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LEPTOSPIROSIS IN URBAN AND SUBURBAN AMERICAN BLACK BEARS (*URSUS AMERICANUS*) IN WESTERN NORTH CAROLINA, USA

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ABSTRACT: American black bear (*Ursus americanus*) populations in North Carolina, US have recovered significantly in recent decades and now occupy much of western North Carolina, including urban-suburban areas. We used the black bear as a potential sentinel for leptospirosis, a bacterial zoonotic disease caused by *Leptospira* spp., which is maintained by domestic and wild mammals. We determined whether *Leptospira* spp. were present across a gradient of housing densities in the urban and suburban black bear population in and around Asheville, North Carolina using serologic and molecular surveys. We collected blood from captured black bears (n=94) and kidneys and bladders from carcasses (n=19). We tested a total of 96 (47 females, 47 males, and 2 unknown) serum samples by microscopic agglutination test (MAT) and had positive results (titer >1:100) for *L. kirschneri* serovar Grippotyphosa (L. Grippotyphosa) in 4 females (8%) and 5 males (10%). No other serovars showed elevated titers in MAT. We tested a total of 125 samples using PCR (n=96 serum, n=20 kidney, and n=9 bladders) and obtained positive results from one serum (1%), one kidney (5%), and one bladder (11%). The presence of *Leptospira* spp. in black bears occupying an urban and suburban landscape may indicate a more extensive occurrence of the bacteria among animals in the study region because black bears are the top carnivore in that ecosystem. Potential threats of widespread contamination during natural events such as flood or drought must be considered.

Key words: Black bears, carnivores, *Leptospira* spp., leptospirosis, reservoir host, urban and suburban areas, *Ursus americanus*, zoonotic disease.

INTRODUCTION

Leptospirosis is a bacterial zoonosis which is common worldwide and is caused by various species of *Leptospira*, an aerobic spirochete (Plank and Dean 2000; Palaniappan et al. 2002). Over 300 known serovars of *Leptospira* are endemic to and maintained by feral and domestic mammals as well as reptile and amphibian hosts (Plank and Dean 2000; Fouts et al. 2016). Infection in humans can be caused through contact with contaminated soil, water, or infected animal tissue or from infected animal bites (Lecour et al. 1989; Evarard et al. 1995; Faisal et al. 2012).

Kidney disease, liver disease, or reproductive dysfunction are the clinical signs of leptospirosis in animals. Infected animals can shed leptospires in urine while remaining subclinical (Plank and Dean 2000). In the US, cattle (*Bos taurus*), dogs (*Canis lupus familiaris*), and rats (*Rattus norvegicus*) are the most-common carriers of *Leptospira* spp. (Vinetz et al. 1996). Leptospirosis is considered a re-emerging disease in domestic dogs, where infection can occur by contact with either the reservoir hosts or their urine or by contact with water sources contaminated with infected urine, often with spatial clusters associated with urban-suburban areas (Gautam et al. 2010).

Wild animals, domestic animals, and humans can serve as potential sources of infection by *Leptospira* spp. for each other (Leighton and Kuiken 2001). *Leptospira interrogans* has been identified in a wide variety of wildlife species including: red fox (*Vulpes vulpes*; Clark et al. 1960), gray fox

(*Urocyon cinereoargenteus*; Clark et al. 1961), coyote (*Canis latrans*; Drewek et al. 1981), black-tailed deer (*Odocoileus hemionus columbianus*; Cirone et al. 1978), white-tailed deer (*Odocoileus virginianus*; Ingebrigtsen et al. 1986), moose (*Alces alces*; Bourque and Higgins 1984), wolf (*Canis lupus*; Zarnke and Ballard 1987), and American black bear (*Ursus americanus*; Zarnke and Ballard 1987). Also, *Leptospira kirschneri* serovar Grippotyphosa has been identified in black bears (Zarnke 1983).

