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Analysis of bacterial communities associated with Mountain Chickadees (*Poecile gambeli*) across urban and rural habitats

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Abstract: Host-associated microbial communities play important roles in wildlife health, but these dynamics can be influenced by environmental factors. Urbanization has numerous effects on wildlife; however, the degree to which wildlife-associated bacterial communities and potential bacterial pathogens vary across urban–rural/native habitat gradients remains largely unknown. We used *16S rRNA* gene amplicon sequencing to examine bacterial communities found on Mountain Chickadee (*Poecile gambeli*) feathers and nests in urban and rural habitats. The feathers and nests in urban and rural sites had similar abundances of major bacterial phyla and dominant genera with pathogenic members. However, richness of bacterial communities and potential pathogens on birds were higher in urban habitats, and potential pathogens accounted for some of the differences in bacterial occurrence between urban and rural environments. We predicted habitat using potential pathogen occurrence with a 90% success rate for feather bacteria, and a 72.2% success rate for nest bacteria, suggesting an influence of urban environments on the presence of potential pathogens. We additionally observed similarities in bacterial communities between nests and their occupants, suggesting bacterial transmission between them. These findings improve our understanding of the bacterial communities associated with urban wildlife and suggest that urbanization impacts the composition of wildlife-associated bacterial communities.

Key words: urbanization, Mountain Chickadee, avian pathogens, microbial communities, avian microbiome.

Résumé : Les communautés microbiennes associées à l'hôte jouent un rôle important dans la santé de la faune sauvage, mais cette dynamique peut être influencée par des facteurs environnementaux. L'urbanisation a de nombreuses conséquences sur la faune sauvage ; toutefois, le degré de variation des communautés bactériennes et des agents pathogènes bactériens potentiels associés à la faune sauvage à travers des gradients d'habitats urbains et ruraux/naturels reste largement inconnu. Les auteurs ont utilisé le séquençage de l'amplicon du gène de l'*ARNr 16S* pour examiner les communautés bactériennes trouvées sur les plumes et dans les nids des mésanges de Gambel (*Poecile gambeli*) dans des habitats urbains et ruraux. Les plumes et les nids des sites urbains et ruraux présentaient des abondances similaires de phylums bactériens majeurs et de genres dominants ayant des membres pathogènes. Cependant, la richesse des communautés bactériennes et des agents pathogènes potentiels sur les oiseaux était plus élevée dans les habitats urbains, et les agents pathogènes potentiels expliquaient certaines des différences de présence bactérienne entre les environnements urbains et ruraux. Ils ont prédit l'habitat en utilisant la présence d'agents pathogènes potentiels avec un taux de réussite de 90 % pour les bactéries des plumes et de 72,2 % pour les bactéries des nids, ce qui suggère une influence des environnements urbains sur la présence d'agents pathogènes potentiels. Ils ont en outre observé des similitudes dans les communautés bactériennes entre les nids et leurs occupants, ce qui suggère une transmission bactérienne entre eux. Ces résultats améliorent notre compréhension des communautés bactériennes associées à la faune urbaine et suggèrent que l'urbanisation peut avoir un impact sur la composition des communautés bactériennes associées à la faune. [Traduit par la Rédaction]

Mots-clés : urbanisation, mésange de Gambel, agents pathogènes aviaires, communautés microbiennes, microbiome aviaire.

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Introduction

Over 50 000 species assessed by the International Union for Conservation of Nature (IUCN) are threatened by activities associated with urbanization. These activities include residential and commercial development (McKinney 2002), and the construction and use of transportation and service corridors (Coffin 2007). Additional threats result from natural system modifications (Coyner et al. 2002; Coffin 2007; Grimm et al. 2008; Wilson and Chakraborty 2013), pollution (McKinney 2002; Longcore and Rich 2004; Gaston et al. 2013; Wilson and Chakraborty 2013; Luther et al. 2016), climate change (McDonald et al. 2018), severe weather (Paul et al. 2018; Zhang et al. 2018), and human intrusions on wildlife (Hadidian 2015; Larson et al. 2016; Liu et al. 2016). Remnant habitat in urban areas is fragmented (Liu et al. 2016; McDonald et al. 2018), especially near the urban core, as land is replaced with artificial surfaces and structures, managed vegetation, and bare ground (McKinney 2002). These factors pose a global threat to biodiversity, with decreases in species richness of over 50% for many plants and animals in urban cores (McKinney 2002).

Because of its effects on ecosystems, urbanization alters the behavior of infectious diseases and may encourage them to spread in local wildlife and human populations (Bradley and Altizer 2007). Urban species loss has been linked to the increased prevalence of malaria, schistosomiasis, Lyme disease, hantavirus pulmonary syndrome, and West Nile virus (Pongsiri et al. 2009). Densification of pathogens, vectors, and hosts in urban environments can result in increased disease transmission due to the concentration of infected individuals around food sources, such as garbage piles and bird feeders (Bradley and Altizer 2007). Arthropod vectors carrying diseases such as Lyme disease may propagate in cities, because reduced seasonality resulting from the heat-island effects may increase their success during mild winters (Lindgren and Gustafson 2001). The presence of pollutants and stressors in urban environments can negatively impact the immunity of wildlife, leading to increased susceptibility to disease (Bradley and Altizer 2007). By establishing human–wildlife interfaces, urban environments can promote the transmission of these wildlife diseases to local humans populations (Daszak 2000; Hassell et al. 2017). These zoonotic infections are often severe (Akritidis 2011; Christou 2011; Pappas 2011), resulting in epidemics (Breithaupt 2003). For example, all of the major influenza epidemics in the past century (Alexander and Brown 2000; Smith et al. 2009) and the COVID-19 pandemic (Zhou et al. 2020) originated in wildlife.

