

Faculty of Science

**THE IMPACTS OF URBANIZATION ON THE BACTERIAL COMMUNITIES OF  
MOUNTAIN CHICKADEES (*POECILE GAMBELI*)**

2020 | COLTON ROBERT ALEXANDER STEPHENS

**B.Sc. Honours thesis – Biology**



**THE IMPACTS OF URBANIZATION ON THE BACTERIAL COMMUNITIES OF  
MOUNTAIN CHICKADEES (*POECILE GAMBELI*)**

by

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## **ABSTRACT**

Urbanization is a global process with numerous consequences on wildlife, including the amplification of certain infectious diseases. No studies have yet determined if this pattern exists across all detectable pathogenic bacteria, and few have investigated how bacterial communities change across urban to rural/native habitat gradients. We used whole-community sequencing of partial 16S rRNA gene amplicons to examine relationships between urban and rural bacterial communities found on Mountain Chickadee (*Poecile gambeli*) feathers and nests in Kamloops, British Columbia, Canada. Between urban and rural sites, we observed mostly similar abundances of major bacterial phyla, and dominant genera with pathogenic members, on both bird feathers and their nests. However, urban habitats tended to increase the richness of both bacterial communities and potential pathogens on birds, and accounted for some of the differences in bacterial occurrence between urban and rural environments. Similarities in bacterial communities between nests and their occupants indicated some degree of transmission occurred between them, or that shared environments result in similar community assemblages. We predicted habitat using potential pathogen occurrence with a 90% success rate for feather bacteria, and a 72.2% success rate for nest bacteria, indicating an influence of urban environments on potential pathogen presence. Our findings show that urban environments result in significant differences between urban and rural bacteria associated with Mountain Chickadees, with potential indications towards diverging disease dynamics across urban and rural gradients.

Thesis Supervisor: Associate Professor Matt Reudink (Ph.D.)

## **ACKNOWLEDGEMENTS**

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## TABLE OF CONTENTS

ABSTRACT.....	ii
ACKNOWLEDGEMENTS.....	iii
LIST OF FIGURES.....	v
LIST OF TABLES.....	v
INTRODUCTION.....	1
MATERIALS AND METHODS.....	5
Mountain Chickadee nest and feather sampling.....	5
Estimating urbanization.....	7
DNA extraction, amplification, and sequencing.....	7
Statistical analyses.....	9
Ethics Statement.....	12
RESULTS.....	12
Community composition and abundance of potential pathogens.....	12
Bacteria dissimilarity and modelling.....	16
Discriminant functions analysis.....	19
DISCUSSION.....	20
Community composition of feathers and nests.....	20
Effects of urbanization on mountain chickadees and their nests.....	23
Differences in urban classifications.....	25
Detection of potential avian pathogens using community filtering.....	11
Recommendations for future work.....	26
Opportunities in urban ecology.....	<b>Error! Bookmark not defined.</b>
LITERATURE CITED.....	28
SUPPLEMENTAL DATA.....	35
Urbanization Index.....	35
Project Data and R Code.....	35

## LIST OF FIGURES

- Figure 1.** Overview of nest boxes monitored for use by Mountain Chickadees (*Poecile gambeli*) in Kamloops, British Columbia, Canada. Nest boxes were located in Kenna Cartwright Park (red), in suburban Kamloops neighborhoods (green), or at Thompson Rivers University (blue).. 6
- Figure 2.** Community phylum-level composition for bacteria found on Mountain Chickadee (*Poecile gambeli*) feathers and nesting material in urban and rural areas. OTU abundances were grouped by phylum and samples were merged based on environment sampled from. Phylum that contributed to less than 1% of each sample's abundance were grouped together as Other..... 13
- Figure 3.** Abundance of the five most dominant genera containing pathogenic members found on Mountain Chickadee (*Poecile gambeli*) A) feathers and B) nests in urban and rural environments. .... 15
- Figure 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). .... 17

## LIST OF TABLES

- Table 1.** Student's t-test scores between urban and rural environments, and between feathers and nests, for bacterial communities and genera with pathogenic members from Mountain Chickadee (*Poecile gambeli*). .... 14
- Table 2.** PERMANOVA results of Bray-Curtis distances for bacterial communities and genera with pathogenic members from Mountain Chickadee (*Poecile gambeli*) feathers and nests, with urban metrics as included variables. .... **Error! Bookmark not defined.**
- Table 3.** Linear mixed modeling for bacterial diversity metrics predicted by age of birds, sex, body size, and habitat sampled from (urban or rural). .... 19
- Table 4.** Linear mixed modeling for community metrics between nest and feather bacteria. .... 19

## 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 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 (Colbert 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). Urbanization is a global threat to biodiversity, with decreases in species richness of over 50% for many plants and animals at urban cores (McKinney, 2002). This reduction in diversity is caused by several factors including land-use change, which replaces natural habitats with pavement and buildings (McKinney, 2002). The vegetation in these areas is degraded or less abundant due to removal of native species through trampling, erosion, mowing, pollution, and other human-related activities, and the invasion of non-native species, both intentionally and unintentionally introduced (McKinney, 2002). Remnant habitat in urban areas is fragmented (Liu et al., 2016; Colbert et al., 2018), especially near the urban core as land is replaced with artificial surfaces and structures, managed vegetation, and bare ground (McKinney, 2002). Urbanization directly leads to bird mortality through collisions with glass windows and tall buildings, and through consumption of the toxic materials that appear in cities (Burton and Doblal, 2004). Domestic urban animals also prey on native wildlife, with, for example, house cats killing 1.3 to 4.0 billion birds annually in the United States (*Felis catus*; Loss et al., 2013). Aronson et al. (2014)

found that native bird density decreased by a mean of 92% in urban areas for 147 cities worldwide, when compared to the density of these species found in non-urban environments.