Globally, urban expansion into wildlands increases potential exposure of wildlife to unprecedented conditions and presents more opportunities for wildlife to become dependent on anthropogenic food resources (Baruch-Mordo et al. 2014). Spatially concentrated, highly productive, and temporally predictable (anthropogenic food) resources in urban or suburban areas attract wildlife to these novel environments (Beckmann and Berger 2003; Schochat 2004; Rodewald and Shustack 2008). Bears successfully exploit the anthropogenic resources in urban and suburban areas by virtue of their behavioral plasticity, intelligence, and omnivorous food habits (McCullough 1982; Gilbert 1989). Reported human-black bear interactions have increased due to presence of garbage disposal units in urban-suburban areas, which are perennial resources for bears because of available and reliable food regardless of season or environmental conditions (Beckmann and Berger 2003).

Since 2010, the human population in Asheville, North Carolina, US has increased from 83,403 to approximately 91,902 (9.3% increase), resulting in urban and suburban encroachment into areas occupied by black bears. Detection of *Leptospira* spp. in black bears may present a possible transmission risk between other wildlife and domestic species as well as to humans. Also, it may indicate how human encroachment can increase the exposure of certain diseases to otherwise healthy wildlife populations. Therefore, the objective of our study was to conduct a serologic and molecular survey to determine the presence of different serovars of *Leptospira* spp. across a gradient of housing densities in an urban-suburban black bear population. Our objectives were to determine if black bears with greater housing density/km^2 within their occurrence distribution (OD) had a greater likelihood of testing positive for leptospirosis; and if younger bears would have a greater likelihood of testing positive than would older adult bears due to their inexperience and being forced into less-optimal habitats.

MATERIALS AND METHODS

Project location and study area

The study was located in western North Carolina and centered on the urban-suburban area in and around the city of Asheville, North Carolina (35.5951°N, 82.5515°W). The Asheville city limits encompass 117 km^2 with approximately 91,000 people, located in Buncombe County in the southern Appalachian mountain range (Kirk et al. 2012).

Western North Carolina is characterized by a heterogeneous topography (500–1,800 m elevation), mild winters, cool summers, and high annual precipitation (130–200 cm/yr), mostly in the form of rainfall. Black bears occur throughout the region. The major forest types include mixed deciduous hardwoods with scattered pine (*Pinus* spp.; Kirk et al. 2012) and pine-hardwood mix (Mitchell et al. 2002).

Bear capture and sample collection

From April 2014 through September 2015, we used reports of black bears on private property to identify trap sites. We followed a spatially balanced design to deploy 10–14 culvert traps within or adjacent to Asheville city limits. Traps were baited with day-old pastries. We checked traps daily from 0800–1100 hours and 1830–2130 hours.

Once captured, we immobilized bears with 5 mL of tiletamine-zolazepam (5 mL at 100 mg/mL; MidWest Veterinary Supply, Norristown, Pennsylvania, USA), 4.0 mL of ketamine hydrochloride (MidWest Veterinary Supply), and 1.0 mL of xylazine hydrochloride (MidWest Veterinary Supply). We placed uniquely numbered ear-tags in both ears, applied a matching tattoo to the inside of the upper lip, removed an upper first premolar for age determination from all bears $\geq$12 mo-old (Willey 1974), and inserted a passive integrated transponder tag (PIT tag, Biomark, Boise, Idaho, USA) between the scapulae. Additionally, we collected ectoparasites and hair as well as blood for serum from the femoral artery. We recorded

body mass, sex, reproductive status (based on evidence of lactation, estrus, or descended testicles), external morphometric measurements, and the date and capture location for each bear. We fitted bears with a GPS radio collar (Vectronic, Berlin, Germany) that did not exceed 3% of the animal's body weight (Samuel and Fuller 1996; Cattet 2011). We administered a long-lasting analgesic and an antibiotic to each bear and then reversed the anesthesia using yohimbine hydrochloride (0.15 mg/kg; Diamondback Drugs, Scottsdale, Arizona, USA) within approximately 60 min of immobilization. All animal handling techniques were approved by the Institutional Animal Care and Use Committee at North Carolina State University (14-019-O) and followed guidelines provided by the American Society of Mammalogists (Gannon and Sikes 2011).

We collected kidneys and the bladder (when intact) from vehicle-killed bears on roadways in Buncombe County. Field necropsies were performed on any carcass obtained within 24 h of the animal's death, prior to decomposition. All biological samples were frozen at –20 C and shipped frozen to the Cornell Animal Health Diagnostic Center, Ithaca, New York every 2–3 mo.

