Inside the guts of the city : urban-induced alterations of the gut microbiota in a wild passerine

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ABSTRACT

Urbanisation represents one of the most radical forms of terrestrial land use change and has been shown to lead to alterations in ecosystem functioning and community dynamics and changes in individual phenotypic traits. While the recent surge in microbiome studies has brought about a paradigm shift by which individuals cannot truly be considered independently of the bacterial communities they host, the role of gut microbiota in organismal response to human-induced environmental change is still scarcely studied. Here, we applied a metabarcoding approach to examine the impact of urbanisation on the gut microbiota of *Passer domesticus*. We found urbanisation to be associated to lower microbiota species diversity, modifications in taxonomic composition and community structure, and changes in functional composition. The strength of these relationships, however, depended on the spatial scale and season at which they were considered. Such spatio-temporal effect suggests that urbanisation may dampen the natural seasonal variation of the gut microbiota observed in more pristine habitats, potentially influencing the fitness of urban organisms. Our results hence shed light on a hitherto little considered perspective, i.e. that the negative effects of urbanisation on city-dwelling organisms may extend to their microbiomes, causing potential dysbioses.

Key words: microbiome, urbanization, house sparrows, global change, anthropogenic perturbations, 16S metabarcoding

1. INTRODUCTION

Human activity is widely recognized as a dominant cause of many contemporary environmental changes (Lewis and Maslin, 2015), and urbanisation represents one of the most radical forms of land use alterations in terrestrial ecosystems (Alberti, 2015) with a predicted threefold increase in urban land use by 2030 (Seto et al., 2012). Urbanisation induces a number of substantial environmental modifications, such as loss and fragmentation of natural habitats (Er et al., 2005), increased atmospheric, soil and water pollution (Grimm et al., 2008), increased temperature (Rizwan et al., 2008) and changes in food resources (Anderies et al., 2007). Such changes consequently lead to alterations in ecosystem functioning and community dynamics, including predator-prey (Rodewald et al., 2011) or host-parasites interactions (Calegaro-Marques and Amato, 2014).

While urbanisation effects on species distribution patterns and community structuring are increasingly well documented, how individual organisms may successfully cope with - or adapt to - urban environments, remains less well understood (see e.g. Shochat, 2004). Moreover, the recent surge in microbiome studies has brought about a paradigm shift by which individuals cannot truly be considered independently of the bacterial communities they host (McFall-Ngai et al., 2013; Zilber-Rosenberg and Rosenberg, 2008). Indeed, these host-associated bacteria sustain multiple essential functions for the hosts. Gut microbiota, for instance, are involved in host digestion or nutrient synthesis (Cummings and Macfarlane, 1997), protection against pathogens (Fukuda et al., 2011) and more widely shape host immunity (Round and Mazmanian, 2009), life history traits (Sison-Mangus et al., 2015) and even behaviour (Ezenwa et al., 2012), underlining its vital importance for host fitness. The characteristics of the gut microbiota are first and foremost shaped by the host, which constitutes its proximate environment (Bevins and Salzman, 2011). Gut microbiota diversity

and composition are thus related to traits such as host genotype (Spor et al., 2011), sex (Martin et al., 2010), or immune status (Hansen et al., 2010). It is also influenced by factors linked to the host's environment with variations according to, for example, salinity (Sullam et al., 2012), habitat type (Bletz et al., 2016) or geographical location (Linnenbrink et al., 2013). Major changes in the environment, including anthropogenic perturbations, may alter host-associated microbiomes and potentially result in abnormal interactions between the host and the microbiota (dysbioses sensu Logan et al., 2016) and in adverse effects on hosts.