Although many species have not adapted to urban environments, some may tolerate, or even thrive in these areas (McKinney, 2002). Species are classified as urban avoiders when they rarely appear in cities, urban utilizers when they occur in cities but rely on surrounding natural habitat, and urban dwellers when they live in urban areas with no reliance on traditional natural environments (Fischer et al., 2015). Moderate disturbance, as seen in suburban habitats, may lead to increased biodiversity (intermediate disturbance hypothesis; McKinney, 2002). This increase in biodiversity often results from the introduction (accidental or purposeful) of non-native species, species which may provide ecological resources for wildlife; for example, fruit and seed-bearing plants. Non-traditional resources exist in suburban habitats, including bird feeders and refuse generated by human activities, both of which increase food availability for urban-tolerant species.

Despite these beneficial resources provided, urbanization can increase exposure of wildlife to infectious diseases (Bradley and Altizer, 2007). Urban species loss has been linked to 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, as reduced seasonality resulting from the heat island effect may increase their success during mild winters (Lindgren and Gustafson, 2001). Urban environments also promote the spread of infectious diseases in wildlife as stress from interspecies competition, or heavy metal and organic contaminant pollution, may have deleterious effects on immunity (Bradley and Altizer, 2007). This

amplification of infectious diseases may place populations at risk of local extinction, especially when other compounding factors already threaten them.

Increased wildlife disease rates in urban areas may also place human populations at risk. By establishing human-wildlife interfaces, wildlife diseases can transmit to humans resulting in zoonotic infections (Daszak, 2000; Hassell et al., 2017). These infections are often severe (Akritidis, 2011; Christou, 2011; Pappas, 2011), resulting in epidemics (Breithaupt, 2003). For example, all major influenza epidemics in the past century were zoonotic (Alexander and Brown, 2000; Smith et al., 2009), including the 1918 Spanish Flu (Taubenberger et al., 2005), the most lethal pandemic in recent history (Taubenberger and Morens, 2006). Birds contribute to the spread of human diseases due to the great distances avian communities disperse and migrate (Smith et al., 1996; Brinkerhoff et al., 2011). Controlling pathogen abundance in urban birds may, therefore, reduce disease emergence events in human, and other animal, populations.

Several studies highlight how urban areas increase pathogen abundance in wild birds. For example, stagnant waters and nutrient-rich runoff in cities can promote mosquito abundance, leading to proliferation of West Nile virus, as has been observed in both Chicago (Hamer et al., 2012) and Atlanta (Bradley et al., 2008). Pathogenic *Yersinia* spp., some of which are causative agents of the bubonic plague, were more prevalent in House Sparrows (*Passer domesticus*) in urban environments than rural environments in Flanders, Belgium (Rouffaer et al., 2017). Recently, both Dhondt et al. (2007) and Adelman et al. (2015) found that feeders may facilitate the transmission of *Mycoplasma gallisepticum*, the causative agent of mycoplasmal conjunctivitis. Feeders may also lead to increased avian pox infections, conjunctivitis, cloacal infections, and fungal skin disease in birds (Wilcoxon et al., 2015). However, not all bird species will respond

equally to urban-amplified diseases, as differences in feeding behavior and ecology partly determine their risk (Morishita et al., 1999).

Here we examine if urbanization is associated with the occurrence and relative abundance of pathogenic bacteria on birds and their nests in the south-central interior of British Columbia, Canada, a region currently experiencing increased population growth and expansion of urban and suburban environments (Environmental Reporting BC, 2018). Some avian species, such as Mountain Chickadees (*Poecile gambeli*), readily nest and breed in urban and suburban habitats and do not experience detrimental breeding success when nesting in urban environments (Marini et al., 2017a, 2017b). However, it is not known if urbanization may result in reduced adult survival if urban nesters experience greater pathogen exposure.

Mountain Chickadees are small songbirds found in the coniferous forests of western North America (McCallum et al., 1999). They often nest in tree cavities created by other species, and have been observed to use artificial nest boxes in both natural forests and urban environments (Marini et al., 2017a, 2017b). During the non-breeding season, urban chickadees regularly visit bird feeders, which may act as vectors for pathogen transfer. During the breeding season however, feeder visits are uncommon, but pathogen 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 these rural and urban habitats, we predict that chickadees living in urban environments will be exposed to different bacterial communities and pathogens than rural chickadees.

Advances in DNA sequencing and nucleic acid detection have allowed for studies on microbial communities, pathogen detection, and quantification. In the context of avian species, published reports include those examining feather-degrading bacteria (Lee et al., 2015; Fellahi et