Laboratory methods

The microscopic agglutination test (MAT) is the gold standard for serodiagnosis of leptospirosis because of its high diagnostic specificity (Ye et al. 2014a, b). In our panel, we used five leptospire species and serovars: *L. interrogans* serovar Canicola (L. Canicola), *L. interrogans* serovar Icterohaemorrhagiae (L. Icterohaemorrhagiae), *L. interrogans* serovar Pomona (L. Pomona), *L. interrogans* serovar Hardjo (L. Hardjo), and *L. kirschneri* serovar Grippotyphosa (L. Grippotyphosa) obtained from the US Department of Agriculture-National Veterinary Services Laboratory, representing the circulating serovars from the US. We mixed a dilution series of each bear's serum with a suspension of live leptospires in microtiter plates. After incubating for about 2 h at 30 C, we read results under the dark-field microscope. The titer was the last dilution in which 50% of the leptospires had remained agglutinated. Seroconversion or a fourfold rise in titer in paired sera was considered consistent with current leptospirosis. We used the MAT as the reference method to determine serum titers, using live *Leptospira* serovars as antigen, as previously described (Ye et al. 2014a, b). The MAT testing performed in this study is a National Veterinary Services Laboratory-validated standard Leptospirosis MAT test with annual proficiency testing and constant monitoring and periodic titration of live leptospire antigens.

Samples from kidneys and bladders could not be tested by MAT. We tested tissues and serum using PCR. All samples were processed using a DNeasy Blood and Tissue Extraction Kit (no. 69506 or 69504, Qiagen, Mansfield, Massachusetts, USA). We placed 100 µL of serum or 20–50 mg of kidney or urinary bladder in a clean microcentrifuge tube and added 180 µL of tissue lysis buffer (ATL) and 20 µL proteinase K (Sigma, St. Louis, Missouri, USA) to each sample tube. Samples were incubated overnight at 37 C, then 200 µL of lysis buffer (AL) and 200 µL of 95% ethanol were added to each sample. The sample tubes were mixed well, and samples were passed through a spin column (1 min, 10,000 × G). The flow through was discarded, and the columns were washed with 0.5 mL of AW1 wash buffer. Samples were washed once more with AW2 wash buffer, and each step was followed by a 10,000 × G spin for 1 min. The washed spin columns were spun dry and then transferred to fresh microcentrifuge tubes. Lastly, we added 40–50 µL of sterile, double-distilled water to each column; these were spun at 6,000 × G for 3 min for the DNA elution step. The eluted DNA samples were used as templates in a subsequent PCR run.

The DNA isolates were tested for the presence of *Leptospira* spp. using a GeneAmp 9700 thermocyler (Applied Biosystems, Carlsbad, California, USA). The PCR recipe totaled 25 µL per sample and consisted of the following: 2.5 µL of 10× buffer, 0.5 µL of Taq, 1.5 µL of $MgCl_2$ (no. 18038-042 Invitrogen, Carlsbad, California, USA), 1 µL of (10 mM) dNTP, and 0.5 µL of 20-µm primer. Sterile, double-distilled water made up the balance of the recipe. About 5 µL of DNA sample were used as a template; each PCR run included both a positive and negative control. The primers had the following sequences: G1 forward 5′-CTG AAT CGC TGT ATA AAA GT-3′, G2 reverse 5′-GGA AAA CAA ATG GTC GGA AG-3′. The parameters set for the *Leptospira* PCR run consisted of an initial 5-min denaturing step at 95 C, a three-stage cycling run of 95 C for 30 sec, 50 C for 45 sec, 72 C for 30 sec (35 cycles total), a 5-min extension step at 72 C, and completion with a 4 C cold soak. After PCR, samples were run on 1% agarose gel with ethidium bromide. The gel was placed in an imager equipped with an ultraviolet lamp lightbox; the gel image generated was saved digitally for future viewing.