Although empirical evidence for the role of gut microbiota in organismal response to environmental change is still scarce, the microbiome of various taxa has been shown to be affected by several major types of anthropogenic perturbations. For instance, in honey bees, exposure to commonly-used pesticides was shown to cause compositional and functional changes in their gut microbiota (Kakumanu et al., 2016) while water contamination by heavy metals reduced both the diversity and abundance of the skin microbiota of frogs (Costa et al., 2016). Along the same lines, climate change was shown to considerably reduce gut microbiota diversity and alter composition and structure in lizards (Bestion et al., 2017), while habitat fragmentation proved to result in lower diversity, changes in taxonomic and functional composition in the gut microbiota of an African primate species (Barelli et al., 2015). Because the process of urbanisation integrates several of such environmental perturbations, it can be predicted to cause alterations in the microbiome of urban-dwelling species as well. However, despite the potential scale and magnitude of its impacts on organisms, no study has ever, to our knowledge, explicitly investigated the effect of urbanisation on host-associated microbiomes. A few studies have shown less diverse gut microbiota in urbanised human populations compared to hunter-gatherer or rural populations (Park et al., 2015; Yatsunenko et al., 2012), however these do not test the effects of urbanisation *per se*, but rather the effects of a western life-style (diet, antibiotics, sanitisation, etc.).

89 Here we examined the impact of urbanisation on the gut microbiota of a passerine bird, the
90 house sparrows *(Passer domesticus).* This species constitutes a good model to study the
91 impact of urbanisation because it is found all along the urban gradient, from rural areas to
92 city-centres (Laet and Summers-Smith, 2007). Furthermore, the ongoing decline experienced
93 by many urban sparrow populations (e.g. SE Asia: Ghosh et al., 2010; NW Europe: Shaw et
94 al., 2008) suggests possible adverse (or suboptimal) environmental conditions in urban
95 habitats. We performed a large scale study by sampling the gut microbiota in 36 Belgian
96 sparrow populations characterized by varying degrees of urbanisation, i.e. ranging from rural
97 habitats to city centres. Using an Illumina metabarcoding approach which amplifies the 16S
98 bacterial ribosomal RNA gene, we measured gut microbiota diversity, composition and
99 functional diversity. For each sampled population, we measured the degree of urbanisation of
100 the habitat at two ecologically relevant scales: a local scale and a landscape scale. Accounting
101 for these two spatial scales allowed us to address the question of whether local and landscape
102 urbanisation levels affect the gut microbiota in the same way, and to what extent both spatial
103 scales interact (Concepción et al., 2015; Lindström and Langenheder, 2012). We were
104 particularly interested in discrepancies between local and landscape urbanisation levels as
105 house sparrow may experience different conditions in contrasted vs. homogeneous
106 landscapes.

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2. METHODS

2.1. Sampling sites

House sparrows were trapped in locations that varied in the level of urbanisation at two spatial scales (see further), within a 4655 km^2 polygon demarcated by the cities of Gent, Antwerp, Brussels and Leuven (Flanders, Belgium). First we identified 18 plots (each measuring 3 by 3 km) located within the polygon boundary and varying in extent of urbanisation as calculated with ArcGIS v9.2. These 18 plots were a subset of a larger set of 27 plots chosen for another project focussing on a wide array of taxa, including the house sparrows (Kaiser et al., 2016). As a base layer we used the vectorial Large-scale Reference Database, GRB (an object-oriented reference map of Flanders https://www.agiv.be/international/en/products/grb-en). Cut-off points for the percentage of built-up areas were set at 0-5% for "rural" plots (lowest level of urbanisation), 5-10% for "suburban" plots (intermediate level), and >10% for "urban" plots (highest level). To ensure a more natural environment for the lowest urbanisation class, we only selected plots comprising >20% of ecologically valuable areas, as described by the vectorial 'Biologische Waarderingskaart' (Vriens et al., 2011). Next, we used all available population information (i.e. own inventories conducted during winter 2012-13 complemented with available data from various citizen science projects) to select 36 sparrow populations within these 18 plots (6 in rural plots, 6 in suburban plots and 6 in urban plots) and that were characterized by contrasting levels of urbanisation at a local scale. For each of these populations, we calculated the level of urbanisation at a "local" scale (400 m radius around the center of the main capture site) and a "landscape" scale (1600 m radius around the centre of the main capture site, thereby excluding the 400 m radius of the local scale). The local scale corresponds to the maximum daily mobility range (Vangestel et al., 2010) and the landscape scale (1600m radius) corresponds to the point whereby the effect of genetic drift

exceeds that of gene flow and house sparrow populations can be considered independent from each other (Vangestel et al., 2012). Each sparrow population was sampled twice (Sep-Dec 2013 and Jan-Mar 2014) with a total of 405 trapped individuals that were individually marked, sexed, measured and had their microbiota sampled (figure 1, table S1). The age of birds (juveniles vs. adults) was not determined as the sampling was carried out after house sparrow summer moult, a period at which juveniles cannot be distinguished from adults.

