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Listeria monocytogenes at the human–wildlife interface: black bears (*Ursus americanus*) as potential vehicles for *Listeria*

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Summary

Listeria monocytogenes **is the causative agent of the foodborne illness listeriosis, which can result in severe symptoms and death in susceptible humans and other animals.** ***L. monocytogenes*** **is ubiquitous in the environment and isolates from food and food processing, and clinical sources have been extensively characterized. However, limited information is available on** ***L. monocytogenes*** **from wildlife, especially from urban or suburban settings. As urban and suburban areas are expanding worldwide, humans are increasingly encroaching into wildlife habitats, enhancing the frequency of human–wildlife contacts and associated pathogen transfer events. We investigated the prevalence and characteristics of** ***L. monocytogenes*** **in 231 wild black bear capture events between 2014 and 2017 in urban and suburban sites in North Carolina, Georgia, Virginia and United States, with samples derived from 183 different bears. Of the 231 captures, 105 (45%) yielded** ***L. monocytogenes*** **either alone or together with other** ***Listeria*****. Analysis of 501 samples, primarily faeces, rectal and nasal swabs for** ***Listeria*** **spp., yielded 777 isolates, of which 537 (70%) were** ***L. monocytogenes.*** **Most** ***L. monocytogenes*** **isolates exhibited serotypes commonly associated with human disease: serotype 1/2a or 3a (57%), followed by the serotype 4b complex (33%). Interestingly, approximately 50% of the serotype 4b isolates had the IVb-v1 profile, associated with emerging clones of** ***L. monocytogenes.*** **Thus, black bears may serve as novel vehicles for** ***L. monocytogenes*****, including potentially emerging clones. Our results have significant public health implications as they suggest that the ursine host may preferentially select for** ***L. monocytogenes*** **of clinically relevant lineages over the diverse listerial populations in the environment. These findings also help to elucidate the ecology of** ***L. monocytogenes*** **and highlight the public health significance of the human–wildlife interface.**

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Introduction

Listeria monocytogenes is the causative agent of the severe foodborne disease listeriosis, which can result in stillbirths, meningitis, septicaemia and death in humans and other animals (Painter and Slutsker, 2007; Scallan *et al.*, 2011). *Listeria monocytogenes* is considered ubiquitous in nature, having been isolated from soil, water and vegetation in diverse geographic regions (Welshimer, 1968; Sauders *et al.*, 2012; Vivant *et al.*, 2013; Cooley *et al.*, 2014; Ferreira *et al.*, 2014). *Listeria monocytogenes* in food and food processing environments

has been extensively studied (Kathariou, 2002; Gandhi and Chikindas, 2007; Carpentier and Cerf, 2011; Ferreira *et al*., 2014), but the ecology of this pathogen in the natural environment remains poorly understood (Vivant *et al*., 2013).

Even though there is a notable dearth of information on the prevalence of *L. monocytogenes* and other *Listeria* spp. in wildlife (Ivanek *et al*., 2006; Wesley, 2007; Czuprynski *et al*., 2010; Chlebicz and Śliżewska, 2018), one of the earliest isolations of *L. monocytogenes* was from a lethal case of listeriosis in wild gerbils (Pirie, 1927). The gerbil (*Meriones unguiculatus*) was subsequently shown to be a valuable model for *Listeria* rhombencephalitis and other listeriosis outcomes (Blanot *et al*., 1997; Disson *et al*., 2009). However, while *L. monocytogenes* is capable of causing severe disease and death among wildlife (Wesley, 2007), infected mammalian species such as red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*) may remain asymptomatic (Sasaki *et al*., 2013; Gnat *et al*., 2015; Weindl *et al*., 2016). Importantly, analysis of *L. monocytogenes* from healthy wild bird populations revealed strain subtypes identical to those detected in foods (Hellström *et al*., 2008). Although limited, these results suggest a larger, currently uncharacterized ecological role for wildlife as reservoir and vehicle for *L. monocytogenes*.

American black bears (*Ursus americanus*) are omnivorous habitat generalists (Karelus *et al*., 2017; Moeller *et al*., 2017). In the United States, they use a wide range of habitats and food sources in forested, rural and residential areas. Increasing black bear habitat loss and forest fragmentation due to urban and suburban development have resulted in increased human–bear interactions, as evidenced by observations and property damage (North Carolina Wildlife Resources Commission, 2019). Increased frequency of human–wildlife interactions has major human and animal health implications, as it may lead to greater rates of zoonotic or anthroponotic transmission of pathogens (Patz *et al*., 2000; Daszak *et al*., 2001).

While the prevalence of several zoonotic pathogens in black bears has been investigated, the focus has been primarily on viruses and vector-borne pathogens (Binninger *et al*., 1980; Ruppanner *et al*., 1982; Gage *et al*., 1995; Bronson *et al*., 2014; Stephenson *et al*., 2015). A substantial knowledge gap currently exists regarding the incidence of other human pathogens in bear populations, especially in urban and suburban regions of the United States. There is noticeably limited information on the role of black bears as potential reservoirs or vehicles for human foodborne bacterial pathogens such as *Salmonella*, *Campylobacter* and *Listeria*.

In this study, samples obtained in the course of a wildlife mobility and demographic analysis of black bears in the southeastern United States were analysed for *L. monocytogenes* and other *Listeria* spp. Samples were obtained through the live capture and monitoring of black bear populations in three separate study sites in North Carolina (NC), Georgia (GA) and Virginia (VA). We determined the prevalence of *L. monocytogenes* and other *Listeria* spp. in various types of samples from black bears, including faeces, rectal swabs, nasal swabs and gastrointestinal tracts. To identify important subtypes of *Listeria* and assess their environmental resilience via previously identified adaptations, we analysed the distribution of different *L. monocytogenes* serotypes and the incidence of resistance to heavy metals (cadmium and arsenic) and the quaternary ammonium disinfectant benzalkonium chloride (BC). These results will help address a key knowledge gap in the ecology of *L. monocytogenes* in the natural environment.

Results

Listeria monocytogenes was frequently isolated from black bears in the southeastern United States. We analysed samples from 231 black bear capture events, corresponding to 183 animals. Of the 183 bears, 142 were captured and sampled once, while 38 were sampled multiple times. Most (203/231; 88%) of the capture events involved bears from urban or suburban areas in Asheville, NC (Fig. 1). Thirteen animals were captured in rural areas in GA, and 15 were in temporary captivity at Virginia Tech's Black Bear Research Center (VT-BBRC), Blacksburg, VA. Sample types obtained from the live animals included faeces, rectal swabs and nasal swabs.

Listeria colonies were identified based on typical morphology on Modified Oxford agar supplemented with Difco™ Modified Oxford Antimicrobic Supplement (MOX) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Of the 231 black bear capture events, 55% (127/231) yielded *Listeria* in one or more sample types (Fig. 2). Putative *Listeria* isolates that failed to yield any amplicons with multiplex PCR serotyping (Doumith *et al*., 2004) were not detected, and all non-haemolytic isolates yielded the *prs* band only. *L. monocytogenes* was detected in 105 (45%) of the black bear capture events, either alone (74/231; 32%) or, less commonly, together with other *Listeria* spp. (31/231; 13%) (Fig. 2). Only 22 captures (10%) yielded exclusively *Listeria* spp. other than *L. monocytogenes* (Fig. 2). Among the *Listeria*-positive black bears, the likelihood of animals being positive for *L. monocytogenes* was significantly higher than for other *Listeria* spp. ($P < 0.001$). The rate of recovery of *L. monocytogenes* and other *Listeria* spp. was similar across the three study sites, ranging between 40% and 56% (Fig. 2).

