral serovars detected in raccoons (*Procyon lotor*) and Virginia opossums (*Didelphis virginiana*) sampled in 2008 and 2009 at Robert Allerton Park, Piatt County, Illinois, USA. Statistically significant differences ($P<0.05$) are indicated in bold.

Serovars	2008[a]			2009[a]		
	Raccoons ($n=97$)	Opossums ($n=51$)	*P* value	Raccoons ($n=124$)	Opossums ($n=61$)	*P* value
Autumnalis	39 (4)	34 (1)	**0.002**	36 (4)	20 (1)	0.601
Bratislava	41 (6)	3	**<0.001**	50 (9)	1	**<0.001**
Canicola	14 (1)	3	0.121	4	6	0.084[b]
Icterohemorrhagiae	17 (2)	3	**0.049**	9	2	0.344[b]
Pomona	13 (1)	4	0.313	3	4 (1)	0.221[b]
Grippotyphosa	32 (10)	7 (1)	**0.011**	28 (11)	4 (1)	**0.007**
Hardjo	4	1	0.660[b]	9	6	0.573[b]
Multiple serovars[c]	41	7		38	5	
Positives	55 (12)	34 (1)	0.239	66 (15)	26 (3)	0.175
Negative	42	17		58	35	

[a] (#) = number of hosts with antibody titers of ≥1:800.

[b] Fisher's exact tests *P* values. All other *P* values are derived from chi-square tests.

[c] Multiple serovars = mammalian hosts with antibody titers showing titers to two or more leptospiral serovars.

We detected leptospiral DNA in all three water source types sampled (Table 5). Six of the eight water samples were interpreted as positive (Ct<38) for *Leptospira*; two of four samples at H-sites tested negative. There was no association between seropositive animals and positive/negative water samples (OR=1.0, 95% CI 0.5–2.0, $P=0.927$).

Temporal differences in *Leptospira* seroprevalence

We identified seroconversion in 25 recaptured animals (Table 6). We recaptured 93/351 animals (30 in 2008, 36 in 2009, and 27 in both years). Six animals seroconverted from negative to ≥1:400; one opossum and three raccoons showed antibody titers ≥1:1600 when recaptured; three had a fourfold rise in titers (1–3 mo after the first capture), one raccoon sustained titers of ≥1:800 for two consecutive years. One raccoon in 2008 and two in 2009 exhibited Hardjo antibody titers >1:800, suggesting a recent infection. Hardjo was the only serovar that increased in 2009 compared to 2008 (Table 7). Overall, *Leptospira* seroprevalence was associated with sampling year for all serovars, except Bratisla-

TABLE 3. Number of observations (percent) at the three lowest cutoff titers for seven *Leptospira* serovars detected in raccoons (*Procyon lotor*) and Virginia opossums (*Didelphis virginiana*) sampled in 2008 and 2009 at Robert Allerton Park, Piatt County, Illinois, USA.

Species	MAT[a] cutoff titer	No. (%) leptospiral serovars						
		Autumnalis	Bratislava	Canicola	Grippotyphosa	Hardjo	Icterohaemorragiae	Pomona
Raccoon total[b] (%)	≥1:25	79 (36)	97 (44)	18 (8)	62 (28)	13 (6)	27 (12)	16 (7)
	≥1:50	69 (31)	88 (40)	12 (5)	57 (26)	11 (5)	24 (11)	13 (6)
	≥1:100	52 (24)	72 (33)	9 (4)	50 (23)	10 (5)	18 (8)	11 (5)
Opossums total[b] (%)	≥1:25	65 (58)	4 (4)	10 (9)	16 (14)	8 (7)	5 (4)	8 (7)
	≥1:50	43 (38)	1 (1)	4 (4)	14 (13)	6 (5)	2 (2)	1 (1)
	≥1:100	18 (16)	0	1 (1)	10 (9)	1 (1)	0	1 (1)

[a] MAT = microscopic agglutination test. MAT serum dilutions start at 1:25.

[b] Total = cumulative counts of hosts with antibody titers at three different cutoffs (including blood samples from recaptures). Percentage of hosts in parentheses.

TABLE 4. Within-year comparisons of the number of positive results for seven *Leptospira* serovars per host (Poisson regression) between host species, sex, and age. Raccoons (*Procyon lotor*) and Virginia opossums (*Didelphis virginiana*) blood samples were collected in 2008 and 2009 across Robert Allerton Park, Piatt County, Illinois, USA. Positive serovars per host could range between zero to seven (the total number of *Leptospira* serovars evaluated). Statistically significant differences ($P<0.05$) are indicated in bold.