Statistical analysis

Space use: We used the Brownian Bridge Movement Model (BBMM package) in program R (R Development Core Team 2009) to develop annual OD for black bears. The BBMM is used to develop ODs for wildlife deployed with GPS tracking collars where data collection typically

exceeds 1,000 locations per season. The model uses sequential relocation data, an estimate of the error associated with the GPS location, and a standardized grid cell size for the associated output (Horne et al. 2007). The BBMM package is based on a Brownian bridge; therefore, the probability of an animal being in a particular area (location) is dependent on the area in which it was most-previously located and is based on the elapsed time between consecutive locations. The model fills in the area between consecutive locations, and the width of that Brownian bridge is based upon the elapsed time (resulting in a larger gap width for locations that are farther apart) and GPS collar location error (Horne et al. 2007; Sawyer et al. 2009; Walter et al. 2011). We used the resulting 95% OD to represent the annual occurrence estimate of the actual area used by the animal during the study rather than the estimated home range (Fleming et al. 2015). For ease of interpretation, we divided housing density/km^2 into five categories: very low (5–29 houses/km^2), low (30–168 houses/km^2), medium (169–307 houses/km^2), high (308–446 houses/km^2), and very high (447–1,267 houses/km^2).

For the construction of our BBMM's, we used the average error rate (m) for the collar locations for each individual bear (mean=4,500 locations/bear). When we were unable to retrieve the collar, and thus download the error rate associated with each fix, we used an error rate of 20 m for that bear. None of our averaged error rates exceeded 19 m and, therefore, we used a cell size of 50 m.

Analysis: We calculated housing density/km^2 of each bear's 95% OD as a representation of that animal's exposure to anthropogenic features (e.g., humans, garbage, pets) as potential sources for leptospirosis contamination. We classified the bear population in our study area into three age groups (i.e., 1 yr as yearlings, 2 yr as subadults, and $\geq$3 yr as adults). We used logistic regression in program R and reported the results as odds ratios to ascertain the effect of housing density/km^2 of the 95% OD, sex, and age category on whether or not a bear tested positive for *Leptospira* spp. We limited our candidate model set to four a priori models to avoid including spurious effects due to overparameterization and because our sample size was small. We used Akaike's information criterion corrected for small sample size (AICc) to assess model weights, and we ranked candidate models using ΔAICc (Burnham and Anderson 2002). We used Akaike weights to determine the relative support for each model, and we used model averaging across parameters for housing density, sex, and age, and calculated the unconditional variance estimates and associated 95% confidence intervals (Burnham and Anderson 2002; Anderson 2008). We determined if these parameters influenced whether or not a bear tested positive for leptospirosis by examining whether their confidence intervals overlapped zero.

RESULTS

We tested a total of 96 (47 females, 47 males, and 2 unknown) serum samples by MAT collected between April 2014 and September 2015. Tissue samples from the bladder and kidney of two nonstudy animals were collected outside Buncombe County and thus could not be included in the housing density estimation (Figs. 1, 2). Also, we did not include three additional bears in our modeling, which were positive for leptospirosis, because we could not calculate housing density for those individuals due to lack of relocations. We had positive results (titer >1:100) for L. Grippotyphosa in four females and five males with titers ranging from 1:200 to 1:3,200; there were no elevated MAT titers to any other serovars (Fig. 2 and Table 1).

Using the PCR technique, we tested 55 females (two bladders, six kidneys, 47 serum samples), 68 males (seven bladders, 14 kidneys, and 47 serum samples), and sera from two individuals in which sex was not determined for a total of 125 samples. We had positive results for one serum, one kidney, and one bladder (Table 1). None of the MAT-positive animals had corresponding positive PCR results. Because the MAT for *Leptospira* tests only for specific serovars whereas PCR detects a broad range of leptospires, it is possible the animal might have been infected with a serovar other than the seven we tested for, and we could not rule out a chronic renal colonization. We were unable to obtain cultures from the PCR-positive samples because they had been previously frozen. GenBank analysis of PCR products revealed 99% similarity to most *Leptospira* species. We did not document any significant influence of housing density, age, or sex of bears on the occurrence of leptospirosis among the bear population in