Analysis of the data from the NC population that was monitored from 2014 to 2017 failed to reveal noticeable

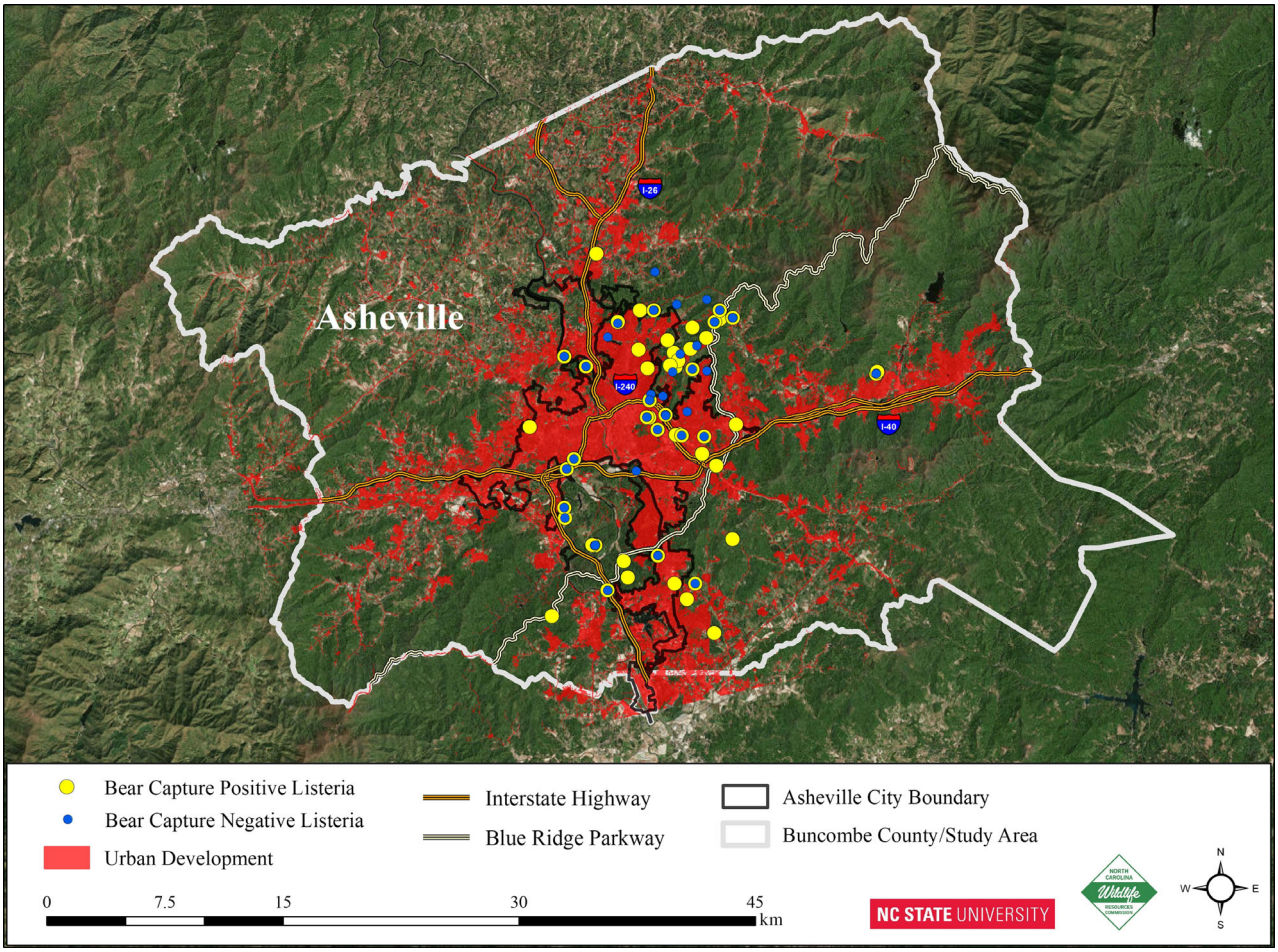

Fig. 1. Black bear capture locations in and around Asheville, NC. Yellow and blue symbols represent bears that were positive and negative, respectively, for *Listeria*. ArcMap 10.5.1 (http://desktop.arcgis.com/en/) was used to generate the map. ArcGIS Desktop: Release 10. Redlands, CA: Environmental Systems Research Institute.

temporal trends in *L. monocytogenes* recovery. The percentage of black bear capture events yielding *L. monocytogenes,* alone or in conjunction with other *Listeria* spp., remained constant throughout all 4 years of the study, varying from 42% to 49% (Fig. 3). The percentage of captures negative for *L. monocytogenes* but positive for other *Listeria* spp. also remained stable, varying between ~ 7-8% in years 1 and 4, and ~10–13% in years 2 and 3 (Fig. 3). Each year, capture events yielding *L. monocytogenes* were significantly more common than those yielding other *Listeria* spp. ($P < 0.01$).

L. monocytogenes *serotype 1/2a (or 3a) and the 4b complex (4b, 4d, 4e) predominated, with significant representation of the variant IVb-v1 multiplex PCR profile*

A total of 777 isolates were characterized over the course of the study. Employment of the multiplex PCR scheme of Doumith *et al.* (2004) to determine serotype designations identified several PCR profiles among these isolates (Fig. 4). Based on haemolytic activity and multiplex PCR results, 537/777 (approx. 70%) of the isolates were *L. monocytogenes*, with the remaining 240 being members of other *Listeria* spp. (Fig. 5). Serotype 1/2a (or 3a) (hereafter designated '1/2a') was the most prevalent serotype (57%), followed by the serotype 4b complex (4b, 4d or 4e, hereafter designated '4b'), which accounted for approximately 33% of the total *L. monocytogenes* isolates (Fig. 5). Interestingly, almost half of the serotype 4b isolates (82/179; 46%) exhibited the variant IVb-v1 multiplex PCR profile (Huang *et al.*, 2011; Leclercq *et al.*, 2011; Lee et al., 2012a,b) (Fig. 5). Examination of the locations for capture events yielding *L. monocytogenes* serotypes 1/2a and 4b in NC showed a relatively even distribution across the capture locations with no obvious geographic bias (Fig. 6A, B). The distribution largely reflected the overall pattern of the capture

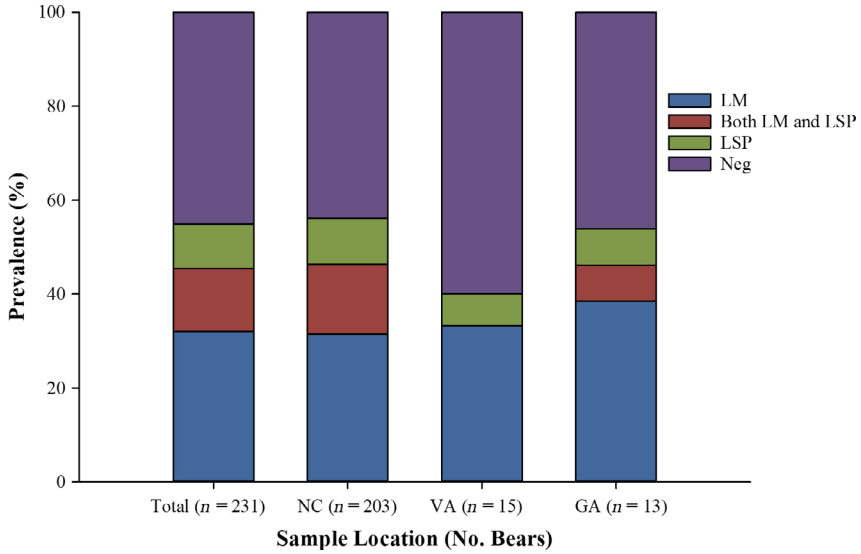

Fig. 2. The prevalence of *L. monocytogenes* and other *Listeria* spp. in American black bears. Samples were collected in 2014-2017 from bears in North Carolina (NC), Georgia (GA) and Virginia (VA). Inset: LM, *L. monocytogenes;* LSP, *Listeria* spp. other than *L. monocytogenes*; Neg, *Listeria*-negative animals.