	2008		2009	
Variables[a]	Mean ratio (95% confidence interval)	*P* value	Mean ratio (95% confidence interval)	*P* value
Species	1.5 (1.1–2.1)	**0.007**	1.6 (1.1–2.2)	**0.008**
Sex	1.5 (1.1–1.9)	**0.005**	1.2 (0.9–1.6)	0.162
Age				
Subadult	0.4 (0.2–0.7)	**0.001**	0.8 (0.6–1.1)	0.233
Juvenile	0.5 (0.4–0.7)	**<0.001**	0.3 (0.2–0.5)	**<0.001**

[a] Reference group: Species = opossums, sex = male, age = adult.

va, which had a higher percent of seropositive animals in 2008 compared with 2009 although it was not significant ($P=0.448$). The decrease in positive opossums in 2009 (Table 2) explained the differences in overall *Leptospira* seroprevalence between 2008 (66.7%) and 2009 (42.6%; Table 7).

DISCUSSION

We surveyed and identified seven *Leptospira* serovars circulating in this natural area located within an agricultural landscape in Illinois. We detected leptospiral DNA in water samples, and following a capture-mark-recapture effort we identified antibody titers in wildlife hosts and multiple serovars within an individual host. Natural areas create opportunities for interspecies interactions that favor *Leptospira* transmission. Humans, pets, and other wildlife species could be at risk of exposure.

The typical minimum accepted positive MAT cutoff titer is 1:100 (1/100 final dilution; OIE 2018). However, some dog studies use 1:200 (Stokes et al. 2007). Although a higher cutoff value for a positive test result might underestimate seroprevalence, it is valuable in the study of *Leptospira*-vaccinated hosts (e.g., dogs) to differentiate immune response to infection from vaccination. In dogs, titers ≥1:1600 suggest recent infection (Animal Health Diagnostic Center 2018). Hosts with chronic infection and antibody titers <1:100 could be renal or genital carriers and might suffer from other clinical symptoms (OIE 2018).

Because wildlife in a natural setting are not vaccinated, we used a cutoff titer ≥1:25 for detection of exposure to *Leptospira*. A study using a cutoff of 1:40 reported 46.1% seroprevalence in raccoons at titers ≥1:80 (Tan et al. 2014). Our seroprevalence in raccoons was 54.8%, comparable to 47% in Indiana (Raizman et al. 2009), 48% in Illinois (Mitchell et al. 1999), and 36% in Connecticut (Richardson and Gauthier 2003); but higher than 11% in Nebraska (Bischof and Rogers 2005). We detected a seroprevalence of 53.6% in opossums compared to Connecticut where *Leptospira* was not detected in 28 opossums (Richardson and Gauthier 2003). Differences between studies might be due to inconsistencies in cutoff titers, serovars evaluated, characteristics of sampling sites, climate, or geographical location and time of the year of the study. We recognized that a low cutoff titer, such as the one used in our study, could result in false positives. Had we decided to consider a higher cutoff titer, a reduction of serovars detected would be evident, but not substantially different for most of the serovars evaluated (Table 3). Despite reduction in serovars detected, the total number of seropositive hosts might not be largely affected, because many hosts were infected by two or more serovars. Therefore, we suggest using a

TABLE 5. Real-time PCR results for the detection of *Leptospira* spp. DNA in water samples taken near the capture sites at Robert Allerton Park, Piatt County, Illinois, USA where raccoons (*Procyon lotor*) and Virginia opossums (*Didelphis virginiana*) were captured for testing for *Leptospira* serovars. Water samples were collected in July 2009.

Site[a]	Water type	Ct	qPCR interpretation
Human sites			
H1	Pond	41.37	Negative
H2	Pond	33.59	Positive
H3	Creek	35.33	Positive
H4	Rain runoff	38.43	Negative
Forest sites			
F1	Rain runoff	37.78	Positive
F2	Creek	36.13	Positive
F3	Creek	29.72	Positive
F4	Rain runoff	29.43	Positive

[a] Human sites are within 300 m of human dwellings and include H1, H2, H3, and H4. Forest sites are located greater than 300 m from human dwellings and include F1, F2, F3, and F4.

[b] Samples with cycle threshold (Ct) <38 were considered leptospiral-positive, samples with Ct>38 were considered leptospiral-negative.

lower cutoff titer of 1:50 to indicate exposure to *Leptospira* in wild mammals.