Owing to the widespread presence of birds in cities, combined with their potential to disperse or amplify diseases, understanding how avian disease dynamics change in urban areas has been the focus on many recent studies. Both Dhondt et al. (2007) and Adelman et al. (2015) found that bird feeders may facilitate the transmission of *Mycoplasma gallisepticum*, the causative agent of avian mycoplasmal conjunctivitis. Bird feeders may also lead to increased cloacal infections and fungal skin disease in birds (Wilcoxen et al. 2015), although disease transmission at these sites appear to vary by pathogen type (Vana et al. 2018). Stagnant waters and nutrient-rich runoff in cities can promote mosquito abundance, leading to the proliferation of West Nile virus in birds and humans, as has been observed in both Chicago (Hamer et al. 2012) and Atlanta (Bradley et al. 2008). Additionally, pathogenic *Yersinia* spp. were found to be more prevalent in house sparrows (*Passer domesticus*) in urban environments than rural environments in Flanders, Belgium (Rouffaer et al. 2017).

Advances in DNA sequencing and nucleic acid detection have advanced studies on microbial communities, pathogen detection, and pathogen quantification. In the context of avian species, published reports include those examining feather-degrading bacteria (Lee et al. 2015; Fellahi et al. 2016) and intestinal microbiota (Fuirst et al. 2018; Teyssier et al. 2018), with few studies on the microbiomes of bird feathers and nests (van Veelen et al. 2017; Fuirst et al. 2018; Musitelli et al. 2018; Javůrková et al. 2019). Studies on bacterial communities in cities have found differences in the community composition of soil bacteria across urban sites (Reese et al. 2016), and *16S rRNA* gene sequencing has been used (Razzauti et al. 2015) to identify potentially zoonotic bacteria in wildlife. Although these studies have reported links between specific diseases and urbanization, to our knowledge, no studies have examined the links between urbanization and bacterial pathogens found on rural and urban birds or their nests.

In this study, we applied high-throughput *16S rRNA* gene sequencing of DNA extracted from Mountain Chickadee (*Poecile gambeli*) tail feathers and nests to examine the microbial community composition and the presence of detectable pathogenic bacteria living on and near these birds across rural and urban habitats in the south-central interior of British Columbia, Canada. This region is currently experiencing increased population growth and expansion of urban and suburban environments (Environmental Reporting BC 2018). Some avian species, such as Mountain Chickadees, readily nest and breed in rural, urban, and suburban habitats and do not experience detrimental effects on their breeding success when nesting in urban environments (Marini et al. 2017*a*, 2017*b*), thereby providing a model with which to examine the impact of urbanization on avian microbiomes. Mountain Chickadees often nest in tree cavities created by other species, but readily use artificial nest boxes in both natural forests and urban environments (Marini et al. 2017*a*, 2017*b*). Outside of the breeding season, urban chickadees regularly visit bird feeders, which may act as vectors for microbial transfer. During the breeding season however, feeder visits are

uncommon, but microbial transfer may occur between these birds and their local environment during foraging activities and nest building. Because the physical habitat (e.g., buildings, roads, artificial lawns, ornamental non-native plants) differs drastically between rural and urban habitats, we predict that bacterial community and pathogen composition will differ between Chickadees living in rural and urban environments.

Materials and methods

Ethics statement

This research was carried out under Thompson Rivers University Animal Care and Use Protocol No. 100846, University of Northern British Columbia Animal Care and Use Protocol No. 2014-06, and under Canadian Wildlife Service permit No. 22806.

Mountain Chickadee nest and feather sampling

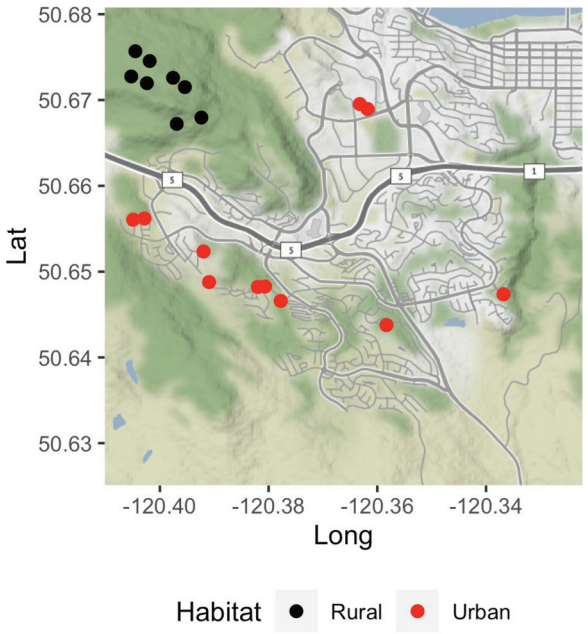
We collected feather samples and nest swabs during the 2018 Chickadee breeding season (May to August) in urban and rural habitats in Kamloops, British Columbia, Canada (50°40.23′N, 120°23.86′W). We monitored urban nest boxes in Kamloops neighbourhoods and rural nest boxes in Kenna Cartwright Nature Park (50°40.232′N, 120°23.855′W) for signs of Chickadee nesting. Kenna Cartwright Nature Park is a protected area encompassing 800 ha, composed of Douglas fir (*Pseudotsuga menziesii*) and ponderosa pine (*Pinus ponderosa*) forests, mixed with shrub–steppe grassland dominated by big sagebrush (*Artemisia tridentata*) and common rabbitbrush (*Ericameria nauseosa*). Some walking paths and single-vehicle roads break up the natural setting of the park, while the remaining area is largely natural. All of the nest boxes were elevated 2 m from the ground, with at least 150 m between the boxes. Nest boxes were composed of untreated wood with either a PVC or wood front panel.