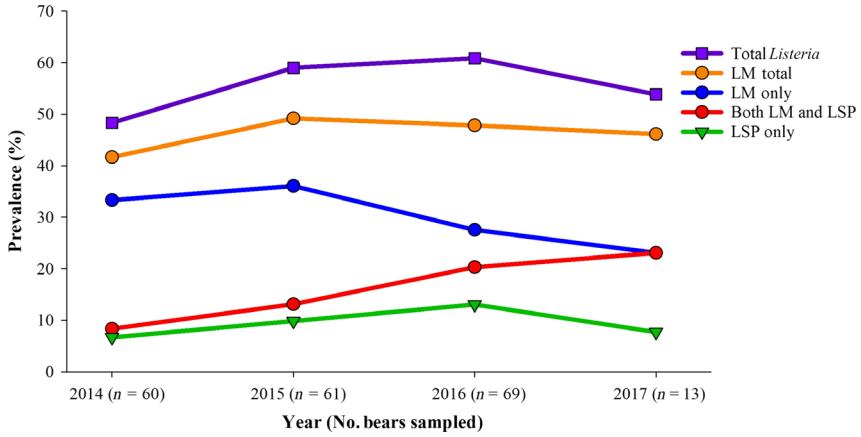

Fig. 3. Annual prevalence of *L. monocytogenes* and other *Listeria* spp. in American black bears in NC. Inset designations are as in legend to Fig. 2.

locations for the *Listeria*-positive animals (Fig. 1). Similar findings were obtained with the locations of capture events yielding serotype 4b strains with the typical multiple PCR profile in comparison to those with profile IVb-v1 (Fig. 6B).

Serotypes 1/2b and 1/2c were rarely encountered. Isolates of serotype 1/2b accounted for only 11/537 (~2%) of the *L. monocytogenes* isolates and were obtained from just five capture events in NC, GA and VA, while the 21 serotype 1/2c isolates accounted for ~ 4% of *L. monocytogenes* and were from nine captures, all in NC (Fig. 5). Lineage III isolates were also uncommon, obtained from only eight captures in NC, GA and VA and accounting for 13 (~2%) of the 537 *L. monocytogenes* isolates (Fig. 5). Multiplex PCR of these isolates for serotype designations yielded only the *prs* band (Fig. 4), and all isolates were PCR positive with *L. monocytogenes*-specific primers for *hly* (data not shown).

The multiplex PCR scheme also revealed a novel multiplex PCR profile in 12 isolates, obtained from six captures in NC in 2014 (n = 4) and 2016 (n = 2). This novel profile (designated 1/2a-1/2b) exhibited a combination of serotype 1/2a and 1/2b amplicons (Fig. 4). All 12 isolates with this profile were PCR positive with *L. monocytogenes*-specific primers for *hly* (data not shown). Capture events yielding isolates with this novel multiplex PCR profile were from several diverse locations (Fig. 6A).

We failed to identify haemolytic isolates that yielded only the *prs* band but were negative for *hly,* as would be expected for *L. seeligeri*, and on blood agar, none of the

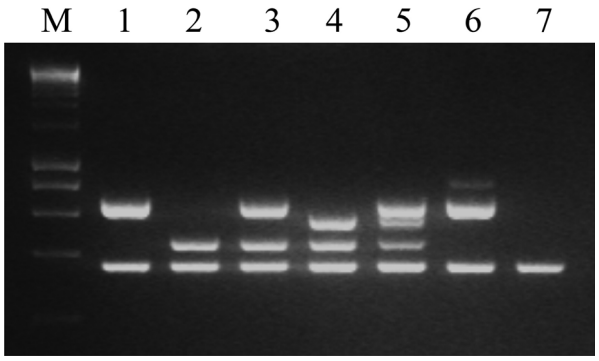

Fig. 4. Multiplex PCR-based serotype designations for *L. monocytogenes*. The multiplex PCR protocol was based on Doumith et al. (2004). Lanes: 1-7, black bear-derived isolates SKB31, SKB781, SKB102, SKB647, SKB341, SKB107 and SKB782 corresponding to serotypes 1/2a, 1/2b, novel multiplex PCR profile with amplicons typical both for serotypes 1/2a and 1/2b (1/2a-1/2b), 4b, IVb-v1, 1/2c and lineage III (only *prs* amplicon), respectively. M, molecular weight marker (HyperLadder 1kb; Bioline, Boston, MA, USA).

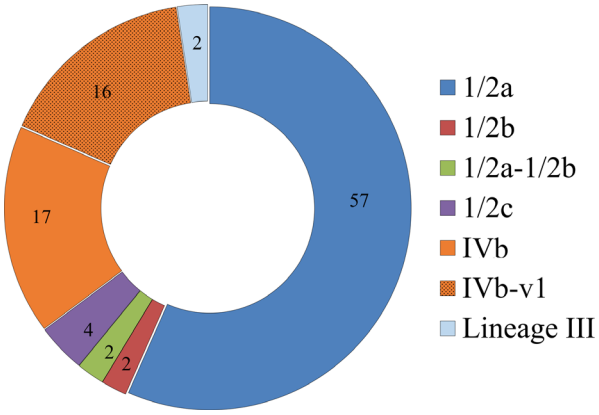

Fig. 5. The prevalence of different serotypes and lineage III among *L. monocytogenes* from American black bears. Numbers indicate the percentage of isolates with a specific multiplex PCR designation. Serotype and putative lineage III designations were determined for 537 isolates by multiplex PCR as described in Experimental Procedures. Bracket indicates total prevalence of serotype 4b (isolates with multiplex PCR profile 4b, indicated as IVb in inset, as well as those with multiplex PCR profile IVb-v1).

isolates exhibited the pronounced haemolysis typical for *L. ivanovii*. As mentioned earlier, we did not identify non-haemolytic isolates with *Listeria*-typical colony morphology on MOX but lacking the *prs* band, as may be the case for some *Listeria* spp. that were identified relatively recently (den Bakker *et al*., 2014) (J. Niedermeyer and S. Kathariou, unpublished).

Temporal stability of dominant serotypes and variant multiplex profile IVb-v1

The prevalence of the leading serotypes 1/2a and 4b remained high during each year of the study, without significant differences in yearly serotype recovery. Serotype 1/2a was encountered among 49–67% of the *L. monocytogenes* isolates, while serotype 4b prevalence ranged between 24% and 41% annually (Fig. 7). Recovery of serotype 4b isolates with the IVb-v1 multiplex PCR profile remained consistent over the study period, ranging between 15% and 17% of the *L. monocytogenes* isolates annually (Fig. 7). *Listeria* spp. other than *L. monocytogenes* accounted for 22–38% of isolates annually (data not shown).

Sample type and enrichment phase impact recovery of listeriae

Listeria was recovered significantly more frequently from primary than secondary enrichments (diff = 0.05, SE = 0.02, $t = 2.39$, $P = 0.04$). The listerial community composition (i.e. relative abundance of *L. monocytogenes*, other *Listeria* spp. and different serotypes of *L. monocytogenes*) was ~ 75% identical between primary and secondary enrichment.

Analysis of faeces, rectal swabs and nasal swabs indicated that sample type had the potential to influence *Listeria* recovery. Recovery of *L. monocytogenes* was similar between faecal samples and rectal swabs (35% and 33% respectively) and significantly higher than from nasal swabs, of which 19% were positive (rectal swabs: diff = 0.1, SE = 0.3, $t = -3.1$, $P = 0.002$, faeces: diff = 0.07, SE = 0.04, $t = -2.1$, $P = 0.04$) (Fig. 8). Interestingly, however, the likelihood of encountering *L. monocytogenes* as opposed to other *Listeria* spp. was significantly higher in nasal swabs than in faeces ($P = 0.0005$) or rectal swabs ($P = 0.02$). Furthermore, the prevalence of samples yielding only *Listeria* spp. other than *L. monocytogenes* was higher in faeces and rectal swabs (16% and 8% respectively) than in nasal samples (4%) (Fig. 8).

