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HOST MICROBE INTERACTIONS

Effects of Urbanization and Landscape on Gut Microbiomes in White-Crowned Sparrows

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Abstract

Habitats are changing rapidly around the globe and urbanization is one of the primary drivers. Urbanization changes food availability, environmental stressors, and the prevalence of disease for many species. These changes can lead to divergence in phenotypic traits, including behavioral, physiological, and morphological features between urban and rural populations. Recent research highlights that urbanization is also changing the gut microbial communities found in a diverse group of host species. These changes have not been uniform, leaving uncertainty as to how urban habitats are shaping gut microbial communities. To better understand these effects, we investigated the gut bacterial communities of White-Crowned Sparrow (*Zonotrichia leucophrys*) populations along an urbanization gradient in the San Francisco Bay area. We examined how gut bacterial communities vary with the local environment and host morphological characteristics. We found direct effects of environmental factors, including urban noise levels and territory land cover, as well as indirect effects through body size and condition, on alpha and beta diversity of gut microbial communities. We also found that urban and rural birds' microbiomes differed in which variables predicted their diversity, with urban communities driven by host morphology, and rural communities driven by environmental factors. Elucidating these effects provides a better understanding of how urbanization affects wild avian physiology.

Keywords Gut microbiome · Urbanization · Bird · Physiology · White-crowned sparrow

Introduction

Urbanization is rapidly transforming habitats around the globe [1], leading to the extirpation of several species [2], and numerous novel selection pressures on animal behavior and physiology [3]. Urban and rural habitats are often different in a number of factors important to native organisms, including food availability [4], environmental stressors [5], and prevalence of disease [6], which can lead to divergence in phenotypic traits, including behavioral, physiological and morphological features [7].

Recent research highlights that urbanization is also changing the gut microbial communities found in a diverse group of hosts, including birds [8], mammals [9], and reptiles [10]. Notably, the effects of urbanization on gut microbiomes have not been uniform—with some studies finding higher microbial diversity and others lower diversity in urban hosts [11, 12]—leaving uncertainty as to how urban habitats are shaping gut microbial communities. There is a clear need to understand these effects, because changes in the gut microbial community can affect an animal's development [13], nutrient absorption [14], and pathogen defense [15], among many other traits likely important to the host persisting in urban environments [16, 17]. A first step is to ask whether certain features of the urban environment, or of host morphology as it varies with the urban environment, can explain differences in the gut microbiome among urban and rural host populations.

Environmental factors associated with urbanization—such as landscape cover—can have both direct and indirect effects on the gut microbiome. The type of landscape cover present can filter which bacteria are present in the environment and

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thus available to colonize an animal's intestinal tract [18]. Additionally, landscape differences influence host diet which can in turn select for different gut bacterial communities [19]. Urbanization can also indirectly affect the gut microbiome via habitat degradation. Increased impervious surfaces, decreased plant diversity, and disruptions in light/dark cycles and noise are all types of habitat degradation which can act as environmental stressors with physiological consequences [20], which could change gut bacterial communities [21]. For example, chronic excessive noise increases the stress hormone corticosterone in birds [22], and corticosterone can affect digestive physiology [23]. Noise can also disrupt social interactions between animals [24], foraging behaviors [25], and predator-prey interactions [26], and all of these behaviors have been associated with shifts in bacterial community structure and membership [27]. However, relatively little is known about how specific urbanization metrics such as landscape cover and noise pollution co-vary with the gut microbiome.

Host morphology is another effect of urbanization that can impact gut bacterial communities. Urbanization can affect some aspects of an animal's morphology, for example urban house sparrows are smaller with lower body condition than their rural counterparts [12]. Urbanization can also cause chronic stress which has lasting, even trans-generational effects on body size [28]. Host size and condition have been associated with bacterial diversity and community structure, although the direction and cause of these relationships is often unclear. Gut volume and animal size predict bacterial diversity across vertebrate taxa, which suggests morphology impacts the microbiome [29]. However, bacterial diversity can also feed back and impact morphology, as evidenced by the induction of obesity in rodents via microbiome transplants [30]. Alternatively, a third variable, such as differences in diet between urban and rural locations, may change both host development and ultimately size, condition, and gut bacteria [19]. Regardless of the direction of the effect, including morphological information in studies of wild gut microbiomes is critical because morphology can vary with urbanization, and morphology is related to gut bacteria.

Here, we investigated how gut bacterial communities vary along an urban-rural gradient of a native species persisting in urban environments, White-Crowned Sparrows (*Zonotrichia leucophrys*). This study expands on a previous study that found urban birds had higher Shannon diversity than rural birds, and an association between Shannon diversity and one measure of territory land cover, but left many questions unanswered regarding how environmental and morphological variables might contribute to gut microbial diversity in urban landscapes. We first assessed differences between urban and rural habitats in a bird's environment, morphology, and gut bacterial community. We then addressed how aspects of a bird's environment and morphology co-vary with their gut bacterial community. We predicted that alpha and beta diversity would vary with environmental and morphological variables. In this species, we have reason to predict that alpha diversity will be higher in more urbanized landscapes (e.g., higher impervious surface and high noise levels), because our previous work suggests that at least one measure of alpha diversity is higher in urban areas [12]. We also predict that higher levels of alpha diversity will be associated with birds in higher condition. We predicted that higher beta diversity would occur between, rather than within, urban and rural populations, reflecting environmental differences between these landscapes and morphological and physiological differences between these populations [31]. Overall, we designed our study to assess how urbanization is shaping gut bacterial communities of a songbird.

Methods

Study Species

The Nuttall's White-Crowned Sparrow (*Zonotrichia leucophrys nuttalli*; NWCS), a sub-species of White-Crowned Sparrow, is an ideal candidate for studying the effects of urbanization on wild avian gut microbiomes. They are a coastal scrub species that breeds in both rural scrub habitats and urban parks, including in the San Francisco Bay Area [32]. Males defend a small territory during the breeding season and are residents year-round, making it feasible to identify important environmental variables that might affect their gut microbiome. They are relatively easy to capture, facilitating taking morphological measurements and collecting non-lethal samples of gut microbial communities (via cloacal swabs) [33].