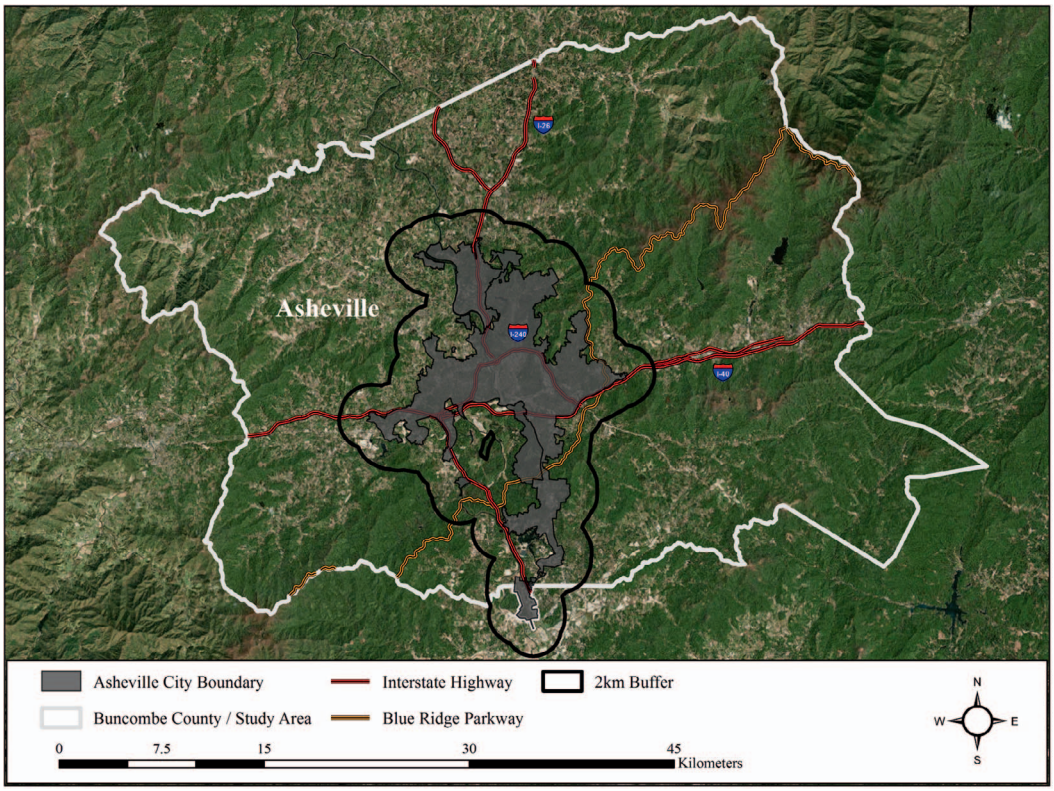

FIGURE 1. The Asheville City and Buncombe County study area boundaries for the North Carolina urban and suburban black bear (*Ursus americanus*) research project, Asheville, North Carolina, USA in 2014–15.

and around Asheville, likely due to our small samples sizes (Tables 2, 3).

DISCUSSION

Our study documented the presence of L. Grippotyphosa in an urban and suburban black bear population across a gradient of housing density and human development around Asheville, North Carolina. Although our sample sizes were relatively small, our observations of the presence of L. Grippotyphosa in very low-density housing areas suggest *Leptospira* spp. are ubiquitous across the urban-suburban gradient of Asheville and not strictly limited to the densely populated areas. Because *Leptospira* spp. can spread through contaminated soil or water, the potential of zoonotic exposure may increase with greater concentration of human development (Plank and Dean 2000). Further, black bears inhabited large areas and may have been incidentally exposed to L. Grippotyphosa present in other mammals or reptile and amphibian species (Plank and Dean 2000; Jorge et al. 2011) in Asheville and the surrounding area. Regardless, black bears should be considered another species capable of perpetuating disease transmission to people and domestic animals.

To elucidate infection versus exposure, we used two tests because MAT is insensitive in early phases of the infection. Infections usually take 8–10 d to obtain a specific antibody response (Merian et al. 1995; Olds et al. 2015). Early infections can be detected by PCR, which is most effective during the first 10 d following exposure (Merien et al. 1995). Our differences in MAT and PCR detection may be explained by the nature of the tests. The PCR detects genetic material from the *Leptospira*, indicating the bacteria