The small number of intestinal samples ($n = 14$; gastrointestinal tracks from vehicle-killed black bears) and the fact that they had been stored frozen prior to analysis prevented accurate assessments of *Listeria* prevalence in these samples. The only positive intestinal samples were from the large intestine of two bears and both yielded *L. monocytogenes* but no other *Listeria* spp.

Sample type and geographical location may impact recovery of certain serotypes and serotype 4b strains with multiplex PCR profile IVb-v1

Sample-dependent differences were noted in the prevalence of certain groups of isolates. Specifically, serotype 1/2a was significantly more common in faeces and rectal

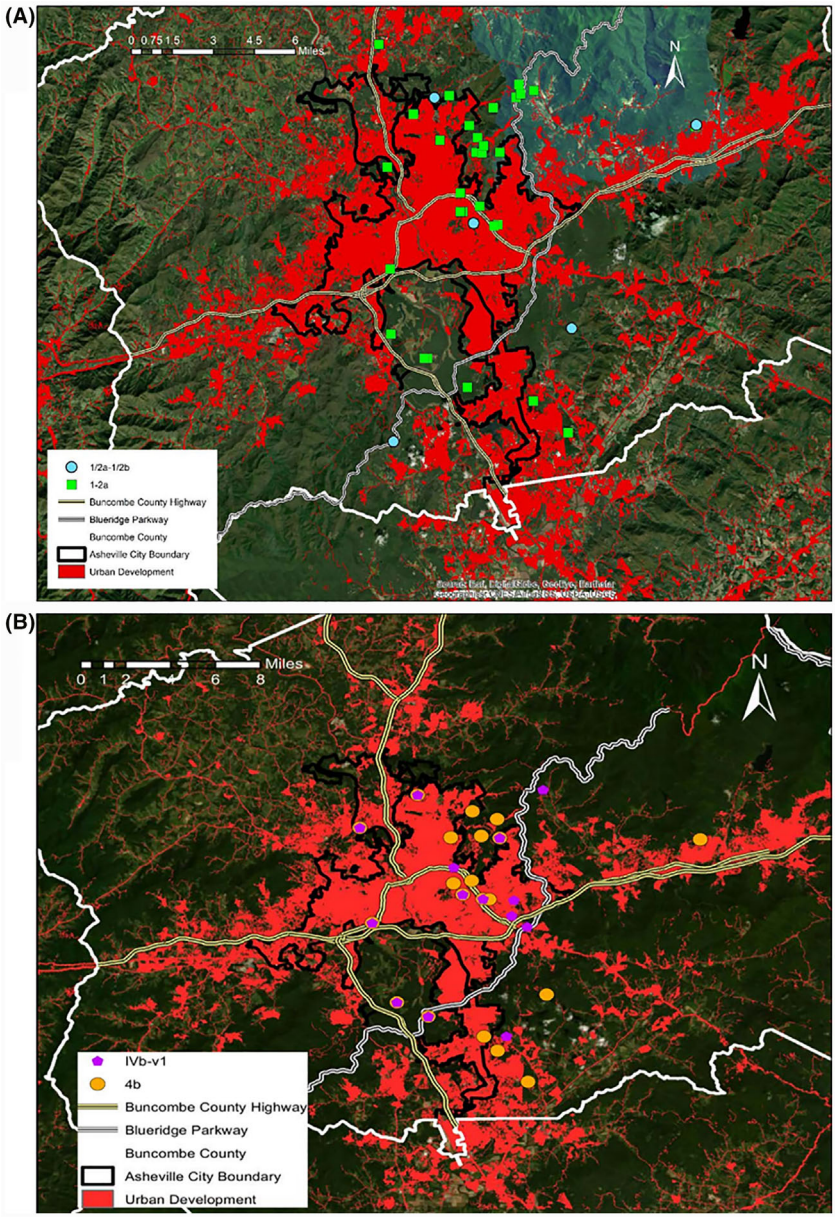

Fig. 6. Capture locations of bears yielding isolates with specific multiplex PCR profiles. (A), isolates with profile typical for serotype 1/2a and novel profile with amplicons typical both for serotype 1/2a and serotype 1/2b (1/2a-1/2b). (B), isolates with profile typical for serotype 4b (designated IVb in the inset) and with variant profile IVb-v1. Mapping software used was as in legend to Fig. 1.

swabs than in nasal swabs (faeces and nasal swabs: diff = −13.1, SE = 6.5, $Z = -2.0$, $P = 0.04$; rectal and nasal swabs: diff = −16.9, SE = 6.7, $Z = 2.5$, $P = 0.01$), with no significant difference in recovery between faeces and rectal swabs (Fig. 9A). Similar trends were noted for serotype 4b isolates with the IVb-v1 multiplex PCR profile (faeces and nasal swabs: diff = −8.1, SE = 3.9, $Z = -2.1$, $P = 0.04$, rectal and nasal swabs: diff = −11.1, SE = 4.3, $Z = 2.6$, $P = 0.01$) and for *Listeria* spp. other than *L. monocytogenes* (faeces and nasal swabs: diff = −20.6, SE = 5.9, $Z = -3.5$, $P = 0.001$, rectal and nasal swabs: diff = −11.9, SE = 5.3, $Z = 2.3$, $P = 0.02$) (Fig. 9A).

Location (NC, GA and VA) did not have significant impacts on the prevalence of serotypes 1/2a and 4b. However, serotype 4b isolates with the IVb-v1 PCR profile were only recovered in NC and GA, while isolates of serotype 1/2c and those with the novel multiplex PCR profile 1/2a-1/2b were only recovered from NC (Fig. 9B).

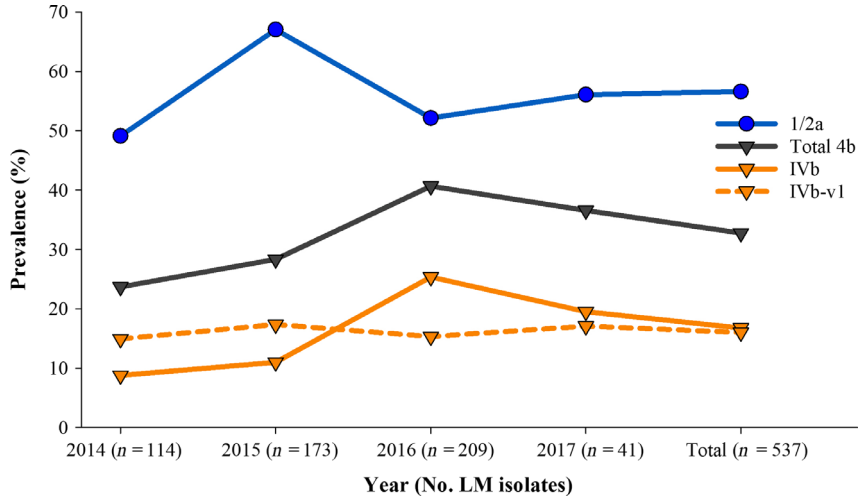

Fig. 7. Annual prevalence of the major serotypes of *L. monocytogenes* recovered from American black bears.

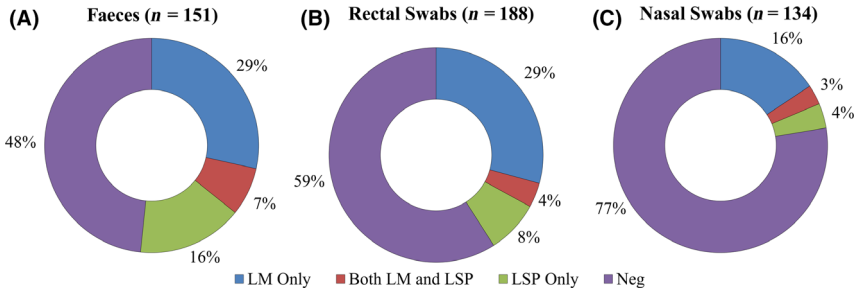

Fig. 8. The prevalence of *L. monocytogenes* and other *Listeria* spp. in different types of samples from American black bears. Sample types include (A) faeces (n = 151), (B) rectal swabs (n = 188) and (C) nasal swabs (n = 134). Numbers indicate per cent of samples. Inset designations are as in legend to Fig. 2.