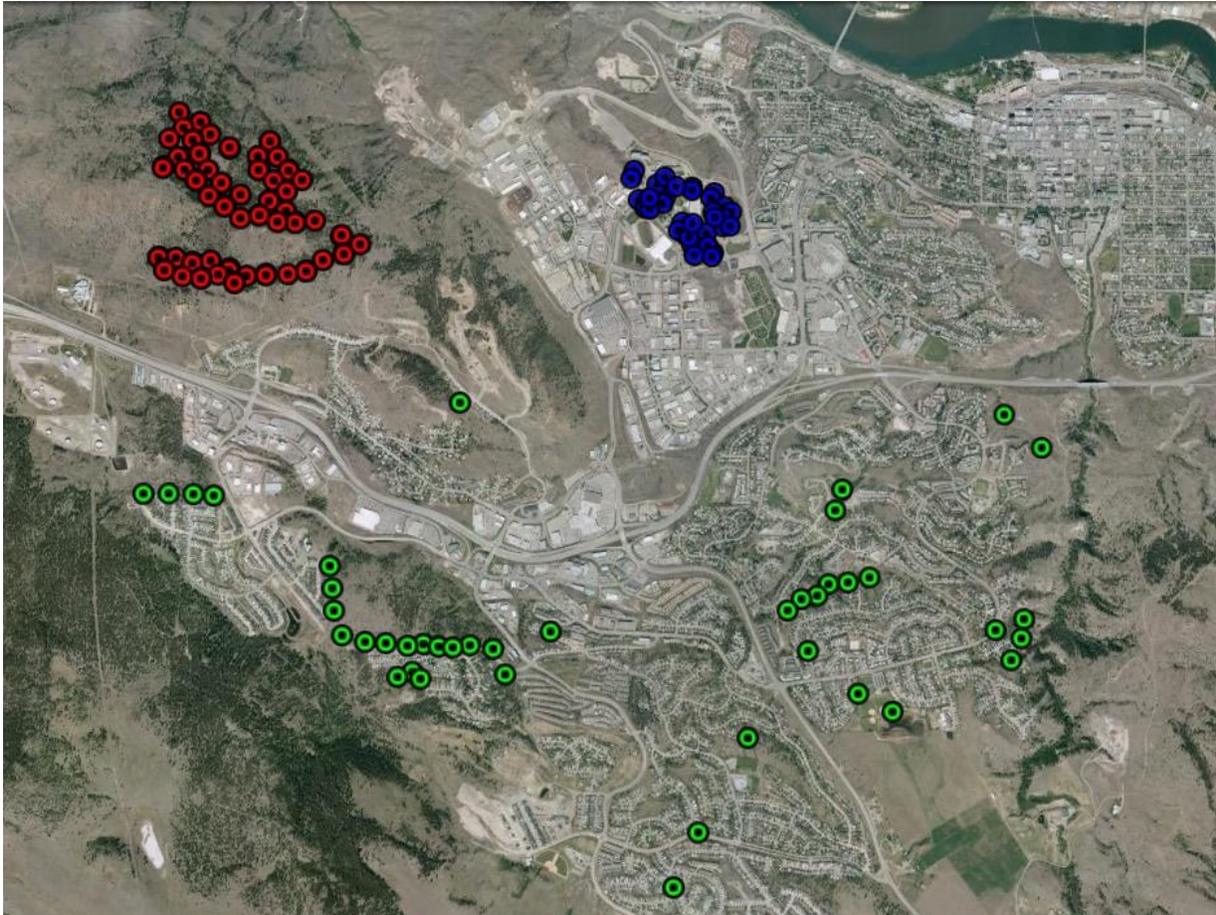
al., 2016) and intestinal microbiota (Furst et al., 2018; Teyssier et al., 2018), with few studies on microbiomes of bird feathers and their nests (van Veelen et al., 2017; Furst et al., 2018; Musitelli et al., 2018; Javůrková et al., 2019). Studies on bacterial communities in cities have found differences in community composition of soil bacteria across urban sites (Reese et al., 2016), and Razzauti et al. (2015) used 16S rRNA gene sequencing to identify potentially zoonotic bacteria in wildlife. While the studies discussed previously have reported links between specific diseases and urbanization, none to our knowledge have examined linkages between urbanization and total detectable bacterial pathogens found on rural and urban birds or their nests. In this study, we apply high-throughput 16S rRNA gene sequencing of DNA extracted from Mountain Chickadee nests and tail feathers to examine the relationship between urbanization and the presence of detectable pathogenic bacteria living on and near these birds.

## **MATERIALS AND METHODS**

### *Mountain Chickadee nest and feather sampling*

We collected feather samples and nest swabs during the 2018 chickadee breeding season (May to August) in Kamloops, British Columbia, Canada (50°40.23' N, 120°23.86' W; Figure 1). Kamloops is a city of approximately 90,000 people, situated in the Interior of British Columbia. We monitored chickadees nesting in 18 nest boxes in suburban Kamloops neighborhoods, 7 boxes at Thompson Rivers University, and 18 nest boxes in Kenna Cartwright park (50°40.232' N, 120°23.855' W). Kenna Cartwright park is located in a suburban area next to the Trans-Canada Highway, and is an 800-ha protected area composed of Douglas-fir 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 boxes were elevated 2 m from the ground with at least 150 m between boxes. Nest boxes were composed of either PVC pipe, pressure-treated wood, or natural logs.



**Figure 1.** Overview of nest boxes monitored for use by Mountain Chickadees (*Poecile gambeli*) in Kamloops, British Columbia, Canada. Nest boxes were located in Kenna Cartwright Park (red), in suburban Kamloops neighborhoods (green), or at Thompson Rivers University (blue).

During the nesting period, we captured chickadees from their nest boxes after donning sterile gloves. We measured the tarsus length, 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  $-80^{\circ}\text{C}$  until processing. At the conclusion of the nesting season, we used sterile 100% cotton swabs to sample the entire top layer of the nest bedding material from each nest box. These were stored and transported under the same conditions

as feather samples. All nest boxes were sprayed on all surfaces with a 10% (v/v) bleach solution at the end of the breeding season.

### *Estimating urbanization*

We estimated urbanization for the 75 m radius area surrounding each nest box according to LaZerte et al. (2017), an area roughly equivalent to an average territory (following Hajdasz et al., 2019). We converted GPS waypoint data for the 75 m radius area of each nest box into KML files using R v3.4.3 (R Core Team, 2017) and the mapproj package v0.9-5 (Bivand et al., 2019), then converted these KML files into aerial images using Google Earth v7.3.2.5776 (Google Earth Development Team, 2019). GIMP software v2.10.8 (The GIMP Development Team, 2019) allowed us to edit each image to categorize urban structures (buildings, pavement, human-made structures, and compacted or disturbed earth), deciduous vegetation, coniferous vegetation, natural grasses, and maintained lawns. Using R and the vegan package v2.5-4 (Oksanen et al., 2019), we performed a principal component analysis (PCA) to generate a continuous habitat index. The first principal component accounted for 67% of the total variation in habitat cover and was used to generate an urbanization index for each nest box. Natural features (coniferous trees and natural grasses) resulted in larger values while more urban features (deciduous trees in Kamloops, maintained lawns, buildings, compacted soils, human-made structures, and pavement) resulted in smaller values.