During the nesting period, we captured 31 adult Chickadees (often nesting pairs) from 18 nest boxes (Fig. 1): 18 Chickadees were sampled from nests in urban areas, and 13 Chickadees were sampled from rural nests in Kenna Cartwright Nature Park. After donning sterile gloves, we measured the tarsus length, then banded, weighed, and sexed each bird. One tail feather from each adult was collected and placed into a sterile centrifuge tube, frozen immediately on dry-ice, transported to the laboratory, and stored at −80 °C until processing. At the conclusion of the nesting season, we used sterile swabs (100% cotton) to sample the entire top layer of the nest bedding material from each nest box. In total, 10 urban nests and 8 rural nests were swabbed. These swabs were stored and transported under the same conditions as feather samples. We sprayed a 10% (*v*/*v*) bleach solution onto all surfaces in the nest boxes at the end of the breeding season.

DNA extraction, amplification, and sequencing

We extracted total DNA from feathers ($n = 31$) and nest swabs ($n = 18$) using the DNeasy PowerLyzer PowerSoil

Fig. 1. Location of Mountain Chickadee nest boxes in Kamloops, British Columbia, Canada, that were sampled in this study. Rural nest boxes were located in the Kenna Cartwright Nature Park (black) and urban nest boxes were located in Kamloops neighbourhoods (red).



Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions, with modifications to improve DNA extraction efficiency. Specifically, we first cut away half of each swab tip, and cut each feather every centimeter along the rachis, and inserted segments into individual tubes. We added bead and lysis buffers to these tubes, being sure that all of the samples were completely submerged in buffer. We added an additional incubation step at 65 °C for 10 min prior to bead beating to increase bacterial cell lysis. Bead beating was completed at maximum speed on a Vortex Genie 2 with a 24-tube platform adaptor (QIAGEN). The manufacturer's protocol was then followed up through the elution step. PCR of the V4 hypervariable region of the bacterial *16S rRNA* gene was completed on an S1000 thermocycler (BioRad, Hercules, California, USA). We prepared 20 μL reaction mixtures containing 1× Go*Taq* Green Master Mix (Promega, Madison, Wisconsin, USA), 0.5 μmol/L of each forward and reverse primer [341 (forward) 5′-TACGGGAGGCAGCAG-3′ and 806 (reverse) 5′-GGACTACVSGGGTATCTAAT-3′), and 5 μL template DNA. Thermocycling conditions consisted of initial denaturation at 95 °C for 4 min, 35 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 55 °C for 45 s, and 72 °C for 2 min, with a final extension at 72 °C for 5 min. Amplicons were cleaned using Agencourt AMPure XP beads (Beckman–Coulter, Brea, Calif.) prior to a second round of PCR with adaptor and Ion Xpress barcoded primers [341 (forward), 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG[barcode]TACGGGAGGCAGCAG-3′; 806

(reverse), 5′-CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGGACTACVSGGGTATCTAAT-3′). Reaction volume, composition, and thermocycling conditions for this second round of PCR were the same as those used for first round PCR, except the annealing temperature was adjusted to 65 °C and the program consisted of 20 cycles. A second clean-up was then completed using Agencourt AMPure XP beads (Beckman–Coulter), followed by gel extraction using a MicroElute® Gel Extraction Kit (Omega Bio-tek, Norcross, Georgia, USA) to remove DNA fragments smaller than 100 bp. We quantified all of the DNA extracts and PCR products using a Quant-iT dsDNA HS Assay Kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) on an ABI QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific). We diluted our cleaned, second-round PCR amplicons to equimolar amounts based on dilution factors obtained through qPCR with an Ion Library Quantitation Kit (ThermoFisher Scientific). We then prepared these pools for sequencing using an Ion 520 and Ion 530 Kit-Chef on an Ion Chef system, and sequenced them on an Ion S5 XL using 400 bp chemistry (ThermoFisher Scientific). Data were processed using AMPtk (Palmer et al. 2018) for quality filtering, OTU clustering at 97% sequence identity, and to assign taxonomies using the Greengenes *16S rRNA* gene database (DeSantis et al. 2006). Of the total 49 samples (31 feathers and 18 nest swabs), 47 samples met the read target number of >10 000 reads, grouping to 3325 OTUs. Sequencing data have been deposited in the European Nucleotide Archive under project PRJEB39008 (http://www.ebi.ac.uk/ena/data/view/PRJEB39008).