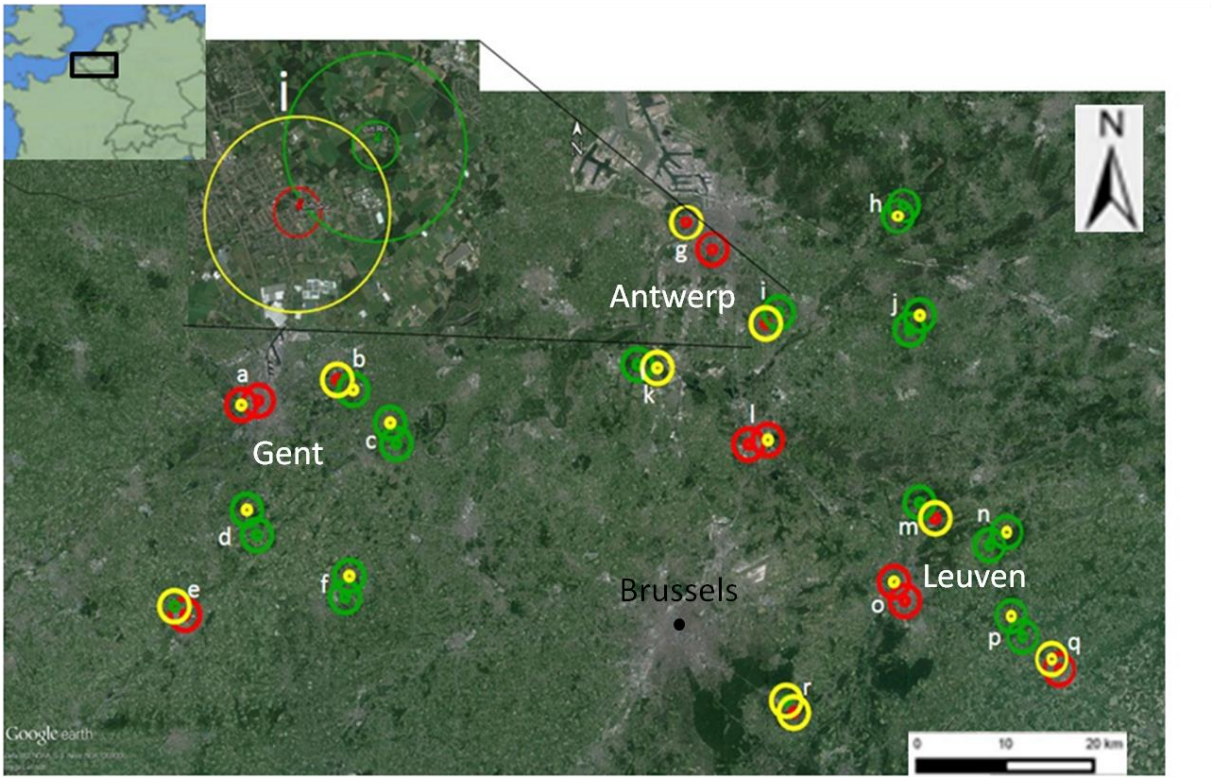

Figure 1: Map indicating locations where house sparrow populations were sampled with inner circle indicating the local (400m radius) urbanisation level (green: low, yellow: intermediate, red: high) and outer circle indicating the landscape urbanisation level (1600 m radius)
Region of Gent (a = Ghent; b = Beervelde; c =Kalken; d = Melsen; e = Oudenaarde; f = Hillegem, Region of Antwerp (g = Antwerp; h = Pulderbos; i = Lint; j = Herenthout; k = Ruisbroek; l = Mechelen) Region of Leuven (m = Wezemaal; n = Houwaart; o = Leuven; p = Kerkom; q = Tienen; r = Overijse).