Black bears are transiently colonized by a diverse population of listeriae

While higher resolution techniques will be required to make strain level distinctions between isolates, determination of *L. monocytogenes*, other *Listeria* spp. and different *L monocytogenes* serotypes/multiplex PCR profiles suggested diversity of the *Listeria* population colonizing bears. As previously stated, 13% of the black bear capture events yielded both *L. monocytogenes* and other *Listeria* spp. (Fig. 2). When strains of diverse serotypes were included, 43% (55/127) of the *Listeria*-positive captures yielded diverse strains, i.e., *L. monocytogenes* of different multiplex PCR profiles and *Listeria* spp. Of the 105 *L. monocytogenes*-positive captures, 45 (43%) yielded multiple serotypes of *L. monocytogenes*. Isolation of *L. monocytogenes* with different serotypes from the same sample was not uncommon; approximately 20% of *L. monocytogenes*-positive samples yielded isolates of multiple serotypes and 11% also yielded other *Listeria* spp. We based the listerial community composition on *Listeria* species, and serotype multiplex PCR profile distribution of all recovered isolates, as calculated by the Jaccard similarity index, was 77% similar between rectal swabs and faeces but similarity was lower between nasal and rectal swabs (45%) or faeces (54%).

As indicated above, 38 of the black bears were captured and sampled more than once. Most (33/38, 87%) of these animals were sampled twice, with four sampled three times and one five times (Table 1). Additionally, two of the bears that had been live-captured were later found to be killed by vehicles one and 4 months after their original capture. Analysis of the samples from the 38 bears sampled on multiple occasions suggested a high degree of transience in the *Listeria* populations of wild black bears (Table 1). Of the two bears sampled while live and again *post-mortem*, one had a *Listeria*-positive rectal swab sample during the live capture but the *post-mortem* small and large intestine samples were negative. In the 38 black bears that were live-captured multiple times, results frequently changed from *Listeria* positive to negative and *vice versa* between one

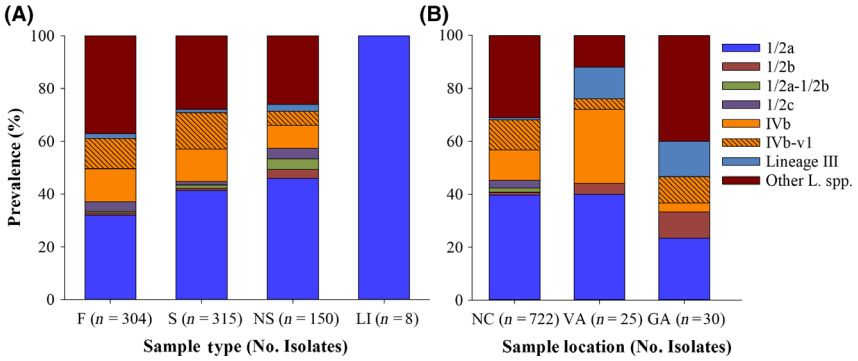

Fig. 9. The prevalence of different serotypes and lineage III among *L. monocytogenes* from American black bears based on (A) sample type and (B) geographical location. Sample types in panel A are faeces (F), rectal swab (S), nasal swab (NS) and large intestine (LI). Geographical locations in panel B are North Carolina (NC), Georgia (GA) and Virginia (VA). Serotype and putative lineage III designations were determined by multiplex PCR as described in Experimental Procedures. Inset designations are as in the legend to Fig. 5. 'Other *L.* spp'. refers to species of *Listeria* other than *L. monocytogenes*.

sampling time and another (Table 1). We identified only one animal (N108) that yielded the same *Listeria*-related data upon recapture (Table 1).

Heavy metal and disinfectant resistance were uncommon among listeriae from black bears

Of the 777 isolates, only 24 (3%; three *L. monocytogenes* and 21 other *Listeria* spp.) were resistant to cadmium (MIC $>$ 35 μg ml^{-1}). The three *L. monocytogenes* isolates were all serotype 1/2a from two black bears in NC, 2015; one was from faeces of one bear, while the other two were from the nasal swab of a different bear. The 21 cadmium-resistant non-*L. monocytogenes Listeria* isolates were from six different bears from NC at various times throughout the study (one each in 2014, 2015 and 2017; three in 2016).

PCR of the 24 cadmium-resistant isolates for the known *Listeria* cadmium resistance determinants *cadA1-cadA4* revealed *cadA1* (n = 6) and *cadA2* (n = 9) in 15 of the 21 non-*L. monocytogenes Listeria* isolates. The remaining nine cadmium-resistant isolates, including the three that were *L. monocytogenes*, were PCR negative and thus may harbour novel, yet unidentified cadmium resistance determinant(s). Resistance to arsenic or benzalkonium chloride was not encountered among any of the 777 isolates from the bears.

Predictors of L. monocytogenes *colonization*

We employed models to assess predictors for listerial colonization, including the percentage of agriculture and forest in each occurrence distribution; averages for housing density, temperature and precipitation were used as fixed effects, with year as a random effect. Human housing density (houses per km^2) was our measure of urban development and was the only predictor in the best model (Table 2) showing a positive, but not statistically significant relationship with *Listeria* colonization (ß = 0.33, P = 0.15). This model did not perform significantly better than the null model, suggesting that 'year' captures most of the variation and that housing density does not add a significant amount of explanatory power. Occurrence distribution overlap was significantly higher (Wilcoxon χ^2 approximation = 81, df = 1, $P < 0.0001$) between capture events yielding *L. monocytogenes* than between those that were negative.

Discussion

Our results indicate that *L. monocytogenes* could be frequently isolated from American black bears in the southeastern United States. We frequently obtained isolates of different serotype-associated multiplex PCR profiles as well as *L. monocytogenes* together with other *Listeria* spp. from the same samples, indicating cohabitation of different *Listeria* strains and species in the same animal. Analysis of samples from three different states over 4 years revealed that each year nearly half of all black bear capture events yielded *L. monocytogenes*. This broad temporal and geographic scope suggests that *L. monocytogenes* was consistently maintained in the black bear population in the study area. Interestingly, occurrence distribution overlap was found to be significantly higher between captures yielding *L. monocytogenes* than between those that were negative. This suggests potential transfer of *L. monocytogenes* among individuals who may be more likely to interact, e.g., through shared habitat, behavioural practices or social structure. In an earlier study of Japanese monkeys, potential spread of *Listeria* spp. among the members of the same troop was also hypothesized (Yoshida *et al.*, 2000).

Table 1. *Listeria* recovery from American black bears that were *Listeria* positive at multiple sampling times.