Statistical analyses

We used phyloseq v1.30.0 (McMurdie and Holmes 2013) to rarefy *16S rRNA* gene sequence reads to an even depth across samples (based on the lowest sequence read number in the dataset), to obtain an even coverage of 11 500 sequences in each sample for subsequent analyses. Bacterial genera containing species pathogenic to wild birds (Willis and Wilkie 1999; Stephens and Hampson 2001; Hubálek 2004; Kilonzo-Nthenge et al. 2008; Tsiodras et al. 2008; Benskin et al. 2009) and clinical pathogens in people (using genera from the 16Spath 2.0 database; Teng et al. 2014) were subsampled from the community dataset to create a new dataset containing only genera with pathogenic members. We filtered pathogens at the genus level because the V4 region of the *16S rRNA* gene may not be reliable for identifying many pathogenic bacteria at the species level (Chakravorty et al. 2007). Feathers and nest swabs were analyzed as independent datasets. After rarefying our data, subsampling pathogens, and separating feathers and swabs, we were left with four datasets: (1) bacterial communities on feathers; (2) bacterial communities on nest swabs; (3) genera with pathogenic members from feathers; and (4) genera with pathogenic members from nest swabs.

We used phyloseq and vegan v2.5-6 (Oksanen et al. 2019) in RStudio v1.2.5019 (RStudio Team 2015) to estimate richness and Shannon–Wiener alpha diversity of bacterial communities and pathogenic genera from feathers and nest swabs. For beta diversity, we first $\log_{10}(x + 1)$ transformed abundances to reduce skew in the sequence data. To visualize similarity among samples, we generated principal coordinates analysis (PCoA) ordinations based on Bray–Curtis distances. We performed PERMANOVA on these distances with an urban or rural category to test the relationship between urbanization on community dissimilarity (Anderson 2001). A permutation test of multivariate homogeneity of groups dispersions (betadisper) was carried out with 9999 permutations, to ensure variances between samples and group medians did not differ significantly ($p < 0.05$) between rural and urban groups, which is an assumption of PERMANOVA (Anderson 2006). In addition to diversity estimates, we graphically compared community composition between urban and rural birds and nest boxes using phyloseq, Dplyr v0.8.3 (Wickham et al. 2019*b*), and GGplot2 v3.2.1 (Wickham et al. 2019*a*). We merged samples based on urban or rural classification according to mean values, then assessed relative abundance of phylum-level community composition in all samples. We also merged urban and rural samples from our pathogen datasets to compare the abundance of the four most common genera (with >50 sequence reads after merging). We fit general linear models to compare differences in phylum or genera abundance for bacterial communities or pathogenic genera, respectively, between feathers, swabs, and habitat (urban or rural).

Given that the geographic area of nest boxes sampled within urban habitats was greater than that for rural habitats, we used Mantel tests to assess spatial autocorrelation in microbial community composition within each habitat. Mantel tests were completed in vegan using the geographic Euclidean distance between samples and the Bray–Curtis dissimilarity in $\log_{10}(x+1)$ transformed microbial community composition between samples with 999 permutations. Mantel tests were completed on both rural and urban data sets to assess spatial autocorrelation in total bacterial community composition on feathers and total bacterial community composition on nest swabs.

We constructed a series of linear mixed models to examine the factors that predicted bacterial community diversity and abundance on chickadee feathers. In separate models, we used the following metrics as response variables: community richness, pathogen richness, pathogen abundance, community Shannon–Wiener diversity index, and pathogen Shannon–Wiener diversity index. For each response variable, we included fixed effects of habitat (urban or rural), age, sex, and body size (PC1) and a random effect of box ID. We then used a stepwise backward procedure to eliminate nonsignificant ($p < 0.05$) effects and arrive at a final model that best predicted the microbial community metric. For nest swabs, we constructed a series of *t* tests with community richness, pathogen richness, pathogen abundance, community Shannon–Wiener diversity index,

or pathogen Shannon–Wiener diversity index as the response variable, and urban or rural habitat as the independent categorical variable. Next, for each microbial community metric, we examined correlations between nest swabs and bird feathers using a linear mixed model with feather microbial community as the response variable, nest feather community as the fixed effect, and box ID as the random effect. Finally, to determine whether we could predict the habitat the birds occupied (urban vs. rural) based on microbial community composition, we used discriminant functions analysis of all OTUs from genera with pathogenic members.

Detection of potential avian pathogens using community filtering

In an effort to survey total bacterial pathogens in an urban environment, we created a dataset containing only genera with pathogenic members from our original community data. This resulted in a dataset of 206 OTUs belonging to genera including members pathogenic to either humans, birds, or both. We further filtered this data to only include potential avian pathogens, identifying 75 OTUs belonging to *Pseudomonas*, *Escherichia/Shigella*, *Clostridium*, *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Rickettsia*, *Mycobacterium*, *Nocardia*, *Erysipelothrix*, and *Campylobacter*. We did not detect any potential avian pathogensfrom the genera *Borrelia*, *Chlamydia*, *Coxiella*, *Ehrlichia*, *Enterobacter*, *Haemophilus*, *Klebsiella*, *Listeria*, *Mycoplasma*, *Pasteurella*, *Riemerella*, *Salmonella*, *Vibrio*, or *Yersinia*.