2.2. Microbiota sampling

To sample the gut microbiota, we flushed the cloaca with 200µL of a sterile saline solution (Phosphate buffer saline). We gently inserted a sterile tip into the cloaca injecting the solution and drawing it out again with a pipette. Samples were immediately put in sterile vials and stored at -20°C. Before sampling, the exterior of the cloaca was cleaned with alcohol to avoid contamination from bacteria outside the cloaca. At each capture site, we also collected control samples to control for possible contamination of the saline solution during sampling and preparation. While each part of the digestive tract harbours specific bacterial communities, there is evidence, in birds, that microbial shifts incurred in the higher intestine lead to concurrent shifts in cloacal communities (e.g. Newbold et al., 2017). Cloacal sampling thus comprises a reliable non-invasive technique to study inter-individual variability in gut communities, which has been successfully used in a number of other studies (see e.g. Bestion et al., 2017; White et al., 2010)

2.3. PCR amplification and high-throughput sequencing

Bacterial DNA was extracted using the Qiagen DNeasy® Blood & Tissue Kit and the standard protocol designed for purification of total DNA from Gram-positive bacteria (Qiagen, Venlo, Netherlands). The V5-V6 region of the bacteria 16S rRNA gene was amplified by PCR using the following universal primers: BACTB-F: 5'-GGATTAGATACCCTGGTAGT-3' and BACTB-R: 5'-CACGACACGAGCTGACG-3' (Fliegerova et al., 2014). To discriminate samples after sequencing, both forward and reverse primers were labelled at the 5' end with a combination of two different 8 bp tags. The PCR amplification was performed in a 25µL mixture containing 3µL of 1/10 diluted DNA extract, 0.4µM of each primers, 1U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 1X of Taq Buffer, 0.24µL of bovine saline albumin (Promega Corporation,

Madison, USA), 0.2mM of each dNTP, 2.5mM $MgCl_2$ and 12.06µL water and following this programme: initial denaturation at 95°C for 10min, 35 cycles of denaturation at 95°C for 30s, hybridation at 57°C for 30s and elongation at 72°C for 30s. All this lab work was done under sterile condition under laminar flux, all materials cleaned with ethanol and sterilized by UV light for 30min. In addition to biological samples, we also used negative and positive controls to check for the PCR effectiveness. PCR products were tested on electrophoresis gel and then 4µL of amplicons per sample were pooled. The library construction (kit Illumina Biooscientific PCR free) and the sequencing (Illumina MiSeq 250 bp paired-end v3 chemistry) were performed at the Genopole of Toulouse (France).

2.4. Bioinformatic analyses

Illumina sequencing data were processed and filtered using the OBITools package (Boyer et al., 2016). First, we aligned paired-end reads in consensus sequences by taking into account the reads overlapping quality and kept consensus reads with overlapping quality higher than 50. Second, we assigned reads to their respective sample by allowing zero error in tags and a maximum of two errors on primers. We further excluded reads containing ambiguous bases (other than A, T, G, C) and reads shorter than 100 bp as they are most likely sequencing errors (Bokulich et al., 2013). Remaining reads were then dereplicated and reads that occur only once in the entire dataset (singleton) were removed. Reads were then clustered into OTUs (Operational Taxonomic Units) using SWARM algorithm with a similarity threshold of 97% of similarity (Mahé et al., 2014). The most abundant sequence of each cluster was considered as the main sequence of this cluster and the representative sequence for the OTU. The taxonomic assignation was then performed on FROGS (Finding Rapidly Otu with Galaxy Solution), a Galaxy pipeline (Escudie et al. 2015). The taxonomic affiliation was done by BLAST using the SILVA 16S gene data bank (Camacho et al., 2009).

From this dataset, we then applied different filters. We first identified contaminant OTUs, bacteria that did not come from biological sample but from extraction or PCR reagents, technical contaminant during lab work by removing OTUs with the maximum and the highest mean abundances in negative controls. Finally, we removed OTUs with a total abundance lower than 0.005% of the dataset total abundance (Bokulich et al., 2013). From the gut samples we identified a total of 298 bacterial OTUs with an average of 78.9±0.9 OTUs per individuals. These OTUs were assigned to 8 phyla, 12 classes and 50 families. The Firmicutes was the predominant phylum with a mean relative abundance per individuals of 78±1.3%, followed by Proteobacteria 17±1.2% and Actinobacteria 2±0.3%. The other phyla were present in very low abundance (< 0.05%). 2% of OTUs were not taxonomically assigned (rarefaction curves, figure S1).