Bear ID	Time between recaptures (Months)[a]	Initial capture[b]	Subsequent captures(s)[b]
N024	9	LM 1/2a	2nd capture: *Listeria* spp.
N052	6	LM 1/2a and LM 1/2a-1/2b	2nd capture: Negative
N059	6	LM 1/2c and *Listeria* spp.	2nd capture: LM 1/2a and LM IVb
N057	19	LM IVb	2nd capture: Negative 3rd capture: LM 1/2a and LM IVb 4th capture: LM 1/2a 5th capture: Negative
N022	10	Negative	2nd capture: Negative
N047	8	Negative	2nd capture: LM 1/2a
N040	8	LM IVb-v1	2nd capture: LM 1/2a, LM IVb and LM 1/2a-1/2b
N043	21	LM 1/2c and LM IVb-v1	2nd capture: LM 1/2a3rd capture: Negative
N048	9	Negative	2nd capture: Negative
N006	12	LM IVb and LM 1/2a	2nd capture: Negative3rd capture: Negative
N046	9	LM 1/2c	2nd capture: Negative
N063	2	LM 1/2a	2nd capture: Negative
N015	13	Negative	2nd capture: LM 1/2a
N016	14	*Listeria* spp.	2nd capture: LM 1/2a
N014	15	Negative	2nd capture: LM 1/2a
N060	11	LM IVb-v1 and *Listeria* spp. and	2nd capture: Negative
N095	7	LM 1/2a, LM IVb-v1 and *Listeria* spp.	2nd capture: LM 1/2a-1/2b
N056	19	*Listeria* spp.	2nd capture: LM 1/2a
N075	11	Negative	2nd capture: Negative
N079	11	LM 1/2a, LM Lineage III and *Listeria* spp.	2nd capture: LM 1/2a
N061	17	LM 1/2a, LM IVb and *Listeria* spp.	2nd capture: LM IVb 3rd capture: LM 1/2a, LM IVb-v1 and *Listeria* spp.
N092	10	Negative	2nd capture: LM IVb-v13rd capture: *Listeria* spp.
N108	2	LM 1/2a and *Listeria* spp.	2nd capture: LM 1/2a and *Listeria* spp.
N051	10	LM IVb and LM IVb-v1	2nd capture: Negative
N121	2	*Listeria* spp.	2nd capture: LM 1/2a and *Listeria* spp.
N109	3	LM 1/2a and *Listeria* spp.	2nd capture: LM 1/2a and LM IVb
N087	13	Negative	2nd capture: LM 1/2a, LM IVb and *Listeria* spp.
N032	21	Negative	2nd capture: Negative
N132	2	*Listeria* spp.	2nd capture: LM IVb and *Listeria* spp.
N138	2	*Listeria* spp.	2nd capture: LM 1/2a and *Listeria* spp.
N151	1	Negative	2nd capture: LM 1/2a
N037	35	*Listeria* spp.	2nd capture: LM 1/2b, LM Lineage III and *Listeria* spp.
BBRC 119	5	LM 1/2a, LM Lineage III	2nd capture: LM 1/2a, LM IVb
BBRC 120	5	Negative	2nd capture: Negative
BBRC 126	4	Negative	2nd capture: Negative
BBRC 127	4	LM IVb and LM Lineage III	2nd capture: Negative
BBRC 128	4	*Listeria* spp.	2nd capture: Negative
BBRC 129	4	Negative	2nd capture: Negative

[a]In the case of animals captured more than twice, time frame spans from first to last capture.
[b]LM, *Listeria monocytogenes; Listeria* spp., *Listeria* species other than LM. Designations 1/2a, IVb, 1/2c, IVb-v1 correspond to multiplex PCR profiles for LM, as in legend to Fig. 5.

One of the unexpected findings from our work was the significantly higher prevalence of *L. monocytogenes* than other *Listeria* spp. The majority (83%) of the *Listeria*-positive capture events yielded *L. monocytogenes*, either alone or together with other *Listeria* spp., and bears found to be positive exclusively for *Listeria* spp. other than *L. monocytogenes* were uncommonly encountered. This apparent bias for *L. monocytogenes* over other *Listeria* spp. was detected in all sample types, but was especially noticeable for nasal swab samples.

A predilection for *L. monocytogenes* in comparison with other *Listeria* spp. has rarely been found in other wildlife studies, where in fact the opposite was frequently observed, with *L. monocytogenes* tending to be noticeably less common than other *Listeria* spp. (Innoue *et al*., 1992; Quessy and Messier, 1992; Yoshida *et al*., 2000; Hellström *et al*., 2008; Wang *et al*., 2017; Cao

Table 2. Coefficients and associated standard error, critical value and p-value from the top model based on AICc from a study of *L. monocytogenes* prevalence in wild American black bears from NC, GA and VA from 2014 to 2017. We fit a generalized linear mixed model to predict the *L. monocytogenes* colonization status of each bear using the average housing density per km^2 within each occurrence distribution with year as a random effect.

Predictor	β	SE	*Z*	*P*-value
(Intercept)	−0.1	0.38	−0.25	0.80
Housing density	0.33	0.23	1.43	0.15

et al., 2019). Only one study of rectal swabs from red fox, beech marten and raccoons in Poland found that, even though overall *Listeria* prevalence was low (approx. 7.4%), most (64.5%) of the *Listeria*-positive samples yielded *L. monocytogenes* (Nowakiewicz *et al*., 2016). Even similar prevalence levels between *L. monocytogenes* and other *Listeria* spp. are uncommonly reported in wildlife surveys. Fenlon (1985) noted *L. monocytogenes*, *L. innocua* and *L. seeligeri* in 8.4, 4.5 and 3.4%, respectively, of faecal samples from seagulls, primarily from sewage disposal sites, with thus similar prevalence between *L. monocytogenes* (8.4%) and other *Listeria* spp. (7.9%). A survey of urban rooks (*Corvus frugilegus*) in France detected *L. monocytogenes*, *L. innocua* and *L. seeligeri* in 33%, 24% and 8%, respectively, of the samples (33% *L. monocytogenes* vs. 32% other *Listeria* spp.) (Bouttefroy *et al*., 1997). Interestingly, *L. seeligeri* was not encountered among our bear isolates. Similarly, *L. ivanovii* accounted for many of the *Listeria* isolates from rodent surveys in China (Wang *et al*., 2017; Cao *et al*., 2019), but was not encountered in our study.

The eco-physiological determinants underlying the apparent association preference of black bears for *L. monocytogenes* remain to be elucidated. Surveys of avian *Listeria* carriage suggested higher prevalence in urban populations, with *L. monocytogenes* accounting for approximately 36–50% of the *Listeria*-positive samples from urban birds (Fenlon, 1985; Bouttefroy *et al*., 1997; Hellström *et al*., 2008), while association with rural bird nesting areas was also noted (Quessy and Messier, 1992). In this study, we sampled both suburban and wild black bears and observed significantly higher *L. monocytogenes* recovery at all locations across the entire study period (Figs 2 and 3). As mentioned above, *L. seeligeri* was not encountered, even though it was overall the predominant species from both urban and relatively undisturbed natural environments in other studies (Sauders *et al*., 2012; Linke *et al*., 2014). It is possible that the foraging behaviour and preferences of black bears preferentially expose them to materials that may serve as reservoirs for *L. monocytogenes* in the natural environment. It is also conceivable that the nasal and intestinal microbiome of the bears may be selecting for the pathogenic species *L. monocytogenes*, despite the more diverse *Listeria* population to which they are exposed.

The prevalence of *L. monocytogenes* in our study (36%) was noticeably higher than in most other wildlife surveys, where prevalence ranged from 0.3% to 18% (Hayashidani *et al*., 2002; Lyautey *et al*., 2007; Wacheck *et al*., 2010; Nowakiewicz *et al*., 2016; Weindl *et al*., 2016; Wang *et al*., 2017; Cao *et al*., 2019;). Only two wildlife studies, involving urban birds in Finland and France, respectively, yielded prevalence levels of 33–36% (Bouttefroy *et al*., 1997; Hellstrom *et al*., 2008), similar to what we found in the bears. The differences in prevalence between our study and others may reflect different animal species, sample types (e.g., faeces, rectal or nasal swabs), geographical regions and methodologies. Nonetheless, the collective findings support the speculation, first articulated in 1975, that *L. monocytogenes* may have a cyclic existence between the gut of animals and the natural environment (Weis and Seeliger, 1975).