### *DNA extraction, amplification, and sequencing*

After protocol optimization, we extracted total DNA from feathers (31) and nest swabs (18) using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer instructions, with some modifications to improve DNA extraction efficiency. Specifically, we first cut away half of each swab tip, and 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 samples were submerged in buffer. We added an additional incubation step at 65°C for 10 minutes to increase bacterial cell lysis. After incubation, we immediately proceeded with bead beating using a Vortex Genie 2 with a 24-tube platform adapter (Qiagen). The manufacturer's protocol was then followed up to the elution step. PCR of the V4 hypervariable region of the bacterial 16S rRNA gene was completed on an S1000 thermocycler (BioRad, Hercules, USA). We prepared 20 µl reaction mixtures by combining 10 µL 2X GoTaq Green Master Mix (Promega, Madison, USA), 1 µL of each forward and reverse primer (341F 5'TACGGGAGGCAGCAG and 806R 5'GGACTACVSGGGTATCTAAT), 5 µL DNA extract, and 3 µl PCR-grade water. Thermocycling conditions consisted of initial denaturation at 95°C for 4 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 45 seconds, and at 72°C for 2 minutes, followed by final extension at 72°C for 5 minutes. A 1.5% agarose gel was exposed to 90 V for 35 minutes in TAE buffer, after our first round of PCR, and revealed amplicons of approximately 400 bp. These amplicons were cleaned using Agencourt AMPure XP beads (Beckman-Coulter, Brea, USA) prior to a second round of PCR with adaptor and Ion Xpress barcoded primers (341F 5'CCATCTCATCCCTGCGTGTCTCCGACTCAG[barcode]TACGGGAGGCAGCAG and 806R 5'CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGGACTACVSGGGTATCTAAT). We used the same reaction volume and component amounts for this second round of PCR. The thermocycler program was also identical to that used for first round PCR, except with an annealing temperature of 65°C. A second magnetic bead clean-up was then completed, followed by gel extraction using the MicroElute® Gel Extraction Kit (Omega Bio-tek, Georgia, USA) to remove non-target DNA fragments smaller than 100 bp. We quantified all DNA extracts and first-

round PCR products using the Quant-iT dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA) on an ABI QuantStudio 3 Real-Time PCR System (Thermo Fisher 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 (Thermo Fisher Scientific). We then prepared these pools for sequencing using an Ion 520<sup>TM</sup> & Ion 530<sup>TM</sup> Kit-Chef on an Ion Chef system, and sequenced them on an Ion S5 XL using 400 bp chemistry (Thermo Fisher Scientific). Data was processed using AMPtk (Palmer et al., 2018) for quality filtering, OTU clustering at 97% sequence identity, and to assign taxonomy: 47 samples met the read target number of >10,000 reads, grouping to 3325 OTUs.

### *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 even coverage for 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 as the V4 region of the 16S rRNA gene is not accurate 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 principle coordinates analysis ordinations based on Bray-Curtis distances. We performed PERMANOVA on these distances with urban metrics (urban or rural category, and urbanization index) to test the influence of urbanization on community dissimilarity. 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., 2019b), and GGplot2 v3.2.1 (Wickham et al., 2019a). We merged samples based on urban or rural classification according to mean values, then generated stacked relative abundance graphs of phylum-level community composition. We also merged urban and rural samples from our pathogen datasets to graphically compare abundance of the four most common genera (with >50 sequence reads after merging). Student's t-tests were used to compare differences in phylum or genera abundance for bacterial communities or pathogenic genera, respectively, between feathers, swabs, and habitat (urban or rural).

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. An identical model was run using the fixed effect of our urbanization index in lieu of Habitat (urban or rural category). We then used

a stepwise backward procedure to eliminate non-significant ( $p < 0.05$ ) 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 a) 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 and b) males and females at the same nest using Pearson correlation. Finally, to ask 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 filtered 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 pathogens from the genera *Borrelia*, *Chlamydia*, *Coxiella*, *Ehrlichia*, *Enterobacter*, *Haemophilus*, *Klebsiella*, *Listeria*, *Mycoplasma*, *Pasteurella*, *Riemerella*, *Salmonella*, *Vibrio*, or *Yersinia*. Although we were only able to broadly categorize these bacteria as potential pathogens, advances in the accuracy of sequencing platforms and pipelines to obtain

species- and strain-level accuracy, such as through amplicon sequence variants (Furst et al., 2018), may allow for absolute pathogen detection in the future.