Functional characteristics of the bacterial communities were analysed using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, (Langille et al., 2013). We first performed closed-reference 97% OTU picking against the Greengenes database (v 13.5), then used the online Galaxy platform (http://huttenhower.sph.harvard.edu/galaxy/) to perform copy number normalisation of each OTU, metagenome prediction of each sample and functional predictions categorized into Kyoto Encyclopedia of Genes And Genomes (KEGG) pathways representing gene counts of each predicted metagenome (Kanehisa and Goto, 2000). Importantly, metagenome predictions depend on whether the bacterial taxa present within the samples are represented in the genome database. We then used the NSTI (Nearest Sequenced Taxon Index) that measures this relationship, with lower values illustrating a closer mean relationship. The average NSTI value for the gut microbiota community was 0.44±0.0007, which indicates a good coverage (Langille et al., 2013). This generated a table with the KEGG pathway abundances for each sample. The KEGG 1 table refers to 6 main functions identified in the

dataset (Table S2). The KEGG 2 pathway refers to specific functions within the 6 main functions identified in the KEGG 1 pathway. The KEGG2 functions from the KEGG1 metabolism main function were summarized in 3 principal components using a principal component analysis (PCA, Table S3).

2.5. Statistical analyses

To study the relationships between gut microbiota characteristics, urbanisation level and host body condition, we first normalized gut microbiota data by the total abundance within each sample. The gut microbiota α-diversity was characterized using two metrics: OTU Richness, which refers to the number of different OTUs present in each sample and the Shannon diversity index (H'), which also takes into account the relative abundance of each OTU within samples. OTU Richness was log-transformed to fit normal distribution and these two diversity indexes were tested with generalized linear mixed effect models (see below). In addition, to control for the differences in absolute abundance between samples (number of reads), we defined a metagenomics coverage value for each sample (Rodriguez and Konstantinidis, 2014). Coverage was calculated as: coverage= 1- (number of singleton/ total number of reads). In this dataset, the average coverage is 0.994±0.004 and the coverage was not correlated with environmental and host factors. Host body condition was calculated using the scaled mass index (SMI) (Peig and Green, 2009), which adjusts the mass of all individuals to that which they would have obtained if they had the same body size, using the equation of the linear regression of log-body mass on log-tarsus length estimated by type-2 (standardized major axis; SMA) regression (Peig and Green, 2009). Two outliers were present in the data (i.e. |standardized residuals| > 3); these two observations were not considered for deriving the SMI relationship. The regression slope was 1.49 and average tarsus length was 18.8 mm. We thus calculated the SMI as body mass x $(18.8/\text{tarsus length})^{1.49}$ (Peig and Green, 2009).

As the local scale is expected to be highly relevant for individuals, metrics of the gut microbiota community structure (OTU Richness, Shannon Index, and relative abundance of the main phyla) were first examined at the local scale only. These first models contained as fixed effects: host body condition, sparrow sex, season, the metagenomic coverage value, and finally urbanisation at the local scale and its interaction with season. The metagenomic coverage variable was always included in the models as a fixed effect. As each location was sampled twice (in autumn and in winter), capture site identity was modelled as a random effect. To avoid pseudo replication, the 21 individuals that were trapped in both autumn and winter (in the same location) were removed from the analyses. A minimal model containing only significant variables was selected through backward elimination of the non-significant variables (R package nlme, Pinheiro et al., 2016). After investigating local scale effects only, we then tested whether these patterns were influenced by landscape-scale processed by adding the urbanisation level at the landscape scale, the interaction between urbanisation at local and landscape scales, and the interaction between season and urbanisation at landscape scale to the local-scale minimal model.