Study Locations

We sampled a total of 82 male birds during the breeding season between May 30 and July 1, 2016. We sampled male birds holding territories along ten transects. These transects occurred in both urban ($n = 7$) and rural ($n = 3$) locations (Fig. 1, Fig. S1; also described in [12]). Each transect was approximately 2 km long (range 1.7–2.6 km), and we sampled approximately 10 males holding territories along each transect (see below). All transects occurred within a sampling area approximately 1400 sq. km. These transects were designed for a separate study investigating the relationship between bird song production and noise levels, and so the transects occurred along noise gradients within both urban and rural landscapes [12]. Noise transects also reflect changes in landscape, including increasing impervious surfaces in urban areas, where the main noise source is roads, and more open areas in rural areas, where the main noise source is ocean surf. There were seven urban transects: five within the Presidio, one in

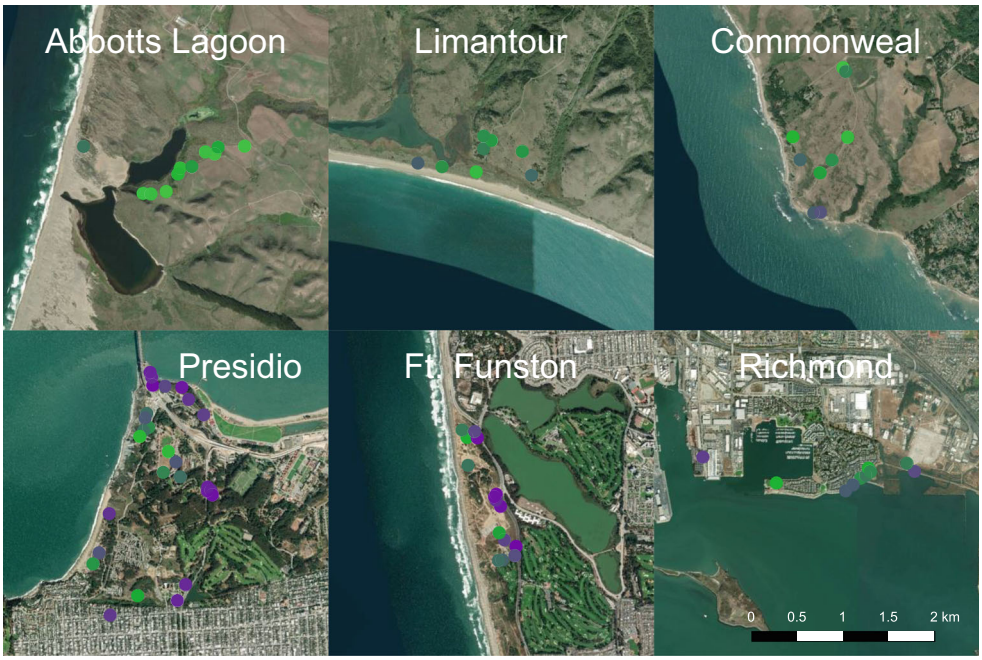

Fig. 1 Transects were sampled in ten locations in urban and rural areas. Transects from Abbotts Lagoon, Limantour, and Commonweal were designated rural, while Richmond, Presidio and Ft. Funston transects were considered urban. Each dot represents a sampled bird. Transects were originally designed to sample across a noise gradient, and so dots are on a color scale from green to purple; light green is quietest and dark purple is loudest (dBA). See supplement Fig. S1 for locations of all transects on one map

Fort Funston near Lake Merced within San Francisco and one in the area of Richmond in the East Bay (Fig. 1). Presidio territories were in heavily trafficked park areas, many near the Golden Gate Bridge and other high traffic roads. Although Fort Funston is within the city of San Francisco and receives recreational foot traffic, it also contains areas closed to foot traffic where a number of sampled sparrows held territories. Richmond territories were largely in or near a suburban park or adjacent to residential yards. There were three rural noise transects: one each in Abbotts Lagoon, Limantour Beach, and Commonweal. All three of these sites occur within the Point Reyes National Seashore (Fig. S1). All rural sites were almost entirely scrub habitat of varying densities. Commonweal territories sometimes experience cattle grazing, Limantour territories were relatively close to the ocean compared to other rural territories, and Abbot's Lagoon territories were inland along a freshwater to brackish pond.

Sampling and Morphological Measurements

Males were captured using mist nets (Avinet Research Supplies; Portland, ME) set up on their breeding territory with playback of a local NWCS song as a lure between 7:00 am and 1:00 pm (inMotion iMT320 speaker (Altec Lansing, New York, NY, USA). North and west coordinates were recorded using a Garmin GPS (Table S1), at the approximate center of each male's territory, as determined by multiple visits and observation of banded birds [34]. We sampled only males because they are the more aggressive defenders of the breeding territory and attacked the speaker more often, making them easier to capture. We did not include any females netted ($n = 4$) because we wanted to achieve a sufficient sample size at each location, and sex differences in the gut microbiome are probable [35]. For each bird, we recorded fat score, plumage wear, plumage fade, wing chord, mass, tarsus length, bill length, bill width, and bill depth measurements, following [36]. We estimated body condition by calculating the scaled mass index using tarsus as the length variable [37]. We collected cloacal swabs by cleaning the cloaca with an aseptic alcohol swab and inserting a sterile swab (Puritan 25-3316-U 6" Sterile Mini-Tipped Nylon Ultra Flocked Swab with Polystyrene Handle) completely into the cloaca and gently turning for 3–5 s. We used RNAlater to preserve swabs and stored them in a − 20 °C freezer within 12 h of collecting. Sampling techniques were approved by Tulane University Institutional Animal Care and Use Committee (protocol 0427-R), Bird Banding Laboratory Permit (23900), California State Collecting Permit (6799), Golden Gate National Recreation Area (GGNRA) Scientific Research and Collecting Permit (GOGA-00079), San Francisco Parks and Recreation Permit (032014), and Point Reyes National Park Scientific Research and Collecting Permit (PORE-0014).

Environmental Measurements

For each bird, we also collected data from their breeding territory on ambient noise level (LAeq dB re: 20 μPa, 8–20 kHz), percent tree, grass, scrub and impervious surface cover, distance to minor road, and distance to freeway. Noise level was recorded using a Larson Davis Model 831 sound level meter with a preamplifier (Larson Davis, Depew, New York, USA). We recorded 1 min of sound in each cardinal direction and calculated the average noise reading for each territory

(following [38]). Readings interrupted by high wind or sudden noise were discarded and re-recorded. NWCS defend a territory with an approximately 50-m radius, so we analyzed land cover data for a 50-m radius around our GPS coordinates to calculate land cover. We created polygons using the polygon measuring tool for each land type (impervious, tree, shrub, grass) within our territory using Google Earth Pro high-resolution imagery (Google, Mountain View, California, USA). To measure distance from freeways and minor roads, we used the Google Earth measuring tool to measure from the center of the territory to the closest small road and major road.

DNA Extraction and 16s Library Preparation

We extracted DNA from cloacal swabs using the MoBio Powersoil extraction kit and recommended protocol, with modifications recommended by Vo and Jedlicka [39]. Additionally, we combined solutions C2 and C3, which precipitate non-DNA substances to increase DNA yield as recommended by MoBio technicians (personal communication 2016). We also included an extraction blank to control for possible contamination, which did not successfully amplify after 6 PCR attempts.