Results

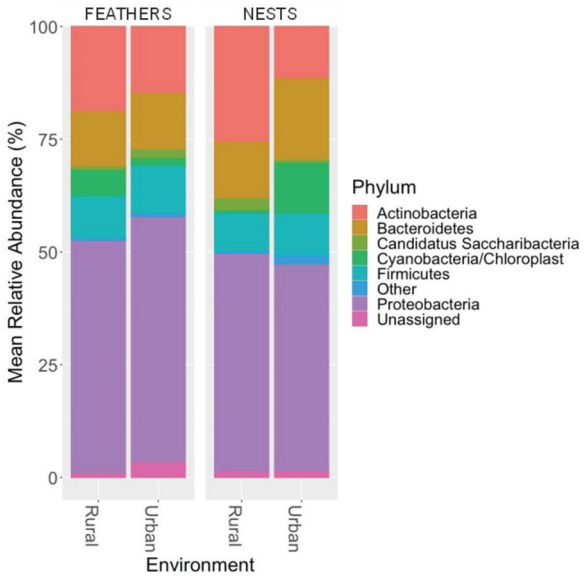
Community composition and the abundance of potential pathogens

Community composition was highly similar at the phylum level for feather and nest bacteria in urban and rural environments (Fig. 2). Proteobacteria was the dominant phylum observed for all categories. Actinobacteria, Bacteroidetes, Cyanobacteria/Chloroplast, and Firmicutes together composed approximately half of each community in different proportions. Candidatus Saccharibacteria, unassigned phyla, and phyla composing less than 1% of each sample (Other), were the least abundant phyla across all feathers, swabs, and environments.

The relative abundance of bacterial phyla found on feathers was not significantly different between urban and rural environments (all $p > 0.05$; Table 1). However, there were more Actinobacteria on rural nests than urban nests ($R^2 = 0.28$, $p = 0.02$). No differences in abundance were observed for all other phyla from nests (all $p > 0.05$). When comparing phylum-level composition between feathers and swabs, there was no difference in relative abundance for any phylum (all $p > 0.05$).

We detected 206 OTUs grouped to genera with pathogenic members. When examining only these genera, we found no differences in abundance between urban and rural environments for both feather and nest bacteria (all $p > 0.05$; Table 1). The five most abundant genera

Fig. 2. Community phylum-level composition for bacteria found on Mountain Chickadee feathers and nesting material in urban and rural areas. OTU abundances were grouped by phylum and samples were merged based on environment sampled. Phyla making up less than 1% of the relative abundance in samples were grouped together as Other.



with pathogenic members were *Clostridium* sensu stricto, *Clostridium* XI, *Enterococcus*, *Escherichia/Shigella*, and the most dominant genus, *Pseudomonas* (Fig. 3). Comparisons between nests and feathers for these genera showed more *Enterococcus* on nests compared to feathers ($R^2 = 0.12$, $p = 0.02$), but no differences for the other genera (Table 1).

Bacterial dissimilarity and modelling

We used PCoA and PERMANOVA analyses to test influences of urban or rural classification on differences in bacterial communities and potential pathogen presence. Bacteria found on feathers and nests appeared to be more similar when found in the same environment (urban or rural; Fig. 4; Table 2). Urban or rural habitat explained some of the differences in bacterial communities on feathers ($R^2 = 0.06$; $p = 0.05$), nests ($R^2 = 0.11$; $p = 0.001$), and potential pathogens from nests ($R^2 = 0.11$; $p = 0.003$). However, this effect was not observed for potential pathogens found on feathers ($R^2 = 0.05$; $p = 0.12$). Permutation tests of multivariate homogeneity of groups dispersions (betadisper) showed the degree of variation did not differ significantly between rural and urban categories for bacterial communities on feathers ($p = 0.15$), bacterial communities on nests ($p = 0.05$), potential pathogens on feathers ($p = 0.39$), or potential pathogens on nests ($p = 0.36$).

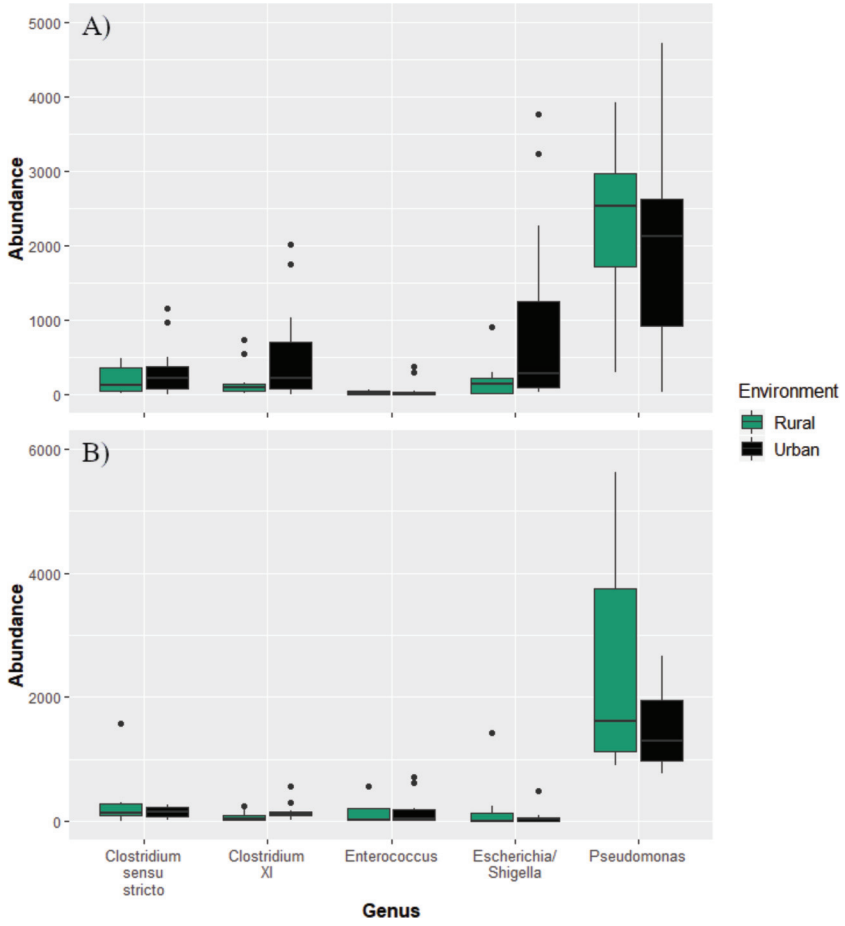
No significant spatial autocorrelation was observed in total bacterial community composition on feathers in rural environments ($r = 0.19$, $p = 0.13$), feathers in urban environments ($r = -0.05$, $p = 0.56$), nest swabs in