Microbiota β-diversity was studied using Bray-Curtis dissimilarity index based on presence-absence community matrices. As dissimilarity analyses typically are non-parametric, we used three different statistical approaches to test for urbanisation gradients in house sparrow microbiota composition (Legendre & Legendre 1998). Microbiota β-diversity was visualized with a non-metric multidimensional scaling (NMDS) and Principal Coordinates Analyses (PCoA) by plotting samples based on their pairwise dissimilarity in a low-dimensional space. Urbanisation ratios at local and landscape scale were then plotted on these ordinations and their correlation with the dissimilarity scores tested with the "envfit" function. We also analysed the variance partitioning due to environmental factors on dissimilarity matrices using Permutational Multivariate Analysis of Variance (ADONIS). ADONIS were done with 1000

permutations and using the "margin" option in order to test for the effect of each variables whilst accounting for the effect of the other variables of the model. Finally, we used Mantel tests to investigate correlations between microbiota dissimilarity matrices (Bray Curtis) and environmental dissimilarity matrices (Euclidean distance). The β-diversity analyses were performed with R using the VEGAN package (Oksanen et al., 2014). All statistical analyses were carried out using R except for KEGG 1 functional profiles which were analyzed using the STAMP software (Parks et al., 2014) with a Bonferroni correction.

3. RESULTS

3.1. Gut microbiota α-diversity

Gut microbiota OTU richness was significanly affected by urbanisation level at the local scale, with sparrows from more urbanised locations hosting fewer bacterial species (GLMM: local_urb: $t_{1,36}$=-3.40 , *p=0.002*; figure2A). We found no significant effect of landscape urbanisation on OTU richness within the gut (GLMM, landscape_urb: $t_{1,36}$ =0.80, *p=0.43*).

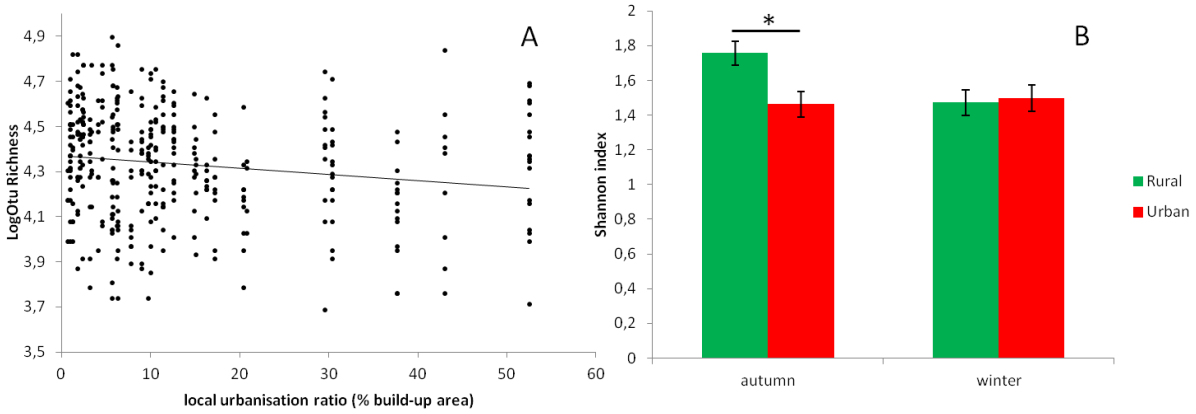

Figure 2: Effect of local urbanisation levels on bacterial species richness (Log of OTU Richness) (A) and on bacterial species diversity (Shannon diversity index) according to two sampling seasons (B). Error bars in figure (B) refer to the standard error and the asterisk to a P-value<0.05.