We amplified the V4 region of the 16s rRNA gene using 515F/806R primers in 25-μl reactions (Integrated DNA Technologies, Coralville, IA, USA) [40]. Each reaction contained: 12 μL sterile, molecular grade water, 1 μL bovine serum albumin, 10 μL 5′ hot Mastermix by Thermo Fisher, 0.5 μL of each primer, and 2 μL of DNA template. Each reaction was performed in triplicate to reduce PCR bias. Water was used as a negative control for each set of reactions. Denaturation of DNA was initially performed at 94 °C for 2 min, then cycling was carried out as follows: 94 °C for 8 s, annealing at 50 °C for 20 s, and extension at 72 °C for 30 s; for 35 cycles. A final elongation was performed at 72 °C for 10 min. PCR success was verified with gel electrophoresis.

We pooled each sample's amplicon triplicates and added dual-end Illumina barcodes in the style of TruSeq HT primers (Illumina Inc., California USA). We used gel electrophoresis alongside untagged PCR product to confirm successful addition of tags. We normalized concentrations of all samples using a SequalPrep normalization kit from Thermo Fisher, then pooled PCR product and purified using Agencourt AmPure beads. GeneWiz, LLC sequenced our library on an Illumina MiSeq platform with v2 reagents, two by 250 base pairs.

Sequence Processing

Sequences were processed in QIIME2 version 2018.4 (qiime2.org) [41]. We used the Divisive Amplicon Denoising Algorithm (DADA) to remove sequence errors, and trim primers from sequences [42]. We aligned sequences, then generated a phylogeny using FastTree, then rooted the tree at the midpoint [43]. Sequences were grouped at 100% similarity (i.e., amplicon sequence variants). We assigned taxonomy using GreenGenes [44]. Finally, we filtered out all mitochondrial, chloroplast, and archaeal sequences. We obtained a total of 5,973,986 sequences (mean = 104,859, SD = 73,186; see Table S1 for sequence and OTU counts for each sample). All sequences are available on the NCBI sequence repository (PRJNA634155). Scripts for processing sequences and replicating all analyses are available on GitHub (https://github.com/mBerlow/urbangutmicrob2020).

Data Analyses

Environmental and Morphological Variables

We analyzed whether our measured environmental and morphological variables varied between urban and rural habitats and among the ten transects using Welch's two-sample *t*-tests.

Bacteria Taxa

To identify bacterial taxa that are differentially abundant between urban and rural populations, we performed linear discriminant analysis effect size (LEfSe). This was accomplished by uploading a rarefied ASV table to the Galaxy project platform, and using the LEfSe module by the Huttenhower lab [45]. LEfSe uses a Kruskal-Wallis rank-sum test to identify taxa that differ between groups and to test for uniformity among groups. Last, to determine effect size of differential taxa, it uses linear discriminant analysis (LDA) and creates a histogram.

Alpha Diversity Analyses

Alpha diversity metrics were calculated from an OTU table rarefied to a depth of 1000 sequences. Alpha diversity was measured using Hill numbers, which provide multiple measures of alpha diversity in the same units (effective number of species). Hill number transformations are calculated as orders of q, written as ${}^{q}D$, with q of 0 (${}^{0}D$) representing bacterial richness, q of 1 (${}^{1}D$) representing exponential of Shannon entropy, including both richness and evenness, and q of 2 (${}^{2}D$) representing the inverse of Simpson's index wherein species are weighted according to their abundance [46]. This means that the effective number of species is less sensitive to rare bacteria (diversity) as q increases. Hill numbers were calculated using the "d" function in the R package "vegetarian" [47]. We also calculated Faith's Phylogenetic diversity in QIIME2 to account for phylogenetic relatedness of bacteria in alpha diversity [41]. Building on our previous work that found differences in Shannon diversity index between urban and

rural gut bacterial communities [12], we assessed whether urban and rural samples are different in all measures of alpha diversity (^{0}D, ^{1}D, ^{2}D, and Faith's pd) using *t*-tests.

We next ran model selection to determine the importance of environmental and morphological variables in explaining variation in bacterial alpha diversity. We did this first with urban and rural samples combined and then second with urban and rural samples treated separately. We *z*-scaled all variables (response and predictor variables) before inputting them into our models. To determine if any of our predictor variables were collinear, we conducted a variance inflation factor analysis (VIF). VIF is a method of measuring multicollinearity between independent variables which can be used to inform adjustments to a model that will increase its ability to detect realtionships between dependant and independent variables. West coordinate, percent scrub, and distance to freeway had VIF scores above 10 (high collinearity). We removed those variables and ran VIF analysis again, at which point no variables had a VIF above 5. We ran model comparison using the remaining 14 variables (8 morphological: fat score, plumage wear and fade, wing chord, condition, bill length, width, and depth; 6 environmental: noise, north coordinate, percent tree, grass and impervious surface cover, and distance to minor road). We ran all possible linear models (total 16,383) using the lm function from the "stats" package in R [48]. We then calculated AICc values and weights for all models using the function aictab from the "AICcmodavg" package in R [49]. From these, we calculated the weights of each predictor variable using the importance function, which adds the weights from all models including each variable to determine their overall contribution. We then unconditionally averaged all models using the modavg function from the "AICcmodavg" package to obtain our final model and variable weights [49]. Important variable scatter plots were generated using ggplot 2 [50].

Beta Diversity—Ordination and dbRDA

We next calculated beta diversity, a measure of how much of a community changes from one point to the next. We calculated four standard measures of beta diversity using QIIME2 [41]: weighted UniFrac, Jaccard, unweighted UniFrac, and Bray-Curtis distances. The former two can be considered to represent microbial community membership, and the latter two to represent microbial community structure [51]. UniFrac distances account for phylogenetic relatedness. Using QIIME2, we created dissimilarity matrixes for each distance measure and reduced dimensionality using principal coordinates analyses (PCoA). To assess dissimilarities in community membership and structure between urban and rural birds, we visualized the first two PCoAs for each distance measure, using ggplot2 [50].

Next, we examined variation in beta diversity in terms of community membership and structure. In this context, we are examining how much microbial community membership and structure are changing from one bird to the next. We examined beta diversity both across all birds and separately for urban and rural birds using all four measures of distance. We first asked whether groups differed according to habitat using a PERMANOVA performed with the function adonis in the R package vegan [52]. We then asked whether environmental and morphological variables can explain beta diversity using ANOVAs within the distance-based redundancy analyses (dbRDA) with 10,000 permutations in the vegan package in R [52].