### *Ethics Statement*

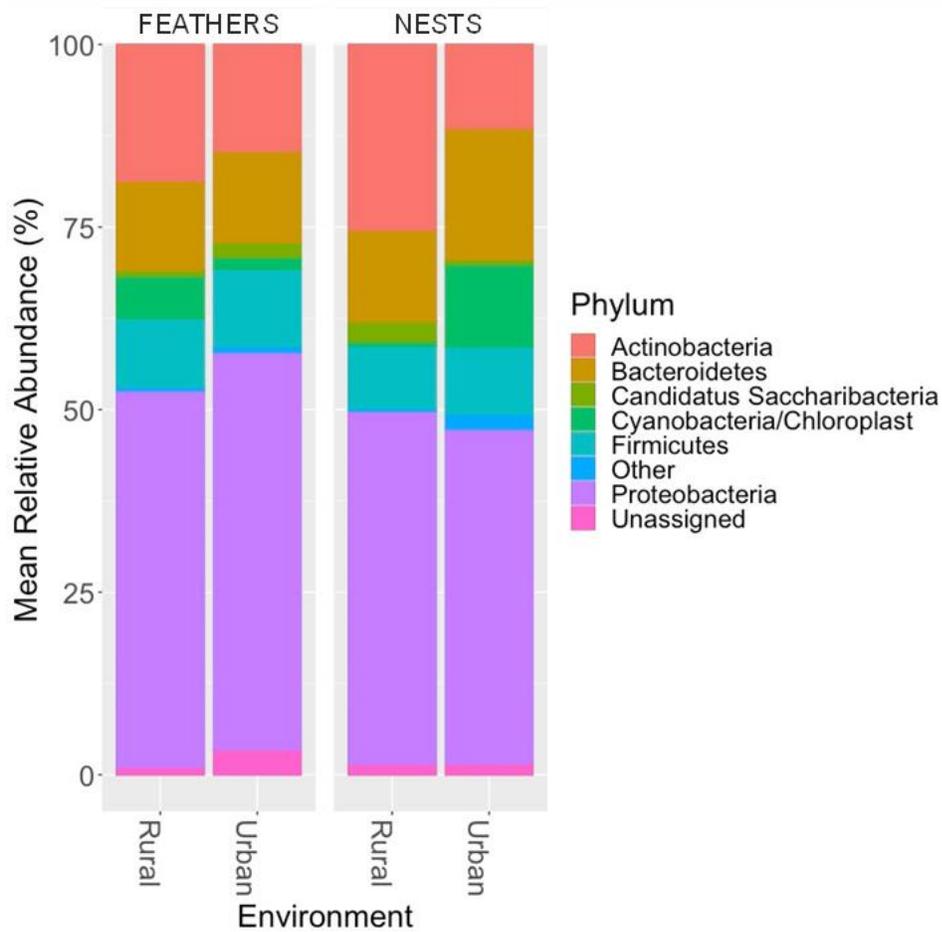
This research was carried out under Thompson Rivers University Animal Care and Use Protocol no. 100846, University of Northern British Columbia Animal Care and Use Protocol no. 2014–06, and under Canadian Wildlife Service collection permit no. 22806.

## **RESULTS**

### *Community composition and abundance of potential pathogens*

Community composition was highly similar at the phylum level for feather and nest bacteria in urban and rural environments (Figure 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 ( $p = 0.05$ ). 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  $p > 0.05$ ).



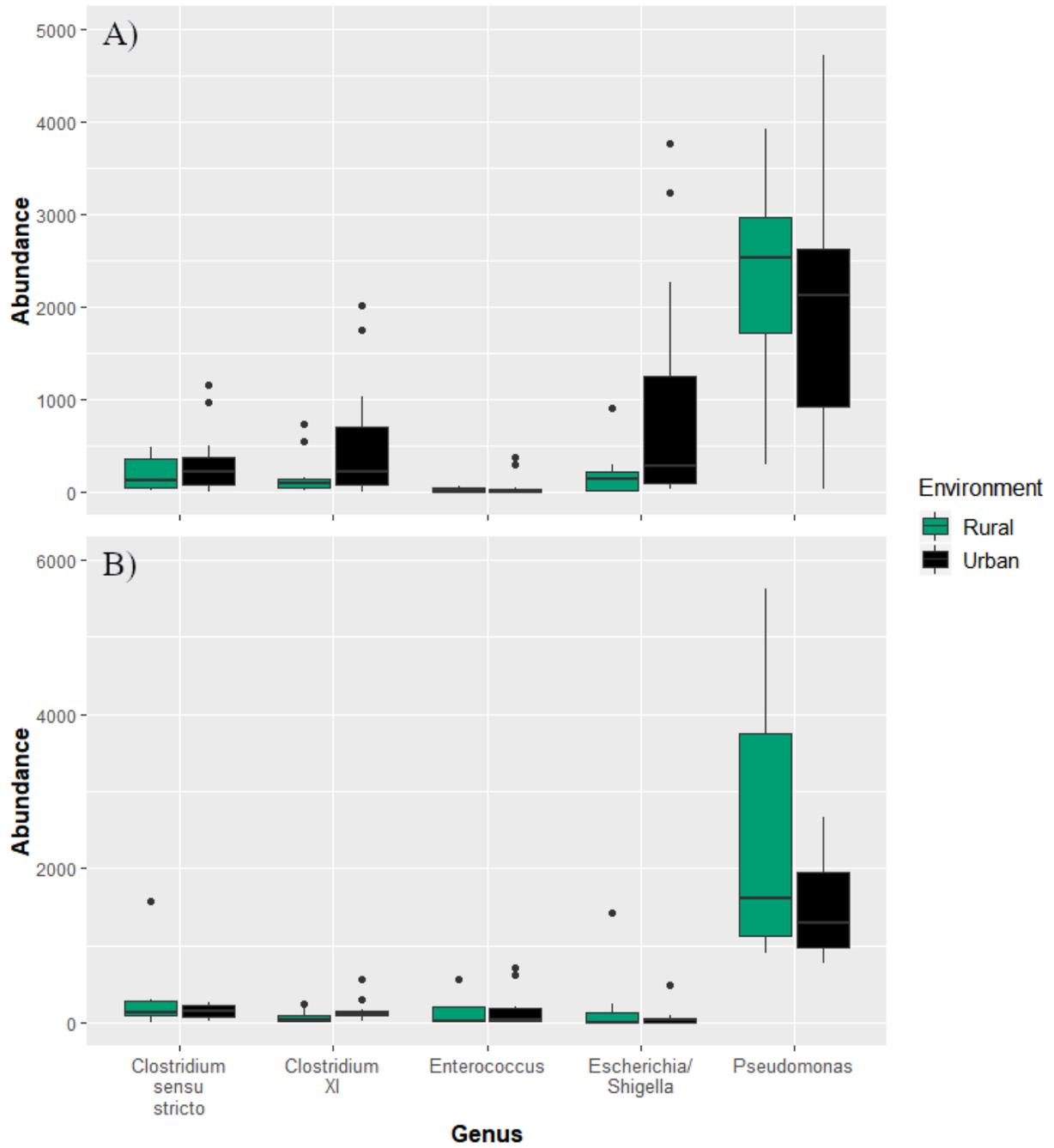
**Figure 2.** Community phylum-level composition for bacteria found on Mountain Chickadee (*Poecile gambeli*) feathers and nesting material in urban and rural areas. OTU abundances were grouped by phylum and samples were merged based on environment sampled from. Phyla that contributed to less than 1% abundance were grouped together as Other.