Table 1. General linear model comparisons between urban and rural environments, and between feathers and nests, for bacterial communities and genera with pathogenic members from Mountain Chickadees.

		p values		
Scale	Phylum/genus	Feathers (urban vs. rural)	Nests (urban vs. rural)	Feathers vs. swabs
Bacterial communities	Actinobacteria	0.16	**0.02**	0.65
	Bacteroidetes	0.92	0.11	0.11
	Candidatus Saccharibacteria	0.14	0.35	0.93
	Cyanobacteria/Chloroplast	0.34	0.07	0.37
	Firmicutes	0.68	0.81	0.58
	Proteobacteria	0.58	0.76	0.16
Pathogen genus	*Clostridium* sensu stricto	0.78	0.31	0.71
	Clostridium XI	0.62	0.20	0.05
	Enterococcus	0.84	0.86	**0.02**
	Escherichia/Shigella	0.83	0.39	0.06
	Pseudomonas	0.95	0.57	0.82

Note: Values in bold font are statistically significant.

Fig. 3. Abundance of the five most dominant genera containing pathogenic members found on Mountain Chickadee (A) feathers and (B) nests in urban and rural environments.



rural environments (r = 0.21, p = 0.18), or nest swabs in urban environments (r = 0.33, p = 0.09).

When we asked which factors best predicted microbial community composition, Habitat (urban vs. rural) was the only factor that explained community richness (R^2 = 0.34, p = 0.04; Table 3) and pathogen richness (R^2 = 0.43, p = 0.03; Table 3), both of which were positively related to urbanization. None of the factors in our model were associated with variation in pathogen abundance, community Shannon–Wiener diversity index, or pathogen Shannon–Wiener diversity index. For nest swabs, we found no differences between urban and

Fig. 4. Principle coordinates analyses (PCoAs) of Bray–Curtis distances between urban and rural feather bacterial communities, nest bacterial communities, potential pathogens on feathers, and potential pathogens on nests. Path lines on feather PCoAs show samples taken from the same site (birds sampled at the same nest box at any point in the season).

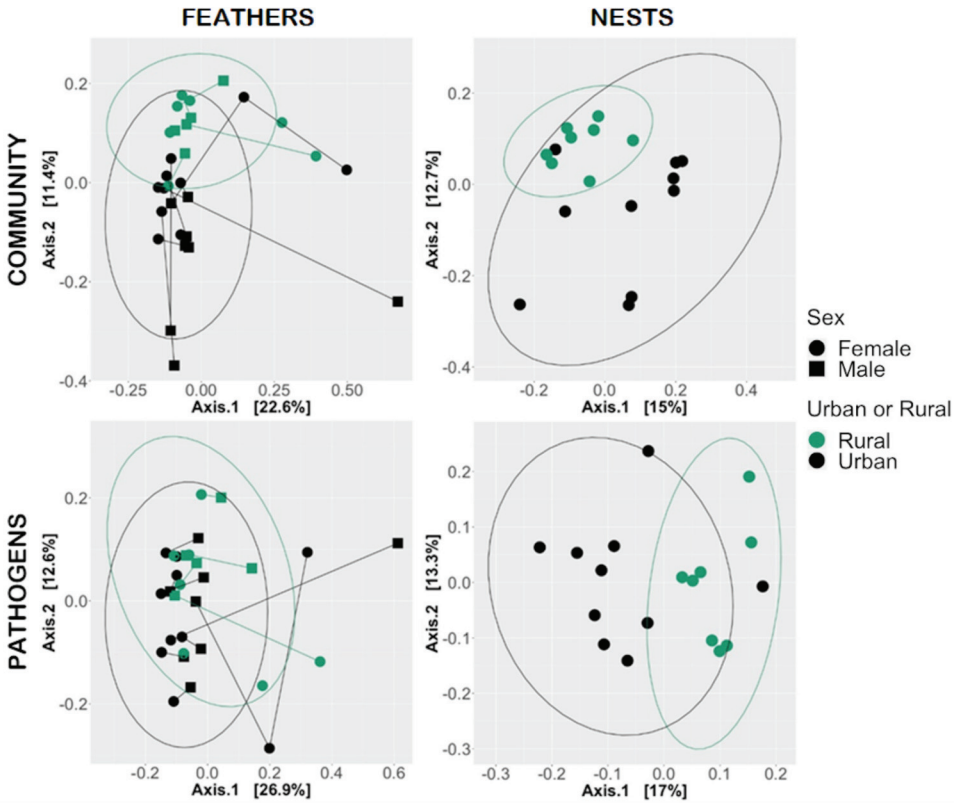


Table 2. PERMANOVA results of Bray–Curtis distances for bacterial communities and genera with pathogenic members from Mountain Chickadee feathers and nests, with urban metrics as included variables.