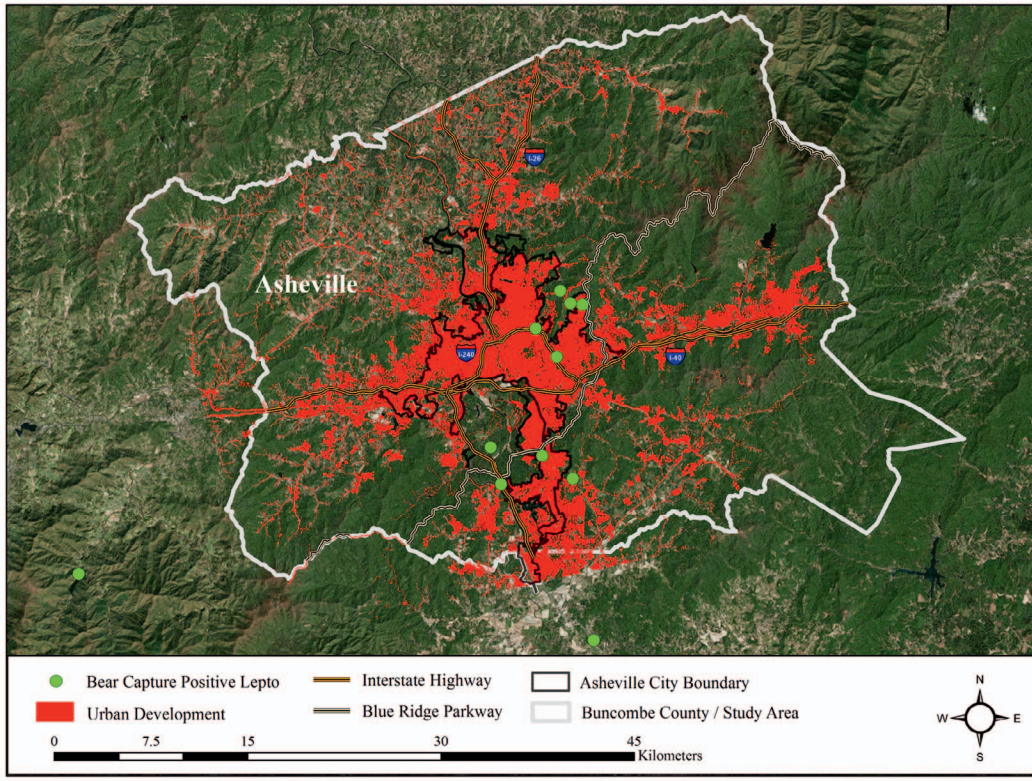

FIGURE 2. The Asheville City and Buncombe County study area boundaries, primary area of human development, and capture or carcass locations with positive test results for *Leptospira* spp., Asheville, North Carolina, USA in 2014–15.

TABLE 1. Titers for *Leptospira* spp. in 12 black bear (*Ursus americanus*) samples collected in 2014 and 2015 along with 95% occurrence distributions (OD) for individual bears and their associated housing density/km^2 within the 95% OD in Asheville, North Carolina, USA.[a]

Bear identification	Sex[b]	Age[c]	Specimen	95% OD (km^2)	House density/km^2	L. Grippotyphosa	PCR
N015	F	6	Serum	15.08	351.3	400	Negative
N018	F	1	Serum	—	—	200	Negative
N018/N091	F	2	Serum	3.77	105.1	<100	Positive
N032	M	3	Serum	24.35	106.8	400	Negative
N040	F	A	Serum	12.66	73.6	200	Negative
N057	F	2	Serum	4.79	79.8	400	Negative
N065	M	1	Serum	35.07	82.0	200	Negative
N068	M	2	Serum	39.93	—	1,600	Negative
N097	M	Y	Serum	—	—	3,200	Negative
N097	M	—	Serum	—	—	400	Negative
Unknown no. 9	M	—	Kidney	—	—	—	Positive
Unknown no. 4	M	—	Bladder	—	—	—	Positive

[a] — = no data available.

[b] F = female; M = male.

[c] A = adult, no tooth obtained; Y = yearling, no tooth obtained.

TABLE 2. Model selection results using Akaike's information criterion (AICc) for the effect of housing density (per km^2), sex, and age class on whether or not a black bear (*Ursus americanus*) in Asheville, North Carolina, USA in 2014–15 tested positive for leptospirosis by microscopic agglutination test or PCR.

Model[a]	Model likelihood					
	AICc	ΔAICc	AICc weight	EXP[b] (−0.5 × ΔAICc)	K[c]	Log like
HDens	42.64	0	0.460	1.00	2	−19.21
Sex	43.01	0.37	0.383	0.83	2	−19.40
Age	45.02	2.38	0.140	0.31	3	−19.29
HDens + sex + age	49.33	6.69	0.016	0.04	5	−19.10

[a] HDens is the density of houses (per km^2) within a bear's 95% OD.