**Table 1.** Student’s t-test scores between urban and rural environments, and between feathers and nests, for bacterial communities and genera with pathogenic members from Mountain Chickadee (*Poecile gambeli*).

<b>Scale</b>	<b>Phylum / Genus</b>	<b>Feathers (p = ) Urban vs. Rural</b>	<b>Nests ( p = ) Urban vs. Rural</b>	<b>Feathers vs. Swabs (p = )</b>
Bacterial	Actinobacteria	0.19	<b>0.05*</b>	0.69
Communities	Bacteroidetes	0.91	0.09	0.13
	Candidatus Saccharibacteria	0.08	0.42	0.94
	Cyanobacteria/Chloroplast	0.44	0.06	0.38
	Firmicutes	0.70	0.83	0.55
	Proteobacteria	0.60	0.40	0.06
	Other	0.40	0.25	0.27
	Unassigned	0.21	0.82	0.39
Pathogen Genera	<i>Clostridium sensu stricto</i>	0.79	0.38	0.73
	<i>Clostridium XI</i>	0.62	0.18	<b>0.02*</b>
	<i>Enterococcus</i>	0.84	0.86	<b>0.05*</b>
	<i>Escherichia/Shigella</i>	0.85	0.45	<b>0.02*</b>
	<i>Pseudomonas</i>	0.95	0.57	0.82

\*Indicates significance

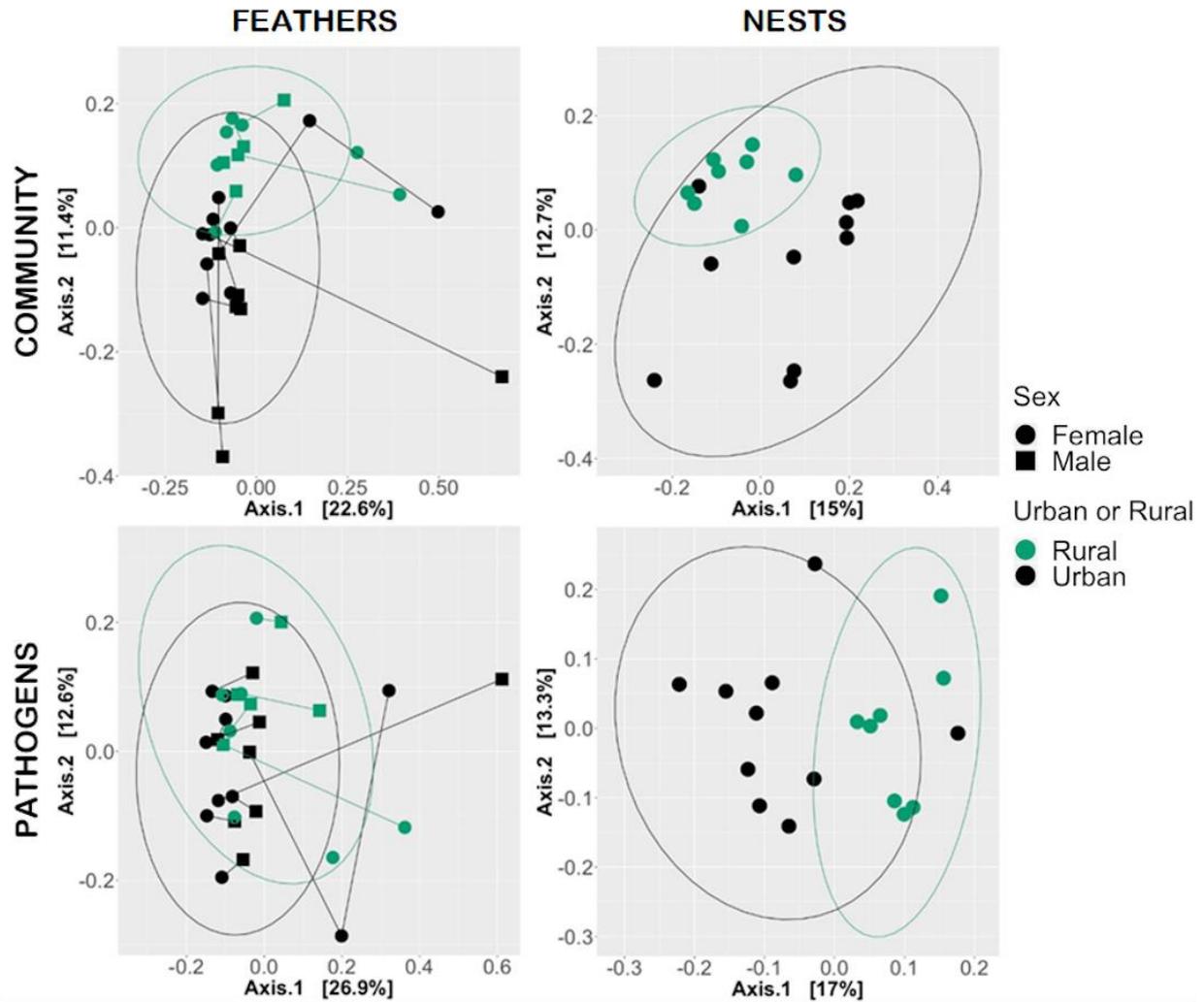
We detected 206 OTUs grouped to genera with pathogenic members. When examining only these genera, we found no differences in abundance between urban and rural environments for both feather and nest bacteria (all  $p > 0.05$ ; Table 1). The five most abundant genera with pathogenic members were *Clostridium sensu stricto*, *Clostridium XI*, *Enterococcus*, *Escherichia/Shigella*, and the most dominant genus, *Pseudomonas* (Figure 3). Comparisons between nests and feathers for these genera showed significant differences in *Clostridium XI* ( $p = 0.02$ ), *Enterococcus* ( $p = 0.05$ ), and *Escherichia/Shigella* ( $p = 0.02$ ) abundance, but no changes in *Clostridium sensu stricto* ( $p = 0.73$ ) or *Pseudomonas* ( $p = 0.82$ ).