Overall, the reported seroprevalence of *Leptospira* in cats is low; with reports of 9.2% positive in Scotland (Agunloye and Nash 1996), 14% in Spain (Millán et al. 2009), and 8.6% in Iowa, US (Palerme et al. 2019). We sampled nine feral cats per year and did not detect *Leptospira* antibodies. Low population prevalence and our small sample size could explain the seronegative results. However, the true prevalence could be as high as 34% because the binomial 95% CI (0–34%) is wide. Wildlife home range overlap is possible in our study. Home ranges for cats are greater than the distances between some of our trapping sites (Horn et al. 2011). A small sample size (n=18) could have limited our ability to detect *Leptospira* serovars in cats, and ecological and regional variations in prevalence could have influenced risk of exposure and serovar diversity (Ward et al. 2004). However, some reports indicate low and short-lived antibody titers to *Leptospira* following experimental infections in cats (Fessler and Morter 1964), suggesting the need for temporal studies to capture seasonal variations. Low- and short-lived antibody titers could explain why only two studies document leptospirosis in free-roaming cats in the US: Markovich et al. (2012) reporting 4.8% seroprevalence, and Palerme et al. (2019) reporting 8.6% seroprevalences. Our study was conducted mostly during summer months (June–October 2008, April–September 2009); there could be seasonal influences impacting seroprevalence detection in cats

TABLE 6. Summary of changes in antibody titers in recaptured animals. Recaptured animals (n=93) were divided into six categories depending on how their titers to *Leptospira* serovars changed upon recapture. A minimum of 2 wk was required before an animal was tested again. Recaptured animals in 2008 and 2009 included raccoons (*Procyon lotor*) and Virginia opossums (*Didelphis virginiana*).

Change in titer	No. animals[a]	Explanation
Second titer 4× higher than original titer	3	Fourfold increase in titer[b]
Negative titer to ≥1:800	7	Recent infection
Titers between 1:25 and 1:800	25	Previous infection[c]
Negative titer to low or moderate titers (<1:400)	25	Seroconversion[d]
Negative titer to significant titer (≥1:400 but <1:800)	6	Incidence
Positive titer to negative titer	26	Recovered

[a] Because some animals had antibody titers to more than one serovar, different changes in antibody titers were observed, thus an animal could have been assigned in more than one category.

[b] Fourfold increase in titer (or seroconversion to >1:1600) is indicative of recent *Leptospira* infection.

[c] Animals that showed antibody titers between 1:25 and 1:400 both times that they were captured.

[d] Seroconversion cases do not account for fourfold increase from their original titer. Therefore, the possibility of a false positive is greater than in the category of incidence cases.

TABLE 7. Comparing *Leptospira* seroprevalence (logistic regression) in raccoons (*Procyon lotor*) and Virginia opossums (*Didelphis virginiana*) sampled between 2008 and 2009 in Robert Allerton Park, Piatt County, Illinois, USA, adjusted by species, sex, or age if significant ($P<0.05$). Statistically significant differences are indicated in bold.

Serovars	Percentage (frequency)[a]		Odds ratio[b] (95% confidence interval)	*P* value
	2008	2009		
Autumnalis	49 (73/148)	30 (56/185)	2.4 (1.5–3.8)	**<0.001**
Bratislava	30 (44/148)	28 (51/185)	1.2 (0.7–2.2)	0.448
Canicola	12 (17/148)	5 (10/185)	2.3 (1.0–5.1)	**0.048**
Grippotyphosa	26 (39/148)	17 (32/185)	2.0 (1.1–3.5)	**0.017**
Hardjo	3 (5/148)	8 (15/185)	0.4 (0.1–1.1)	0.075
Icterohaemorragiae	14 (20/148)	6 (11/185)	2.5 (1.2–5.5)	**0.021**
Pomona	12 (17/148)	4 (7/185)	3.3 (1.3–8.2)	**0.010**
All 7 serovars	60 (89/148)	50 (92/185)	1.6 (1.0–2.5)	**0.043**

[a] Frequency = number of positive samples/total number of samples tested per year.

[b] Reference group: 2009, adjusted for significant covariates.

(Palerme et al. 2019) that we were unable to capture. Pet cats with outdoor access can shed leptospires even when their serology results are *Leptospira*-negative (Arbour et al. 2012). We do not know if seronegative feral cats can shed *Leptospira* in the park.

Autumnalis, Bratislava, and Grippotyphosa were the most common serovars that we detected. There is debate over the pathogenicity of the Autumnalis serovar (Prescott et al. 2002; Moore et al. 2006), especially because dogs vaccinated with Grippotyphosa and Pomona have developed higher and long-persisting titers to Autumnalis, exceeding the titers for the vaccinating serovars (Barr et al. 2005). Wildlife in the study area were not vaccinated, therefore our results were not a response to vaccination but to circulating infective serovars in wildlife. In our study, Autumnalis titers were detected at lower levels (<1:400), whereas Grippotyphosa and Bratislava most frequently showed antibodies indicative of active or recent infection (titers ≥1:800). Grippotyphosa and Bratislava have similar protein profiles and share some degree of serological cross reactions. When testing a battery of *Leptospira* serovars, reaction to various serovars could be seen due to cross-reactivity among antigenically similar serovars, or infection with multiple serovars (Chirathaworn et al. 2014). Having antibody titers to multiple serovars might not mean infection of an animal by multiple serovars, but that additional diagnostic methodologies are required to validate distinct serovar infections.