[b] EXP = exponentiation.

[c] Number of estimable parameters, including intercept and residual.

are present in the tissues or blood and that the animal is currently infected. Conversely, the MAT measures antibodies, indicating the animal has been exposed to the pathogen but may not currently be infected and shedding leptospires.

Studies that relied only on MAT may have overestimated the occurrence of *Leptospira* in wildlife, depending on the duration of an antibody response in a given species. Our prevalence was 9% as determined by MAT and 2% as determined by PCR. Based on the discrepancies of the two test modalities, we believe that our results are conservative and that a larger proportion of black bears may have actually been exposed to *Leptospira* but were not shedding during our study. Further, the presence of *Leptospira* in the samples taken from the kidney and the bladder indicates the animal's ability to shed leptospires. The few PCR-positive samples indicated that bacteria can be located in multiple tissues (serum, bladder, and kidney); however, bears may be incidental hosts capable of eliminating these organisms before they develop antibody titers. It is possible that even if animals do begin shedding, they do not shed for an extended period of time and are able to clear the organism.

The 9% seroprevalence (determined by MAT) in black bears in our study was higher than that observed in black bear studies from Idaho (Binninger et al. 1980), Alaska (Zarnke 1983), and Maryland (Bronson et al. 2014). However, antibody prevalence for *Leptospira* spp. in black bear populations could be considered low when compared to raccoons (*Procyon lotor*; 36%), skunks (*Mephitis mephitis*; 13%), bobcats (*Felis rufus*; 25%), rats (44%), and squirrels (*Sciurus carolinensis*; 100%) in other studies (Heidt et al. 1988; Richardson and Gauthier 2004; Thayaparan et al. 2013). Lack of *Leptospira* spp. epidemiology in our study area indicates the need for

TABLE 3. Model-averaged coefficients and associated odds ratios (OR) for the effect of housing density (per km^2), sex, and age class on whether or not a black bear (*Ursus americanus*) in Asheville, North Carolina, USA in 2014–15 tested positive for leptospirosis by microscopic agglutination test or PCR.

Variable	Estimate	Unconditional variance SE	Unconditional 95% confidence interval	OR	OR 95% confidence interval
Housing density	0.001	0.003	−0.005 to 0.007	1.003	0.994–1.011
Sex (male)	0.025	0.581	−1.114 to 1.163	1.064	0.169–6.696
Age (subadult)	0.016	0.417	−0.802 to 0.833	1.105	0.135–9.070
Age (yearling)	0.074	0.455	−0.807 to 0.955	1.606	0.191–13.508

more-detailed studies to determine the role black bears and other wildlife play in maintaining leptospirosis in western North Carolina.

The serovar L. Grippotyphosa is associated with disease in dogs (Center for Food Security and Public Health 2013), where occurrence of the serovar appears to be common even in vaccinated dogs (Barr et al. 2005; Goldstein 2010; Midence 2012). Domestic dogs can serve as an incidental or "dead-end host" that harbors the pathogen but cannot pass it to another host (Center for Food Security and Public Health 2013). Thus, the presence of serovar Grippotyphosa in the urban and suburban black bear population suggests that dogs may be at risk.

Infected animals usually excrete leptospires in urine, and the survival of the bacteria is dependent on local conditions such as temperature >22 C, moisture, and alkaline soil (Ghosh and Stevenson 1977; Southern 1981). Thus, the bacteria can stay in the environment for a long period of time once introduced. The presence of *Leptospira* spp. in the black bear population in western North Carolina indicated there was long-term environmental persistence of the bacteria. *Leptospira* is commonly transmitted through indirect contact with contaminated water and soil, which is generally associated with occupational, recreational, or avocational activities (Faisal et al. 2012; Haake and Levett 2015). Many of the common outdoor recreational activities in and around Asheville can possibly cause exposures of *Leptospira* in humans.

Our research demonstrated that *Leptospira* is present in black bears around Asheville, North Carolina. Urbanization coupled with potentially changing climates increases the risk of an outbreak of leptospirosis in and around urban and suburban areas (Lau et al. 2010).

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