Employment of both primary and secondary enrichments, multiplex PCR profiling of multiple colonies per positive sample and analysis of multiple sample types from the same animal were important in capturing the diversity of the *Listeria* population associated with the black bears. Even though *Listeria* recovery was significantly higher from primary than from secondary enrichments as also reported by others (Weindl *et al*., 2016), including both enrichments may be useful in capturing diversity. Furthermore, and in agreement with Weindl *et al.* (2016), examination of different sample types from the same animal proved important in assessments of species (*L. monocytogenes* vs. other *Listeria* spp.) and serotype heterogeneity within the *Listeria* population from the animals.

The preferential recovery of *L. monocytogenes* from nasal swabs, in comparison with other *Listeria* spp., was of special interest. It was also noteworthy that even though the overall prevalence of *L. monocytogenes* was lower in nasal swabs than in faeces or rectal swabs, multiplex PCR profile diversity was higher, with all multiplex PCR profiles encountered among nasal swab-derived isolates (Fig. 9). Whether nasal passage colonization is mediated by specific genetic adaptations of *L. monocytogenes* or reflects black bear foraging behaviour that expose nasal passages to materials especially likely to be contaminated by a variety of strains, is worthy of further investigation.

The two serotypes of *L. monocytogenes* dominant in our study (1/2a and 4b) are among the three (1/2a, 1/2b, and 4b) most commonly implicated in human listeriosis (Swaminathan and Gerner-Smidt, 2007). Serotypes 1/2a and 4b were also the leading serotypes in other wildlife studies, including surveys of red deer, wild boar and wild birds (Yoshida *et al*., 2000; Hellström *et al*., 2008; Weindl *et al*., 2016). Serotype 1/2b was only encountered in 2% of *L. monocytogenes* isolates in our study and was also infrequently (5%) recovered by Weindl *et al.* (2016). Lineage III isolates were also uncommon in our study even though they were reported to show proclivity for non-human animals (Jeffers *et al*., 2001; Liu *et al*., 2006). It was noteworthy that the distribution of serotype 1/2a and 4b isolates in the black bears remained relatively constant over the 4 years of the study. The high representation of serotypes 1/2a and 4b

in this and other wildlife hosts is intriguing as it may reflect yet unidentified reservoirs for these serotypes in the natural environment. This high prevalence of serotypes 1/2a and 4b among the bear isolates also has potential public health implications which need to be further assessed, e.g., via high-resolution genotyping and virulence assessments.

Serotype-related multiplex PCR profiling of the isolates yielded two surprising findings. First, serotype 4b strains with the variant multiplex profile IVb-v1 (Leclercq *et al*., 2011) were repeatedly encountered in our study and accounted for almost half of the serotype 4b isolates. They were recovered from both NC and GA, demonstrating a wide geographic distribution. The IVb-v1 profile has been detected in several genetically unrelated, emerging clones of serotype 4b, including those implicated in recent outbreaks (Moura *et al*., 2016; Lee *et al*., 2018). High-resolution genotyping is needed to determine the genetic relatedness of the bear-derived IVb-v1 strains to those implicated in human listeriosis. The second unexpected finding was the discovery of isolates with a novel multiplex PCR profile that included amplicons typical for both serotypes 1/2a and 1/2b. These isolates were from animals captured at diverse times and locations in NC, and to our knowledge, this represents the first reported encounter of this unique PCR profile.

Resistance to the heavy metals cadmium and arsenic is one of the longest-known environmental adaptations of *L. monocytogenes* (Buchanan *et al*., 1991; Lebrun *et al*., 1992; McLauchlin *et al*., 1997), and prevalence of cadmium resistance ranged from 50% to 66% among isolates from food processing facilities (Mullapudi *et al*., 2008; Ratani *et al*., 2012; Ferreira *et al*., 2014; Xu *et al*., 2014). The determinants that confer cadmium and arsenic resistance in *L. monocytogenes* are usually harboured on mobile genetic elements such as plasmids, genomic islands or transposons (Lebrun et al., 1994a,b; Kuenne *et al*., 2010; Dutta *et al*., 2017; Parsons *et al*., 2017). Interestingly, none of the 537 *L. monocytogenes* isolates from the black bears were resistant to arsenic and only three (0.56%) were resistant to cadmium. These low levels of prevalence of resistance may reflect niche-specific factors that deter acquisition or stable maintenance of mobile genetic elements associated with heavy metal resistance. Cadmium resistance was, to our knowledge, examined in only one other study of *Listeria* from wildlife, where 28.6% of *L. monocytogenes* and 7.7% of other *Listeria* spp. from urban rooks were found to be resistant to cadmium (Bouttefroy *et al*., 1997). Further studies are needed to determine whether the surprisingly low incidence of resistance in our study is a niche-specific trait in American black bears.

Our results suggest the potential for black bears to serve as a natural vehicle and perhaps a reservoir for *L. monocytogenes* and other *Listeria* spp. Whether the source for the colonization of the black bears originated from the environment (e.g. soil, water or vegetation) or from anthropogenic sources (e.g. bears scavenging around human habitations or agricultural areas) warrants further investigation. Our microbiological results and the occurrence distributions for the black bears which largely overlapped with humans (and accompanying companion animals) in an urban/suburban community suggest that *L. monocytogenes* may represent an attractive model for the investigation of both anthroponotic and zoonotic transmission of pathogens. Our results also provide novel insights into the complexity of the natural ecology of *L. monocytogenes,* suggesting a potential role for black bears in the transmission, dissemination and adaptations of this pathogen. Given the increasing human–animal interactions due to urban expansion and the implications for both humans and other animal species to become sick from exposure to *L. monocytogenes*, further studies of this nature are of clear significance for human and veterinary health.

Experimental procedures

Project locations and study areas

Black bear populations were investigated in three states, (NC, GA and VA) in the southeastern United States from 2014 to 2017. As indicated above, the animals were live-captured for the purpose of several separate studies focusing on the mobility and demographic patterns of black bear populations in this region. None of the animals were trapped or handled specifically in order to collect samples for isolation of *L. monocytogenes* or other *Listeria* spp.

The majority of the samples were obtained through the study in NC, which focused on populations within or adjacent to the city of Asheville, located in Buncombe County in the southern Appalachian mountain range of western NC (Fig. 1). Asheville encompasses 117 km^2 with a residential population of approximately 92 000 people and is characterized by heterogeneous topography, temperate climate with high annual precipitation (36.95″ rainfall, 13″ snowfall avg.) and mixed deciduous hardwood and pine-hardwood forests (Mitchell *et al*., 2002; Kirk *et al*., 2012).

The study in GA was conducted within Houston and Twiggs Counties in central GA along the Ocmulgee River drainage (Hooker *et al*., 2015). This area is located at the boundary of the Piedmont and Coastal Plain regions and is characterized by hardwood forest that dominates the bottomland region and mixed pine-hardwood forest dominating the uplands. Land west of the study area is dominated by human development including Macon (pop. 91 234), Warner Robins (pop. 72 531)

and Bonaire (pop. 13 999; USCB, 2016), whereas land south and east is primarily agricultural. Common crops, several of which are used by bears, are cotton, corn, peaches, peanuts and pecans.

The VA study included 15 animals that were kept in temporary captivity (6–8 months) at VT-BBRC in Blacksburg, VA (Mesa-Cruz, 2018) and then released into the wild. These black bears were captured from different counties in the Appalachian Mountains of western VA by the VA Department of Game and Inland Fisheries (VDGIF).