Raccoons are presumed reservoirs for *Leptospira* spp. (Hamir et al. 2001), especially for serovar Grippotyphosa (Mitchell et al. 1999). Grippotyphosa, a dominant serovar detected in raccoons in this study, has been associated with human outbreaks of leptospirosis in Illinois (Morgan et al. 2002). However, it is important to determine if the high antibody titers for *Leptospira* found in raccoons are a result of disease, reservoir, and shedding status, or the result of a particularly robust immune reaction.

Raccoons had most of the higher antibody titers (≥1:800) whereas opossums had low to moderate antibody titers (<1:400). Despite observed differences in titer levels for raccoons and opossums, and the lack of detectable antibody titers in cats, raccoons and opossums were exposed to the seven *Leptospira* serovars evaluated, and 35.7% of raccoons and 23.2% of opossums had antibody titers for two or more serovars. Although not known, all sampled mammals could be reservoirs for leptospirosis. Clinically diseased animals are likely to shed leptospires in urine for months to years after initial infection (Guerra 2009). Raccoons held the highest seroprevalence for 2 consecutive years; serology does not allow for inferences about

disease or shedding status, but raccoons could serve as sentinel species (Duncan et al. 2012) for leptospirosis. Opossums exhibited a significantly lower seroprevalence in 2009 (43%) compared to 2008 (67%), indicating temporal changes associated to host species. Age and sex influenced seroprevalence, with higher proportions of seropositive adults and higher seroprevalence for specific serovars in females. Older animals could have been exposed to the pathogen for longer periods of time, developing higher antibody titers than juveniles (Raizman et al. 2009). Seroprevalence differences between hosts might be explained by habitat use and the natural history of these species. Differences in home range could impact exposure to the pathogen, thus, a lower proportion of seropositive juveniles could be expected because juveniles have smaller home range than adults (Mitchell et al. 1999). Social behaviors among females and family groups could affect pathogen exposure in different ways for different host species. Female opossums provide moderate parental care, with time to independence of 3 mo (Martina 2013). Raccoons have longer time to independence (about 10 mo) and can form strong bonds between siblings as they den and feed together, especially during winter (University of Wyoming Raccoon Project 2019). Other contributing factors to survival of *Leptospira* around the park might include soil characteristics, soil and water pH, and temperature (Barragan et al. 2017).

We trapped more animals at H-sites compared to F-sites, suggesting a concentration of wildlife around human areas and wildlife dependence on humans for food (Prange et al. 2003) and shelter (Fredebaugh et al. 2011). Congregations of animals increase the risk of pathogen transmission between species, and could explain the higher proportion of antibody titers, suggestive of recent *Leptospira* infection in animals trapped at H-sites vs. F-sites. Despite that finding, we did not see a statistically significant correlation (P=0.551) between antibody titer levels and capture sites. We trapped animals at night, but they might live in the forest and travel to the human sites at night to find food. We did not use radio telemetry, and cannot identify habitat overlap and associated opportunities for risk of exposure and infection between mammals. Nevertheless, the increased number of animals trapped near human dwellings could increase human, domestic animal, and wildlife interactions, favoring the likelihood of transmission of the pathogen among hosts.

Six of eight water samples tested positive for leptospiral DNA, indicating that all three types of water sources (runoff, creek, and pond) could be potential sources of *Leptospira*. The lack of correlation between trapping location of seropositive animals and leptospiral-positive water sources suggested that temporal evaluation of water sources could help us to understand the ecology of *Leptospira* in contaminated water. Temporal data could help us to integrate weather data (e.g., rainfall and temperature) to bacterial survival in water sources and *Leptospira* infection in wildlife. All water sources were within 6 m of a marked trail, suggesting easy access for dogs. Thus, we recommend bringing drinking water for dogs visiting the natural area rather than allowing them to drink from natural sources.

We did not sample other potential reservoirs such as rodents, cervids, or domestic or wild canids; their contribution to leptospirosis in this natural area is not understood. Unlike the clinical disease seen in canines and humans, the health impact of leptospirosis in wildlife is unclear. Collection of urine sample to detect shedding of *Leptospira* organisms could allow the assessment of an animal's infectious status. Performing a necropsy on fresh road-killed animals to look for kidney lesions (Millán et al. 2009) and collecting tissues for immunohistochemistry would also aid in confirming disease (Shearer et al. 2014). Pairing urine PCR and serology data could help to establish the relation of *Leptospira* antibody titers and shedding of leptospires in urine, thereby helping us establish proper MAT cutoff values to study leptospirosis in wildlife.

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