Urban metric	Community	R^2	p
Urban or rural	Feather community	0.06	**0.05**
	Nest community	0.11	**0.001**
	Feather pathogens	0.05	0.12
	Nest pathogens	0.11	**0.003**

Note: Values in bold font are statistically significant.

rural habitats for any of the microbial community metrics (all $p > 0.14$). When we examined the relationship between microbial communities on nest swabs and feathers, we found no relationships with community richness, pathogen richness, or community Shannon–Wiener diversity index (Table 4); however, we did find positive relationships with pathogen abundance and pathogen Shannon–Wiener diversity index (Table 4).

Discriminant functions analysis

Discriminant functions analysis of bird feathers using 30 OTUs belonging to genera with pathogenic members performed with a 90% success rate; 1 out of 12 rural birds were misclassified as urban, and 2 out of 18 urban birds were misclassified as rural (entropy, $r^2 = 0.44$). For nest swabs, the discriminant functions analysis was less successful, with a 72.2% success rate; 2/8 rural nests were misclassified as urban and 3 out of 10 rural nests were misclassified as urban.

Discussion

Urban landscapes have myriad effects on the wildlife living in or near them, and change ecosystems on a macroscopic scale. Our characterization of the bacterial community composition on Mountain Chickadees and their nests suggests microbiome composition may be related to habitat type across urban and rural gradients. Numerous studies and reviews have highlighted how urbanization can amplify infectious diseases in wildlife (Coyner et al. 2002; Bradley and Altizer 2007; Bradley et al. 2008; Pongsiri et al. 2009; Hamer et al. 2012; Rouffaer et al. 2017; Tian et al. 2018). However, no studies to our knowledge have attempted to examine the differences in detectable pathogens across urban and rural habitats. While recent studies have found changes in intestinal/faecal microbiota across urban environments in birds (Fuirst et al. 2018; Teyssier et al. 2018), none have examined how urbanization is related to the external microbial communities of avian species. Other researchers have further reported on urbanization affecting the

Table 3. Final best fit models from a linear model predicting bacterial diversity metrics based on age of birds, sex, body size, and habitat sampled from (urban or rural).

Habitat metric	Community metric	Final model	DF	*F*	*p*
Urban or rural	Community richness	Habitat	1, 15.91	4.88	**0.04**
	Pathogen richness	Habitat	1, 16.41	5.34	**0.03**
	Pathogen abundance	—	—	—	—
	Community (Shannon–Wiener)	—	—	—	—
	Pathogen (Shannon–Wiener)	—	—	—	—

Note: Values in bold font are statistically significant.

Table 4. Relationships between bacterial community metrics on feathers and swabs.

Metric	DF	F	p
Community richness	1, 16.46	0.47	0.50
Pathogen richness	1, 18.7	0.25	0.62
Pathogen abundance	1, 14.13	7.64	**0.02**
Community (Shannon–Wiener)	1, 18.92	0.71	0.41
Pathogen (Shannon–Wiener)	1, 12.96	7.90	**0.01**

Note: Values in bold font are statistically significant.

assemblage of microbial communities in soils (Reese et al. 2016; Wang et al. 2017), water bodies (Wang et al. 2011; Drury et al. 2013; Newton and McLellan 2015; Hosen et al. 2017), plants (Bartlewicz et al. 2016), and as a result, microbial ecosystem functions (Wang et al. 2011, 2017). Taken together these findings show that urban landscapes not only alter ecosystems on a larger, visible scale, but also influence the characteristics of microbial communities at smaller ecosystem scales.

Community composition of feathers and nests

In this study, we contribute to the growing body of literature characterizing avian microbiomes, and note similarities and differences between species. Bacterial communities on Mountain Chickadee feathers and nests were dominated by the phyla Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. This is similar to the findings of Javůrková et al. (2019) who showed that these 4 phyla were consistently abundant in 7 passerine species from the Czech Republic. These dominant phyla have also been observed in similar proportions on non-passerine birds such as herring gulls (*Larus argentatus*) in the mid-Atlantic coastal US (Fuirst et al. 2018), and woodlarks (*Lullula arborea*) and skylarks (*Alauda arvensis*) in the Netherlands (van Veelen et al. 2017). The consistent occurrence of these dominant phyla across avian species could indicate some conserved elements of avian microbial communities. However, variation in geography and diet of these species likely result in differences between their microbiomes. This might explain why we detected Candidatus Saccharibacteria and Cyanobacteria/Choloroplast as some of our major phyla while other studies did not. Additionally, changes in avian microbiomes due to seasonality may be significant and demands further investigation. For example, house sparrows in the autumn and winter from Belgium have vastly different microbiomes than any of the species discussed, which may result from seasonal differences in community assemblage (Teyssier et al. 2018).