Results

Environmental and Morphological Variables

Our sample sites were chosen as either rural or urban sites, and to quantify this categorization, we measured many potential indicators of urbanization. All of the measured environmental variables were significantly different between urban and rural locations (Welch's *t*-test, $p < 0.05$; Table 1), except for percent grass ($t = -1.53$, $P = 0.134$). Percent tree ($t = 6.14$, $P < 0.001$) and impervious surface cover ($t = 4.83$, $P < 0.001$), and noise ($t = 7.33$, $P < 0.001$) were significantly higher in urban areas, whereas percent scrub cover ($t = -4.46$, $P < 0.001$) was significantly higher in rural areas. The only morphological feature different between urban and rural areas, after Bonferroni correction for multiple tests, was plumage wear (Welch's *t*-test, $p < 0.001$; Table 1). Bill size, plumage fade, wing chord, cloacal protuberance, fat scores, and body condition were not significantly different between urban and rural areas.

Bacterial Taxa

We found 23 families to be differentially abundant between urban and rural birds (LDA scores >3, Fig. 2). Urban birds had significantly higher abundances of Enterobacteriaceae, Campylobacteraceae, Phormidiaceae, and 11 others. Rural birds had significantly more Mycoplasmataceae, Ktedonovacteraceae, Diplorickettsiaceae, and six others.

Bacterial Richness Is Higher in Urbanized Landscapes

We found support for our prediction that alpha diversity is higher in the urban as compared to the rural habitat; however, this pattern was significant only for bacterial richness (^{0}D) ($t = 2.077$, $P = 0.041$, urban mean = 107.2,

Table 1 All morphological and environmental variables included in analyses. Variables were compared between urban and rural sites using Welch's two-sample *t*-test for means. Variables with an asterisk are ones that are significantly different between urban and rural sites (Bonferroni adjusted alpha (0.003))

	Variable	*t*-stat	df	*p*		Urban mean	Rural mean
Morphological	Bill length (mm)	−2.52	65	0.014		12.37	12.64
	Bill width (mm)	1.7	74	0.094		6.23	6.13
	Bill depth (mm)	1.17	56	0.247		6.88	6.81
	Wing chord (mm)	−0.18	72	0.861		71.15	71.23
	Plumage wear	−4.85	46	<0.001	*	1.34	2.19
	Fat	−0.94	63	0.353		1.17	1.35
	Plumage fade	−1.63	76	0.108		0.49	0.71
	Body condition	−1.02	57	0.312		31.16	31.54
Environmental	Noise (dB)	7.33	80	<0.001	*	55.45	46.4
	North coordinate	−12.27	48	<0.001	*	37.8	38.03
	West coordinate	21.04	43	<0.001	*	−122.46	−122.86
	% trees	6.14	59	<0.001	*	0.21	0.02
	% scrub	−4.46	67	<0.001	*	0.43	0.73
	% impervious	4.83	80	<0.001	*	0.31	0.12
	% grass	−1.53	42	0.134		0.07	0.14

rural mean = 80; Fig. 3; Table S2). We did not find a significant difference in ^{1}D, ^{2}D, or Faith's phylogenetic diversity between birds from urban and rural habitats ($t = 1.174$, 0.884, and −1.8484 respectively, $P = 1.174$, 0.379, and 0.071, urban mean = 21.6, 11.6, and 9.31, rural mean = 16, 9.2, and 7.76; Fig. 3; Table S2).

Alpha Diversity Predictors Differ According to Weight of Rare Bacteria

In the final overall averaged model combining urban and rural samples, percent grass cover was the most important predictor for richness ($^{0}D\ w^{+} = 1$; Table S3&4, Fig. 4). North

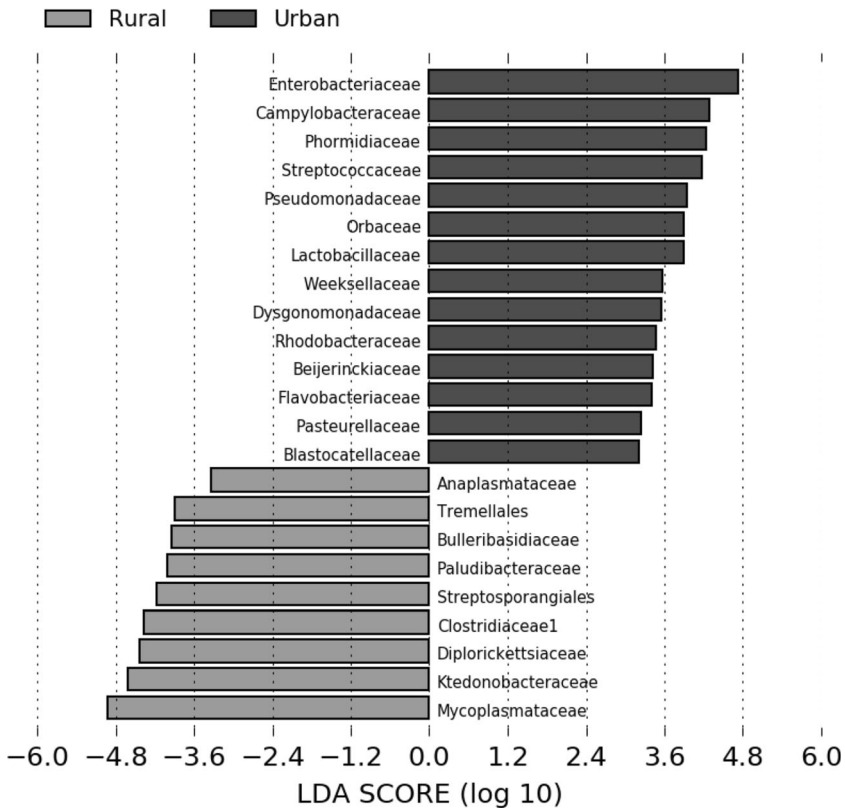

Fig. 2 Linear discriminant analysis effect size (LEfSe) comparing bacterial families between urban and rural groups. Colors correspond to which group was found to have disproportionately more abundance of that bacterial family