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

### *Bacteria dissimilarity and modelling*

We used PCoAs and PERMANOVA analyses to test influences of urban metrics (urban or rural, urbanization index) 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; Figure 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 ( $R^2 = 0.05$ ;  $p = 0.12$ ). Urban bacterial communities and potential pathogens also appeared to show greater variation within groups than rural communities (Figure 4). Our urbanization index only explained some of the differences in nest bacterial communities ( $R^2 = 0.10$ ;  $p = 0.009$ ), and was not significant for feather communities ( $R^2 = 0.05$ ;  $p = 0.11$ ), feather pathogens ( $R^2 = 0.05$ ;  $p = 0.11$ ), or nest pathogens ( $R^2 = 0.07$ ;  $p = 0.22$ ).



**Figure 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 (*Poecile gambeli*) feathers and nests, with urban metrics as included variables.

Urban Metric	Community	R <sup>2</sup>	p =
Urban or Rural	Feather Community	0.06	<b>0.05*</b>
	Nest Community	0.11	<b>0.001***</b>
	Feather Pathogens	0.05	0.12
	Nest Pathogens	0.11	<b>0.003**</b>
Urbanization Index	Feather Community	0.05	0.11
	Nest Community	0.10	<b>0.009**</b>
	Feather Pathogens	0.05	0.11
	Nest Pathogens	0.07	0.22

\*Indicates significance

When we asked which factors best predicted microbial community composition, Habitat (urban vs. rural) was the only factor that explained Community Richness ( $p = 0.04$ ; Table 3) and Pathogen Richness ( $p = 0.03$ ; 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 rural habitats for any of the microbial community metrics (all  $p > 0.14$ ). When using urbanization index as our Habitat fixed effect, Pathogen Richness was also associated for feathers ( $p = 0.04$ ). No other metrics from feather or swabs were explained by urbanization index (all  $p > 0.05$ ). 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). When we examined relationships between male and female feather microbial community composition at the same nest, only pathogen abundance was correlated ( $r_{12} = 0.59$ ,  $p = 0.04$ ; all other  $p > 0.34$ ).

**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	<b>0.04*</b>
	Pathogen Richness	Habitat	1, 16.41	5.34	<b>0.03*</b>
	Pathogen Abundance	-	-	-	-
	Community Shannon-Wiener	-	-	-	-
	Pathogen Shannon-Wiener	-	-	-	-
Urbanization Index	Community Richness	-	-	-	-
	Pathogen Richness	Habitat	1, 15.65	5.01	<b>0.04*</b>
	Pathogen Abundance	-	-	-	-
	Community Shannon-Wiener	-	-	-	-
	Pathogen Shannon-Wiener	-	-	-	-

\*Indicates significance

**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	<b>0.02*</b>
Community Shannon-Wiener	1, 18.92	0.71	0.41
Pathogen Shannon-Wiener	1, 12.96	7.90	<b>0.01*</b>

\*Indicates significance

### *Discriminant functions analysis*

Discriminant functions analysis of bird feathers using 30 OTUs belonging to genera with pathogenic members performed with a 90% success rate; 1/12 rural birds were misclassified as urban and 2/18 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 3/10 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. Here we demonstrate how urbanization also influences the bacterial communities and potential pathogens found on Mountain Chickadees and their nests. 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; Wilcoxon et al., 2015; Rouffaer et al., 2017; Tian et al., 2018). However, no studies have attempted to characterize global changes in all detectable pathogens across between urban and rural habitats. A few studies have also found differences in intestinal/faecal microbiota across urban environments in birds (Furst et al., 2018; Teyssier et al., 2018), but none have questioned how urbanization changes the external microbial communities of avian species. Other researchers have further reported on urbanization influencing 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 change the characteristics of microbial communities at smaller ecosystem scales.

### *Community composition of feathers and nests*

With the recent advancement of microbiome research, researchers have begun characterizing the effects and relationships of entire microbial communities in their environments. This has created numerous opportunities to examine bacterial community relationships with wildlife. In this study, we contribute to the growing body of literature characterizing avian microbiomes, which highlight the similarities and differences between them. 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 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 (Furst 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/Chloroplast 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 (*Passer domesticus*) 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).

During the breeding period, cavity nesting birds like Mountain Chickadees spend much of this time living in their nest alongside their mate. This may indicate that bacterial communities between bird pairs, and their nests, may be similar as contact between them likely results in transmission between these elements.

When we compared the bacterial phyla found on feathers and nests, there were no differences in abundance between them. Similarly, when comparing the abundance of the dominant potential pathogens on nests and birds, 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 as a consequence of living 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 between nests and birds, or that small but significant differences exist between these environments that change the occurrence of some bacteria.