Sample collection

All animal capture and handling protocols were approved by the Institutional Animal Care and Use Committee at North Carolina State University (14-019-O), the University of Georgia (A2011 10-004-A1) and Virginia Tech (12-112 and 15-162). Bears were live-captured from April 2014 to August 2017 in NC and June to July 2014 in GA. In VA, the bears were sampled from October 2014 to March 2015 and from October 2015 to February 2016. Live-captured bears were chemically immobilized with a combination of 5 ml of tiletamine-zolazepam (100 mg ml^{-1}) with 4.0 ml of ketamine hydrochloride and 1.0 ml of xylazine hydrochloride. Bears captured in Georgia were immobilized with Telazol® (equal parts tiletamine and zolazepam; Fort Dodge Animal Health, Fort Dodge, IA, USA) or large animal xylazine (100 mg ml^{-1}) combined with Telazol® delivered intramuscular (IM) via blowdart. Each bear in the NC and GA study was given uniquely numbered ear tags, matching lip tattoo, passive integrated transponder (PIT) tags and GPS radio collars (Vectronic, Berlin, Germany or Lotek Wireless Inc., Newmarket, ON, Canada) to track movement. Weight, sex, reproductive status and body measurements were recorded for each bear. Temperature, pulse and blood oxygen levels were monitored throughout immobilizations to ensure the health/stability of the bears. Anaesthesia was reversed using a reversal agent such as yohimbine hydrochloride (0.15–0.3 mg kg^{-1}, I.M. or I.V.). Anaesthesia of bears immobilized with Telazol in GA was reversed approximately 45 min after initial immobilization using latipamazole hydrochloride (5 mg ml^{-1}; Antisedan®, Orion Pharma, Orion Corporation, Espoo, Finland) and diazepam (5 mg ml^{-1}) delivered IM via hand injection.

When available, fresh faecal samples were collected in Ziploc® bags and shipped overnight on ice to North Carolina State University (NCSU). Rectal and nasal swabs were collected, while the animals were anesthetized, using sterile techniques with pre-sterilized swabs inside collection tubes (Becton, Dickinson, and Company) and similarly shipped to NCSU.

A small number ($n = 14$) of bears killed by vehicular collisions in Buncombe County (NC) were also sampled. GI tract samples from these bears were kept frozen at −20°C and shipped on ice in batches to the NCSU laboratory.

Microbiological sample processing and enrichment

Samples were enriched for *Listeria* using the ISO Method as described (Azizoglu *et al*., 2014) with minor alterations. Specifically, faecal samples were diluted 1:10 (1.25 g 11.25 ml^{-1}) in Half Fraser Broth supplemented with Half Fraser Selective Supplement (Oxoid, Hampshire, UK) and incubated at 30°C for 24-48 h for primary enrichment. Swab samples were similarly enriched in 11.25 ml of the primary enrichment broth. The primary enrichment (0.1 ml) was then transferred into 10 ml of Full Fraser Broth supplemented with Fraser selective supplement (Oxoid) and incubated at 37°C for 48 h for secondary enrichment. Putative positive primary or secondary enrichments, evidenced by colour change in the medium, were streaked (20 μl) on MOX and incubated at 37°C for 48 h. Up to six putative *Listeria* colonies from the MOX plates were streaked on tryptic soy agar (TSA) with 5% sheep blood (Remel, San Diego, CA, USA) and incubated at 37°C for 36-48 h to purify and assess haemolytic activity. Pure cultures were preserved at −80°C in brain heart infusion (BHI) with 20% glycerol (Fisher Scientific, Fairlawn, NJ, USA). Cultures were routinely grown on Tryptic Soy Broth (Becton, Dickinson and Company) supplemented with 0.7% yeast extract (Fisher Scientific) and 1.2% agarose (Becton, Dickinson and Company; TSAYE).

Determination of serotype designations and susceptibility to heavy metals and benzalkonium chloride

Listeria spp. verification and serotype designations of 1/2a (or 3a), 1/2b (or 3b), 1/2c (or 3c) and 4b (or 4d, 4e) were determined via multiplex PCR (Doumith *et al*., 2004), as was the serotype 4b variant profile, IVb-v1 (Huang *et al*., 2011; Leclercq *et al*., 2011; Lee et al., 2012a,b). Haemolytic isolates yielding only the *prs* PCR product were further tested with PCR using *L. monocytogenes*-specific *hly* primers for Listeriolysin O, to determine whether they were *L. monocytogenes* lineage III isolates serotype 4a or 4c or *L. seeligeri* (Eifert *et al*., 2005). Susceptibility to cadmium and arsenic was determined on Iso-Sensitest™ Agar (Oxoid) with 35 and 70 μg ml^{-1} cadmium chloride or 500 μg ml^{-1} sodium arsenite, respectively, while BC susceptibility was determined on TSAYE with 10 μg ml^{-1} BC; positive and negative control strains were used for each agent in each test for quality assurance, as described (Lee *et al*.,

2013). Isolates exhibiting resistance to cadmium were tested by PCR for known resistance determinants using previously described primers (Lee *et al*., 2013; Parsons *et al*., 2017).

Assessing predictors of Listeria *colonization*

Black bear capture and trap locations in and around Asheville, NC, are shown in Fig. 1. We analysed movement data from GPS-collared bears in this area, as it accounted for the preponderance of the samples, and we lacked movement data for the GA and VA collection sites. We restricted movement data to within 2 weeks after the sample collection date and used the ctmm package (Calabrese *et al*., 2016; Fleming and Calabrese, 2016) in R (v. 3.3.1) (R Foundation for Statistical Computing, 2016) to construct 95% occurrence distributions (OD). Thus, we estimated the area that each animal actually used during each 2-week period as opposed to constructing a home range. We used the raster package (Hijmans *et al*., 2016) in R to determine the percentage of agriculture and forest (reclassified from GAP land cover to include all forest types) (U.S. Geological Survey, 2011) in each OD and the average housing density (houses per km^2, SILVIS) (Hammer *et al*., 2004). We obtained daily temperature and precipitation data from Weather Underground using the rwunderground package in R (Shum, 2018). Initial assessments using a chi-squared test for independence in R indicated that sex ($\chi^2 = 437.6$, df = 1, $P < 0.001$) and age ($\chi^2 = 286.2$, df = 1, $P < 0.001$) of each bear were not strong predictors of *L. monocytogenes* colonization and these were omitted from our models.

We ran a generalized linear mixed model to predict the *L. monocytogenes* colonization status of each bear using the percentage of agriculture and forest in each OD, the average housing density, average temperature and average precipitation as fixed effects, with year as a random effect. Correlation between covariates was assessed using a Pearson correlation matrix in R to verify that all covariates were correlated < 50% and to also verify that where > 50% those predictors did not appear in the same model. We ran a suite of seven models and chose the best model for the data based on Akaike's information criterion (AIC) (Burnham *et al*., 2002). All continuous predictors were centred and scaled prior to running the model.

Because contact with other *L. monocytogenes*-positive bears could lead to pathogen spread, either through direct contact or via the faecal contamination of shared spaces, we assessed whether *L. monocytogenes*-colonized black bears shared space with other non-colonized black bears. We used the adehabitatHR package in R (Calenge, 2006) to compute indices of home range overlap from locations as described in Fieberg and Kochanny (2005), specifically estimating the proportion of the home range of one animal covered by the home range of another, separately for each year. We separated the black bears into those that yielded at least one *L. monocytogenes*-positive sample and those from which all tested samples were negative for *L. monocytogenes*, then determined whether the average home range overlap between the groups was significantly different using a Wilcoxon rank-sum test in R.

Statistical analysis of black bear sampling data

Differences in recovery based on sample type were assessed using a Wilcoxon rank-sum test in R. Similarity in *Listeria* community composition (*L. monocytogenes*, other *Listeria* spp. and serotype) was assessed via a Jaccard similarity index where 1 is no similarity and 0 is identical, using the vegan (Oksanen *et al*., 2017) package in R. Tests for overall positive or negative status for sample type or enrichment type were performed using a logistic regression with bear ID as a random effect and sample type or enrichment as fixed effect ($\alpha = 0.05$). Differences in yearly recovery were assessed using a chi-squared test. Differences in recovery of *L. monocytogenes* vs. other *Listeria* spp. were assessed using a Pearson's chi-squared test in SPSS (IBM, Armonk NY, USA). Differences in mean yearly prevalence of *L. monocytogenes* vs. other *Listeria* spp. were assessed using an independent samples t test in SPSS (IBM).

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Conflict of interest

None declared.

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