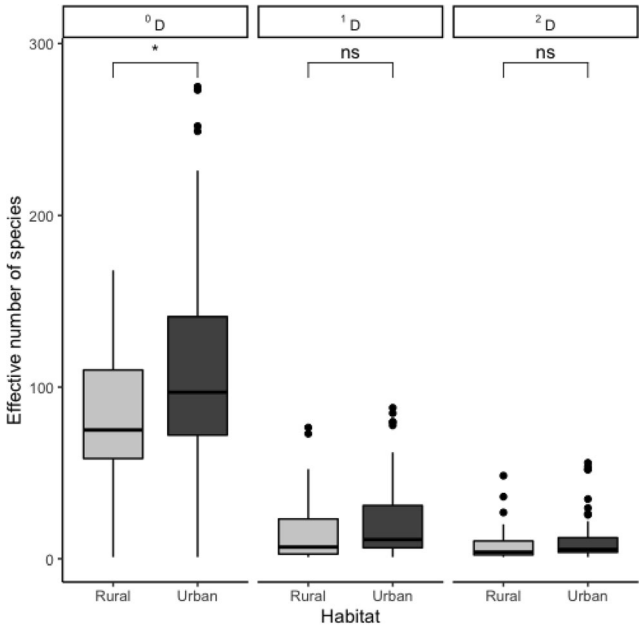

Fig. 3 Variation in alpha diversity (effective number of species) measured in Hill numbers (three orders of q). Effective number of species is less sensitive to rare bacteria as q increases. ^{0}D is bacterial richness, ^{1}D is the exponential of Shannon entropy, and ^{2}D is the inverse of Simpson's Index. Color corresponds to habitat type for sampled birds. * indicates significance difference ($p < 0.05$)

coordinate, i.e., geographic location, was the most important predictor for ^{1}D and ^{2}D (^{1}D $w^+ = 0.71$; ^{2}D $w^+ = 0.61$; Table S3&4, Fig. 4) The next three most important predictors for bacterial richness (^{0}D) were bill depth ($w^+ = 0.73$), north coordinate, ($w^+ = 0.7$), and noise ($w^+ = 0.58$). For both ^{1}D and ^{2}D, the next three most important predictor variables after north coordinate were wing chord ($w^+ = 0.56$, 0.56; respectively), noise ($w^+ = 0.54$, 0.52), and bill depth ($w^+ = 0.51$, 0.46). All other predictor variables for all levels of q were less than 0.5. Three of these environmental variables (percent grass, north coordinate, and noise) drop in importance, as rare species are down-weighted. We also ran our models with Faith's PD as the response variable, but the results did not differ notably from those of the hill numbers (Table S2, S3).

To further examine patterns in alpha diversity, we plotted important variables separately against orders of q, for all birds combined (Fig. S2; Fig. S3). These ordinations showed us that although some variables—such as percent grass cover—were the most important variable in our averaged model, most of these variables alone were not tightly correlated with alpha diversity. Richness (^{0}D) had a relatively high R^2 value when plotted against bill depth.

Predictors of Alpha Diversity Differ for Urban and Rural Birds

We next ran all possible models separately for urban and rural samples. We found that the most important predictors of alpha diversity were not the same for urban and rural samples (Fig. 4). For bacterial richness (^{0}D) in urban models, bill depth was the most important variable ($w^+ = 0.72$), followed by bill length ($w^+ = 0.58$), percent tree cover ($w^+ = 0.57$), and noise ($w^+ = 0.54$). However, in rural samples, percent grass ($w^+ = 0.99$) and tree cover ($w^+ = 0.57$) best predicted species richness. When rare bacteria are down-weighted (^{1}D or the exponential of Shannon index, ^{2}D or inverse Simpson's index) urban gut bacterial alpha diversity is best predicted by bill length (^{1}D $w^+ = 0.93$, ^{2}D $w^+ = 0.93$) and depth (^{1}D $w^+ = 0.59$, ^{2}D $w^+ = 0.58$), and wing chord (^{1}D $w^+ = 0.67$, ^{2}D $w^+ = 0.65$). In contrast, in rural areas, alpha diversity is best predicted by percent grass (^{1}D $w^+ = 0.95$, ^{2}D $w^+ = 0.79$) and tree cover (^{1}D $w^+ = 0.5$, ^{2}D $w^+ = 0.57$), north coordinate (^{1}D $w^+ = 0.89$, ^{2}D $w^+ = 0.83$), and distance to minor road (^{2}D

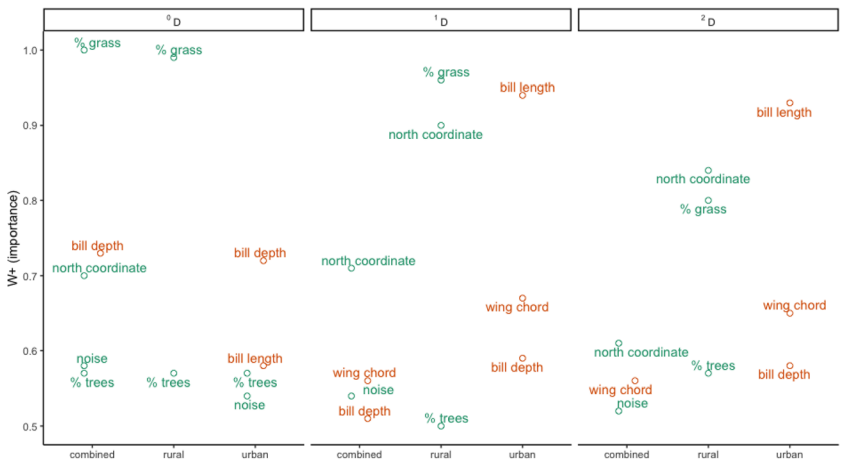

Fig. 4 Important variables ranked by importance for averaged model for all data (combined) and urban and rural locations separately. Shown are all variables above .5 importance. Points and labels colored by variable type (environmental = green; morphological = orange). For example, for ^{0}D combined dataset, the most important variable is % grass, followed by bill depth, north coordinate, noise, and finally % trees. Overall, urban gut bacterial communities are best predicted by morphological characteristics, whereas rural gut bacterial communities are better predicted by environmental factors

$w^+ = 0.78$) as rare bacteria are down-weighted. Altogether, we find that urban gut alpha diversity is best explained by morphological variables whereas rural gut alpha diversity is best explained by environmental variables.

We then considered how predictor variables change in importance, as rare species are down-weighted within urban and rural samples. We find that for urban samples, the morphological variables increase or stay the same in importance as rare species are down-weighted. In contrast, the environmental variables are only above 0.5 in importance for ^{0}D and drop precipitously for higher orders of q. We find that for rural samples, percent grass, and percent tree cover decrease slightly as q increases but north coordinate increases in importance as q increases. This latter pattern is nearly the opposite of what was found for environmental variables when urban and rural samples were combined.

To further examine patterns in alpha diversity, we plotted important variables separately against orders of q, for both rural and urban birds (Fig. S2, Fig. S3). These ordinations showed us that although some variables—such as percent grass cover—were the most important variable in our averaged model, most of these variables alone were not tightly correlated with alpha diversity. Richness (^{0}D) had a relatively high R^2 value when plotted against bill depth and bill length for urban birds, such that richness increases with increase in bill size, whereas percent tree cover had relatively high R^2 for all orders of q in rural birds, but the direction of the relationship varied with Hill number. This pattern is consistent with the overall pattern we see in model averages, that urban gut bacterial communities are best predicted by morphological variables, whereas environmental variables are more important for rural gut bacteria.