When we compared the bacterial phyla found on feathers and nests, there were no differences in relative abundance between them. Similarly, when comparing the relative abundance of the dominant potential pathogens on nests and chickadees, we found no changes in *Clostridium* sensu stricto or *Pseudomonas*, but did find differences for *Escherichia/Shigella*, *Enterococcus*, and *Clostridium XI*. We also found that the abundance and diversity between feathers and nests for all potential pathogens was related. However, community or potential pathogen richness, and community diversity, was not related between feathers and nests. This shows Mountain Chickadees share much of their bacteria communities with the nests they live in, although some differences do exist. This similarity may result from increased transmission between these elements (Brandl et al. 2014; van Veelen et al. 2017), or simply as a consequence of both bacterial communities existing in the same environment. Other studies have found similar microbial community assemblages between larks and their nests (van Veelen et al. 2017), and between some reed warbler (*Acrocephalus scirpaceus*) nest components (Brandl et al. 2014), which supports this pattern across different avian species. The differences we did observe between feathers and nests may indicate that not all bacteria transmit readily between nests and chickadees, may reflect differences in environmental interactions and time spent in the nest by males and females, or may result from differences between feather and nest environments that shape microbial community composition.

Effects of urbanization on Mountain Chickadees and their nests

Urbanization is a global driver of biodiversity and ecosystem change. Although we are aware of some of these changes in animal and plant populations, few studies have examined whether urbanization is related to microbial community composition. If variation in bacterial community composition occurs across urban landscapes, then birds living in these areas may have different bacterial assemblages and possibly be exposed to different pathogens.

We first assessed whether the microbial communities of Mountain Chickadees and their nests varied between

urban and rural habitats. Using broad scale investigations on phylum composition and community dissimilarity, combined with linear mixed modelling of community diversity and richness, we found that habitat type was related to some aspects of bacterial community composition. We found no differences in the relative abundance of dominant phyla between urban and rural environments, except for increased relative abundance of Actinobacteria in rural nests. This contrasts with the findings of Fuirst et al. (2018), who observed significant differences between phyla and families of intestinal bacteria found in herring gulls (*Larus argentatus*) across urban environments of the mid-Atlantic coastal US. However, differences resulting from geography, ecology of our study species, type of urban environments sampled, and microbiome source (exterior vs. intestinal) may account for these contradictions. Although no effect was found for the relative abundance of dominant phyla, urbanization was related to a slight increase in community richness, but not Shannon–Wiener diversity, for chickadee feather microbiomes. Teyssier et al. (2018) found an opposite effect for gut microbiomes in house sparrows (from Belgium, with urbanization decreasing the richness of these communities. Finally, we found that urbanization explained some (5%–10%) of the differences in bacterial community composition on both chickadee feathers and nests. This influence of urbanization on community dissimilarity was also found for cloacal microbiomes in house sparrows by Teyssier et al. (2018). These results suggest, at least for Mountain Chickadees, that bacteria on feathers and nests of birds in urban and rural habitats are not distinct at the phylum level. However, bacterial species richness on chickadee feathers was slightly higher in urban habitats, and accounts for some of the differences between communities across these gradients at finer taxonomic resolution. These effects may be similar for other species and urban environments, although we were unable to determine whether inconsistencies between our findings and other studies resulted from differences in experimental design and methodology, or from the ecology of the study species.

Next, we compared the relative of abundance of bacterial genera with pathogenic members, which we broadly grouped as potential pathogens, associated with Mountain Chickadees from urban and rural habitats. We carried out comparisons between urbanization on the abundance of dominant potential pathogens, as well as their richness, diversity, and the total abundance of all potential pathogens grouped together. Furthermore, we used these potential pathogens to predict the urban or rural environments from which they originated using discriminant functions analysis. When looking at the five most dominant potential pathogens, we found no differences in their relative abundance between urban and rural environments. Urban or rural habitat also had no effect on the abundance of all potential pathogens grouped together, or the diversity of potential pathogens. However, we did see that urbanization was related to an increased richness of potential pathogens on Chickadees, but not nests. Urbanization also accounted for 11% of the differences between potential pathogens on nests, but not feathers. Finally, our discriminant functions analysis could predict the environment source location of samples (urban or rural) with 90% success using potential pathogens on Chickadees and 72.2% success using potential pathogens on nests. Similar to our findings with bacterial communities, habitat type was not related to the abundance of the most common potential pathogens or the abundance of all potential pathogens grouped together. However, habitat type accounted for some differences in the prevalence of these organisms. Our discriminant functions analysis specifically shows that certain members of these genera may associate with urban landscapes, and our models revealed an increased number of potentially pathogenic species found on Chickadees in urban areas. Whether these bacteria are confirmed pathogens and pose a significant risk to urban Chickadees is beyond the ability of our current analytical approaches. Future studies would benefit from analysis of multiple regions of the *16S rRNA* gene (particularly the V2, V3 and V6 regions; Chakravorty et al. 2007) to enable a more complete view of bacterial pathogens in environmental samples.

Conclusions

Systematic poor land-use planning and overly permissive development regulations have resulted in large-scale urban sprawl without a full understanding of its environmental impacts (Wilson and Chakraborty 2013) and consequences on biodiversity and wildlife (McKinney 2002; Aronson et al. 2014). Here, we report that microbial community composition, including composition of potentially pathogenic bacteria, on Mountain Chickadees nesting in rural and urban areas. We observed differences in microbial community composition related to habitat type and additionally found feather microbial community composition to be related to nest microbial community composition. Shifts in microbiome composition may have important consequences on animal health, and transmission of pathogens through avian vectors is a health concern for both wildlife and human populations. Improved understanding of the impact of urbanization on wildlife associated microbiota will be necessary to inform wildlife management and urban development strategies to address these health concerns.

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