When we examined differences in bacterial communities between male and female birds, we found the abundance of potential pathogens was connected, but not the diversity or richness of microbial communities and potential pathogens. As no studies (to our knowledge) have compared microbiomes of bird pairs during the breeding season, we can only speculate as to why these results occurred. We suggest that direct contact between these birds may result in similarities for their microbiomes, while differences between these communities could result from the allocation of tasks during the breeding season. Male chickadees spend much of the early breeding season outside the nest foraging food for the young, while the females spend most of their time incubating eggs and nestlings (McCallum et al., 1999). This could result in male birds being exposed to different bacteria and environmental conditions outside the nest box, which may explain some of the differences observed between male and female microbiomes.

### *Effects of urbanization on mountain chickadees and their nests*

Urbanization is a global threat to biodiversity and ecosystems due to the alterations they make to native habitats. Although we are aware of some of these changes in animal and plant populations, few studies have examined if urbanization alters microbial communities. If changes in bacteria occur across urban landscapes, then birds living in these areas may have different bacterial assemblages and possibly be exposed to different pathogenic species.

We first determined if urbanization changes the microbial communities of Mountain Chickadees and their nests. Using broad scale investigations on phylum composition and community dissimilarity, combined with linear mixed modelling of community diversity and richness, we found that urbanization does influence some aspects of bacterial community assembly. We found no changes in the relative abundance of dominant phyla between urban and rural environments, except for an increase in Actinobacteria in rural nests. This contrasts with the findings of Furst 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, and type of urban environments sampled, 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 diversity, for bird feather microbiomes. Teyssier et al. (2018) found an opposite effect for gut microbiomes in House Sparrows (*Passer domesticus*) 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 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 show

that, at least for Mountain Chickadees, urbanization does not drastically alter community composition at a phylum level for bacteria on feathers or nests. However, urbanization does increase species richness slightly for bacterial communities on chickadee feathers, and accounts for some of the differences between communities across these gradients. These effects may be similar for other species and urban environments, although we are unable to determine if 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 examined effects of urbanization on bacterial genera with pathogenic members, which we broadly grouped as potential pathogens. 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. From these analyses we found that urbanization alters the prevalence of some potential pathogens found on birds and their nests. 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 tied to an increased richness of potential pathogens on birds, 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 urban or rural environment with 90% success using potential pathogens on birds and 72.2% success using potential pathogens on nests. Similar to our findings with bacterial communities, urbanization does not determine the abundance of the most common potential pathogens or the abundance of all

potential pathogens grouped together. However, when differences exist in the prevalence of these organisms, urbanization does account for some of these differences. 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 birds in urban areas. Whether these bacteria are confirmed pathogens and pose a significant risk to urban birds is beyond the ability of our current sequencing methods.

#### *Differences in urban classifications*

We classified urbanization in two ways, as a binomial variable (urban/rural) or as a continuous variable using an urbanization index. This index is useful for assigning an unbiased and objective measurement of urbanization. Unfortunately, these indexes do not yet account for all aspects of urban environments that a broad urban or rural classification inherently includes, which could explain why we found stronger effects from urbanization when considering a broader urban/rural classification. Our index is based on the territory size of the chickadee species being studied here, but urban influences from outside home ranges, like heat islands and changes in air quality (McKinney, 2002; Wilson and Chakraborty, 2013; Aronson et al., 2014) may contribute to what makes these areas urban. Both Rouffaer et al. (2017) and Teyssier et al. (2018) also showed this problem of scaling when studying House Sparrows, as landscape and home-range scales to classify urbanization resulted in different relationships with community metrics. As a result, we concluded that the use of our urbanization index was not suitable for this study, and instead found the results of an urban or rural category to be more robust and inclusive of the many nuances of urban environments.

### *Recommendations for future work*

Based on our results, we have identified some recommendations for future studies. Researchers should account for differences in both bacterial communities and pathogens across seasons, as some known pathogens, such as *Mycoplasma gallisepticum*, change in abundance depending on the season (Hartup et al., 2001; Hosseini et al., 2004). As nest swabs were taken at the end of the breeding season, while feathers were sampled roughly at the midpoint of the season, we cannot rule out that the differences observed between feathers and nest did not result from differences in sampling period. We therefore suggest sampling in similar timeframes when comparing feather and nest microbiota to minimize temporal influences on communities. Studies could also investigate effects of urbanization on multiple bird species across environments to better understand how ecology and geography influence these relationships. For detecting all pathogenic bacteria from a community, approaches that utilize multiple regions of the 16S rRNA gene (particularly the V2, V3 and V6 regions; Chakravorty et al., 2007) may allow for a more complete view of bacterial pathogens in environmental samples. Finally, approaches that survey non-bacterial pathogens would only further increase our understanding of the role of urbanization in the spread of wildlife diseases.

As cities continue to expand, research that identifies the environmental roles of these novel ecosystems will become increasingly important. Systematic poor land-use planning and overly permissive development regulations has allowed unregulated urban sprawl without a full understanding of its environmental impacts (Wilson and Chakraborty, 2013). We do however know that urban areas have substantial consequences on biodiversity and wildlife (McKinney, 2002; Aronson et al., 2014). Yet, some rare species now inhabit cities allowing new opportunities for wildlife conservation because of urbanization (McKinney, 2002). It is these opportunities and

unknowns of urban systems that demand more attention in future research, in particular the role of urbanization and its impacts on disease in wildlife.

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## **SUPPLEMENTAL DATA**

### *Urbanization Index*

Urbanization Index code available at <https://github.com/steffilazerte/urbanization-index>, following the updated methods in the Read-me file.

### *Project Data and R Code*

All code and data for this project is made freely available on Google Drive at <https://drive.google.com/open?id=1yLDF8NEwIKw22vBeF-xEpZDZjfWRV5V7>