Beta Diversity Is Greater in Community Membership than Structure between Urban and Rural Birds

Our principal coordinate ordinations of community membership showed some grouping of urban versus rural birds (Fig. 5a, b; unweighted UniFrac and Jaccard). In other words, the beta diversity in gut microbial community membership is higher between urban and rural birds than it is within those groups, as we predicted. This visualization is supported by the PERMANOVA results that show a significant difference in community membership beta diversity between urban and rural birds (Table 2). In contrast, there was no obvious clustering according to urban versus rural sites for distance measures of community *structure* (Fig. 5c, d). This means that the beta diversity in gut microbial community structure is not greater

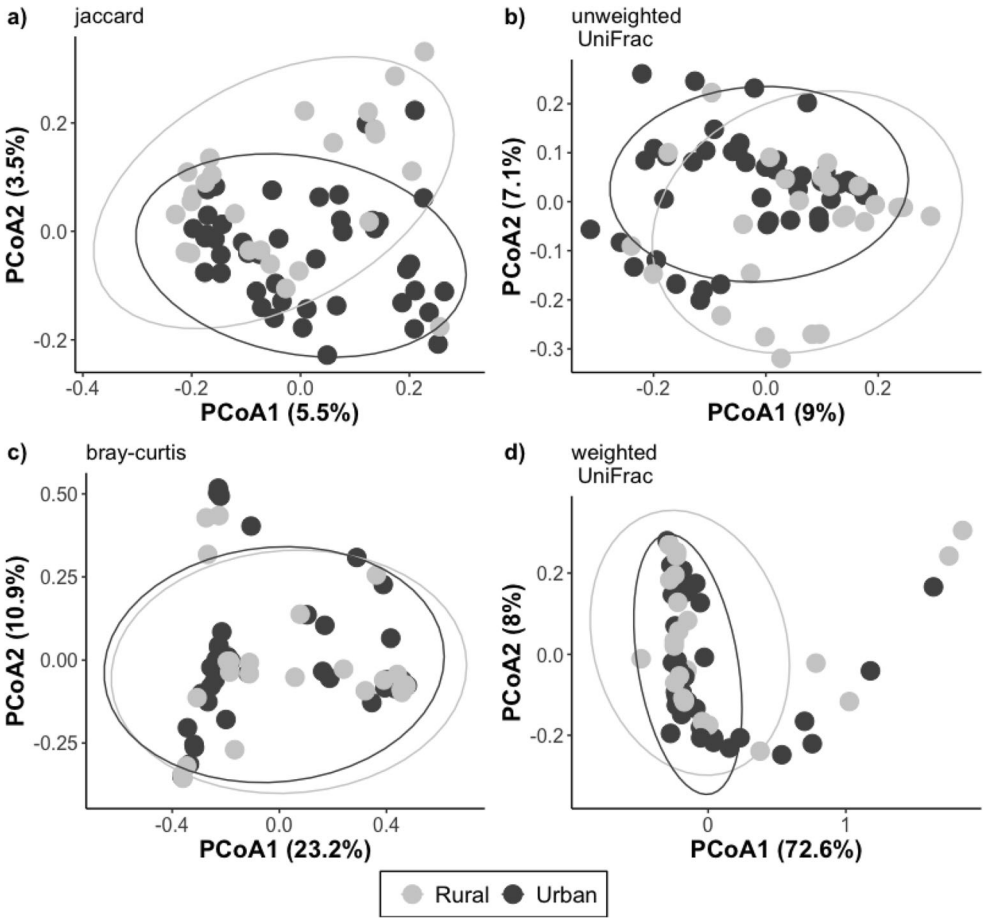

Fig. 5 Principal coordinates plot (PCoA) of **a** Jaccard, **b** unweighted UniFrac, **c** Bray-Curtis, and **d** weighted UniFrac distances. Variation explained by each axis is provided in parentheses. Each data point represents one sample

Table 2 PERMANOVA results on beta diversity distances between communities in urban and rural habitats

Distance measure	df	Pseudo-F	*p*
Jaccard	1	1.408	0.001
unweighted UniFrac	1	1.978	<0.001
Bray-Curtis	1	0.914	0.5044
Weighted UniFrac	1	1.018	0.3296

between urban and rural birds as compared to within those groups. This visualization is supported by PERMANOVA results. We found no significant differences between communities in different habitat types when phylogenetic relatedness was accounted for (i.e., Bray-Curtis and weighted UniFrac) (Table 2). Altogether, urban and rural birds differ more in terms of beta diversity of community membership (i.e., presence/absence of taxa), and less so in terms of beta diversity of community structure (i.e., accounting for relative abundance).

We ran distance-based redundancy analysis (dbRDA) with a full model for all samples together including all non-covarying variables (as determined by VIF above) for each of our four distance measures (Jaccard, Bray-Curtis, unweighted and weighted UniFrac distances, Table 3). For all distance measures, the full model consistently explained about 17% of variation in beta diversity of gut microbial communities among individuals (Table S5a-d). We found that percent tree cover explained a significant amount of variation in phylogenetic community membership (unweighted UniFrac, ANOVA $P = 0.03$). No variable explained variation in beta diversity of community structure (Jaccard, Bray-Curtis, weighted UniFrac). When urban and rural populations were examined separately, only urban weighted UniFrac distances were significantly explained by one of our variables (bill width, ANOVA $P = 0.04$).

To examine if drivers of bacterial beta diversity differ for urban and rural birds, we repeated these dbRDA analyses for urban and rural birds separately for both community membership and structure. A model including all non-covarying environmental and morphological variables explained approximately 25% of the variation in beta diversity of gut bacterial communities for urban sites, and about 55% of variation in beta diversity for rural sites, for all measures of community membership and structure. None of the environmental or morphological variables explained beta diversity in bacterial community membership or structure among rural birds (dbRDA ANOVA, all $P > 0.05$; Table 3; Table S5a-d). In contrast, bill width explained some of the beta diversity in bacterial community structure among urban birds (dbRDA ANOVA $P = 0.04$; Table 3; Table S5d). However, overall, not much of the variation in beta diversity among urban birds or among rural birds is explained by the environmental or morphological variables measured in this study.

Discussion

Our intent was to understand how gut bacterial communities vary along an urban-rural gradient for a native species persisting in urban environments (Fig. 1), and whether the host's environment and morphology co-vary with their gut bacterial community. As predicted, we found significant differences between urban and rural locations in hosts' gut microbial communities (abundance of different bacterial families (Fig. 2), alpha (Fig. 3) and beta diversity (Fig. 5), and these differences appeared driven mainly by rare bacteria (Fig. 3). We also found significant differences among urban and rural birds in their territory's land cover (such as tree cover) and degree of urbanization (such as noise levels) as well as in their morphological and physiological characteristics (mainly measures of beak size and body condition) (Table 1). We also

Table 3 dbRDA both combined and separately on rural and urban birds. Results showing percent explained for each distance measure, and significant variables if any. Jaccard and unweighted UniFrac distances represent community membership, Bray-Curtis and weighted UniFrac distances represent community structure, and UniFrac distances are corrected for phylogenetic relatedness. Full model used in each case: distance measure ~fat + wear + fade + wing chord + noise + bill length + bill width + bill depth + body condition + % trees + % grass + % impervious + % scrub

	URBAN			RURAL			COMBINED		
Diversity measure	dbrda1% explained	dbrda2% explained	Significant variables	dbrda1% explained	dbrda2% explained	Significant variables	dbrda1% explained	dbrda2% explained	Significant variables
Jaccard	4%	3%	Condition	8%	5%	None	2%	2%	None
unweighted UniFrac	4%	4%	None	9%	86%	None	3%	2%	% trees
Bray -Curtis	9%	3%	None	10%	7%	None	4%	2%	None
Weighted UniFrac	20%	2%	Bill width	49%	4%	None	80%	6%	None

found evidence that some of these environmental factors and morphological characteristics can explain variation in host gut microbial communities. Consistent with our previous work [12], alpha diversity was higher in urban areas (Fig. 3); however, not for the reasons we expected. We predicted that environmental factors associated with urbanized landscapes, such as impervious surface and noise levels, would be most important in explaining higher alpha diversity in urban areas. Instead, environmental factors such as grass and tree cover were more important (Figs. 4 and 5), with urban areas having higher tree cover and higher levels of alpha diversity (Table 1). Further, we found no association between alpha diversity and body condition, counter to our predictions. Most unexpected was finding that different types of variables explain variation in alpha diversity within urban versus within rural areas—specifically environmental factors (such as grass cover) appear more important within rural areas whereas morphological factors (such as bill size) are more important within urban areas in explaining host gut microbial community diversity (Figs. 4 and 5). As we predicted, beta diversity (beta diversity) was greater between, rather than within, urban and rural populations, but only for community membership, not structure (Table 2 and Fig. 5). The key difference among urban and rural habitats that appeared to explain this beta diversity was an environmental variable, percent tree cover (Table 3) similar to our findings for alpha diversity. On further examination of predictive variables for beta diversity within urban and rural areas separately revealed subtle but informative associations between beta diversity across urban individuals and host body condition and bill width (Table 3), also in line with our findings that morphological factors are important in explaining alpha diversity in urban gut microbiomes. Altogether, our analyses provided insight into how urbanization is shaping gut bacterial communities of a songbird that persists in both urban and rural habitats.

Urban and Rural Songbirds Differ in Morphology, Physiology, and Territory Features

Our previous work in this system provided good evidence that we would find significant differences among urban and rural White-Crowned Sparrows in their morphology and physiology; however, we had never before compared together this number of different morphological, physiological, and environmental factors. Urban and rural territories were significantly different along nearly all of the dimensions of environmental variables we considered, including noise levels and different types of cover (scrub, tree, and impervious surfaces; Table 1). Only the relative level of grass cover was similar, on average, between urban and rural territories. We found many fewer significant differences among morphological features and condition measures, with only bill length slightly longer and plumage wear greater in more rural populations. These comparisons demonstrate the many dimensions along which these urban and rural locations differ from one another, all of which could contribute to differences in host gut microbial communities.

Urban and Rural Songbirds Differ in Bacterial Communities

Urban and rural male White-Crowned Sparrows share many of the same bacterial taxa, despite holding territories in different locations, consistent with findings from other studies comparing urban and rural songbirds [8]. However, we did find 23 families to be differentially abundant between urban and rural birds (Fig. 2). Because our data on bacterial taxa comes from 16S sequencing, we cannot make functional interpretations. However, urban birds did have a significantly higher abundance of Camplylobacteraceae, and some members of this family are pathogenic [53]. This finding is broadly consistent with that of other work in urban songbirds. For example, urban house sparrow gut microbial communities are enriched with microbes from the phylum *Proteobacteria*, which can cause intestinal diseases in mammals [8]. Future work is needed to examine potential functional differences, or differences that might impact host health, in the gut microbial communities of urban and rural songbirds.

Urban birds also have higher gut bacterial richness (^{0}D) than rural birds (Fig. S3). These findings are also similar to those of urban Eastern Water Dragons (*Intellagama lesueurii*), which have higher bacterial richness than eastern water dragons in native habitats [10]. However, the opposite is the case in other songbirds, including house sparrows (*Passer domesticus*) [11] and Darwin's finches (*Geospiza fuliginosa* and *Geospiza fortis*) [54]. Both of these songbirds have lower bacterial richness in more urbanized areas. It is particularly interesting that although both house sparrows and White-Crowned Sparrows persist and to a certain extent thrive in urban environments, they show different effects of urbanization on bacterial richness. These differences may be due to how selection pressures for each species vary across urban and rural habitats. Additionally, there is a wide range of landscape compositions among urban areas around the world with some comprised of more greenspace than others; therefore, it may not be useful to draw comparisons between urban areas with drastically different environmental features. More work is needed to assess why some hosts have higher bacterial richness in urban environments while others have lower richness. Our results on bacterial richness add to the growing number of studies investigating gut bacterial communities across urbanization gradients, which could contribute to future meta- or comparative analyses.

When we examined beta diversity (beta diversity), we found significant differences between urban and rural males (Fig. 5 and Table 2). These differences were driven by

community membership (i.e., presence/absence), not structure (i.e., accounting for relative abundance). Other studies that have examined beta diversity in urban versus rural locations have found similar results. For example, in both Darwin's finches and Eastern water dragons, there are more differences in beta diversity between urban and rural areas in community membership than structure [10, 54], similar to our findings. Finding differences in membership and not structure suggest that rare bacteria drive these differences. Thus, rare bacteria may be a key component of the gut bacterial community to examine in urban environments.

Differences between Urban and Rural Bacterial Communities Driven by Rare Bacteria

Our results show differences between urban and rural populations in alpha diversity and beta diversity decreased as rare species were down-weighted (i.e., higher orders of q). This means that rare bacteria drive the differences found in gut microbial communities between urban and rural White-Crowned Sparrows (Figs. 3 and 6). A similar pattern is seen in house sparrows, such that urban and rural birds are not different in alpha diversity as rare species are down-weighted [8]. Therefore, because measures of alpha and beta diversity that account for relative abundance show weakened differences between urban and rural populations, our results suggests that dominant bacteria are present in relatively similar proportions across birds in these populations. Why might rare species drive observed differences in gut microbial communities? Rare bacteria may be transient and sourced from the environment. If urban and rural habitats have different environmental bacteria, then this could explain why rare species are driving these apparent differences in gut bacterial communities. Urban areas have been shown to host different bacterial communities on different surface types, and thus differences in urban and rural surfaces may explain differing rare gut bacteria [55]. We do not know what role these rare and potentially transient bacteria play in host health and development; the differences we see may be neutral or could be the result of novel pathogen exposure in urban birds. Future research is needed to examine functional components of shared bacterial communities between urban and rural birds to determine what essential functions ubiquitous bacteria might serve, and if perhaps more rare bacteria that differ are occupying similar or different functional roles.

Drivers of Gut Diversity Reveal Potential Mechanisms of Urban Impacts

Our central question was whether any environmental or morphological characteristics of these wild songbirds might explain differences in their gut microbial communities between urban and rural habitats. A number of the factors we measured were important in explaining bacterial alpha diversity, specifically bill size (length and depth), territory noise level, percent tree cover, and percent grass cover (Fig. 4). Bacterial richness (^{0}D) increased with bill size and percent tree cover and decreased with territory noise levels and percent grass cover (Fig. S3). When we examined how these factors varied between urban and rural hosts, we found that rural birds tend to have larger bills, lower noise levels, and slightly higher amounts of grass whereas urban birds have territories with significantly more trees (Table 1). Taken together, the higher levels of alpha diversity in urban hosts seem most likely to be associated with differences in tree cover between urban and rural territories. Urban birds have more trees on their territories, and bacterial richness increases with tree cover in both urban and rural habitats (Fig. S3). Grass cover may also contribute to this pattern, as bacterial richness decreases with grass cover, and rural birds have slightly more grass cover, but this relationship is not as strong, even though grass cover is important, overall, in explaining variation in bacterial richness. Bill size and noise levels seem unlikely to explain divergence in bacterial richness between urban and rural hosts, as urban birds have higher richness but also higher noise levels on their territories as well as smaller bills. Originally, we predicted that it would be urbanized features of the landscape (such as distance from roads or noise levels) that would explain differences in bacterial richness between urban and rural habitats, but our results suggest the opposite. Instead, shifts in more "natural" features of the landscape seem to be most important. White-Crowned Sparrows are thriving in the big city, but their territories are becoming more and more restricted to urban park boundaries over time [34]. This means that the composition and management of urban parks is becoming ever more important to their persistence and the types of selective pressures these sparrows are experiencing.

When we examined urban and rural birds separately, however, we found that different factors are important for each type of habitat—morphology in urban hosts and environment in rural hosts. Alpha diversity in urban males was best predicted by morphological traits like bill length and depth (Fig. S3). Although Knutie et al. did not find an association between body mass or bill size and the gut microbiome of Darwin's Finches, they did find that body mass was impacted by human activity [54]. Urban birds may have a more diverse diet available to them as a result of human development as with human activity comes food litter, and the insects that follow. Which food sources a bird can exploit can be determined by bill morphology [56], and diet is likely a good predictor of gut microbiome. For example, in both lizards (*Liolaemus* sp.) and desert woodrats (*Neotoma lepida*), the gut microbiome has significant overlap with plants and insects comprising their diets [57, 58]. Altogether, this may explain the link between urban microbiomes and urban host morphology. Another possible explanation is that a more diverse diet affects the

microbiome as we see here, which then affects developmental growth, leading to changes in bill morphology. While our study does not provide data on how specific bacteria affect bird development, this would be a ripe direction for future research. In contrast, alpha diversity was best predicted by environmental traits like percent of territory covered by grass or trees for rural males (Fig. S3). A number of other studies in non-urbanized habitats—including in fish, birds, and salamanders—have also found evidence for environmental factors explaining alpha diversity in gut microbial communities. Experimental work in fish, such as carp (*Hypophthalamichthys* sp.), has demonstrated that the gut microbiome is often sourced from the environment [59]. Nest environment has been shown to be more important for cloacal bacterial community assemblage than genetic relationships in great tits (*Parus major*) and in blue tits (*Parus caeruleus*), providing evidence that a bird's environment plays a large role in shaping their bacterial communities. A habitat signature was also found in fire salamanders (*Salamandra salamandra*), such that a change in environment induced a change in gut bacteria (Bletz 2016). Availability of certain food sources may be accurately reflected in landscape cover measures in rural areas, where the ground cover types occur naturally. However, in urban areas much of the landscape is artificially comprised, which may reduce the association between the gut microbiome and the host's environment. Thus, if landscape cover more closely maps onto diet for rural birds than urban ones, this could explain why landscape predicts the gut microbiome only for rural birds. Overall, our sampling of multiple sites within both urban and rural habitats allowed us to further elucidate the potential associations between landscape, host morphology, and gut microbial alpha diversity. However, our findings also highlight the need to further investigate the link between urbanization, landscape cover, and diet.

Gut bacterial community beta diversity was significantly correlated with a morphological feature (bill width) and a physiological one (body condition), although these features only explained a small portion of the variation in beta diversity between urban and rural hosts (Table 3). The other few studies of songbirds have not found any association between host morphology and beta diversity. For example, barn swallow (*Hirundo rustica*) morphology was not correlated with beta diversity [60] and in Darwin's finches (*Geospiza fuliginosa* and *fortis*), bacterial beta diversity was explained by host species but not bill morphology or body mass [54]. These results tell us that bacterial beta diversity between songbirds in general may not be sensitive to differences in host morphology, although our results offer an intriguing suggestion that such associations may be detectable with enough sampling. Within urban birds alone, measures of condition (body condition and plumage fade) also explained significant albeit small amounts of variation in beta diversity across urban individuals. One reason plumage fade might be important is that urban birds have higher tree coverage (e.g., lower levels of sunlight) on their territories which might explain why urban birds have lower plumage fade. A number of experimental studies suggest condition and gut microbial communities should be associated, and our study of a free-living bird finds some evidence of this predicted association.

Conclusions

Together, our results present a detailed picture of the potential drivers of avian gut biodiversity in urbanized landscapes. Our approach of sampling multiple transects in urban and rural locations across an environmental gradient allowed us to tease apart the relative contribution of environmental factors and morphological traits in explain alpha and beta diversity of the gut microbiome. Although a growing number of studies are beginning to examine urban wildlife gut microbiomes, few have examined as many potential contributing variables limiting our understanding of what is driving gut microbial variation across urbanized landscapes. Perhaps most notable is our work found that different factors are important in urban versus rural landscapes, suggesting that the selective forces shaping avian gut microbiomes are different in cities than in the rural landscapes in which these species evolved. More studies similar to this are needed to understand the degree to which these patterns are consistent or not across species as well as experimental work to begin to test causal relationships.

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