When considering the Shannon diversity index, we found an effect of local urbanisation in interaction with the season (GLMM, season*local_urb: $t_{1,325}$=2.25, *p=0.025*, fig2B). More specifically, gut diversity decreased with urbanisation in autumn (GLMM, local_urb: $t_{1.35}$=-3.18, *p=0.003*) but not in winter (GLMM, local_urb: $t_{1.34}$=-0.73, *p=0.47*). In addition, we found that the gut microbiota diversity of rural birds decreased in winter (GLMM, season: $t_{1.169}$=2.5, *p=0.013*) whereas such a seasonal difference was not observed for urban birds (GLMM, season: $t_{1.155}$=-0.18, *p=0.85*, figure 2B). Interestingly, we found that local

urbanisation does not impact microbiota diversity in the same way according to the landscape urbanisation levels (GLMM, local*landscape: $t_{1,36}$=2.1, *p=0.04*,figure 3). We observed the lowest microbiota diversity within birds living in highly urbanised local habitat surrounding by a rural landscape whereas the microbiota diversity was higher in homogeneous environments (very urbanised or rural environment). A focus on situations with discrepancies between local and landscape urbanisation levels showed similar results, with significantly lower diversity in birds living in very urbanised patches within rural landscapes than birds from 'green patches' within urbanised landscapes (see Urbanisation Contrast Index in SI and Fig S2).

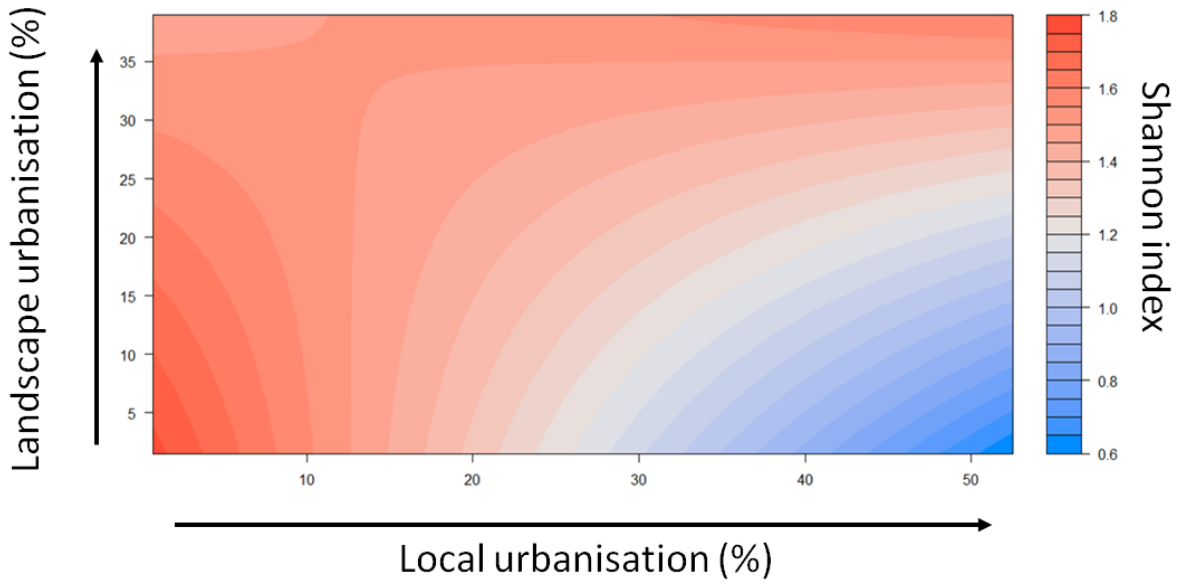

Figure 3: Interacting effect of urbanisation levels (% built-up area) measured at local and landscape scales on the gut microbiota species diversity (Shannon diversity index)

3.2. Gut microbiota β-diversity (community dissimilarity)

We found a strong effect of the season on gut community dissimilarity. Season accounted for 7% of the Bray-Curtis distance variance between birds (Adonis permutation test F=27.9, *p=0.001*) and also strongly correlated with the community dissimilarity matrix (Mantel

318 R=0.25, *p=0.001*). We further performed an environmental fitting test and found that season
319 was significantly correlated with NMDS structure (envfit: R^2=0.19, *p=0.001*).

320 When accounting for season, we found that both local and landscape urbanisation
321 significantly explained gut dissimilarity between birds in autumn (Table 1A, Figure 4A) and
322 winter (Table 1B, Figure 4B). Overall, we found a relatively stronger influence of local
323 urbanisation on community dissimilarity in autumn whereas landscape urbanisation was more
324 influent in winter (table 1).

325 *Table 1. Variance of community distance explained by local and landscape urbanisation*
326 *levels in autumn (A) and winter (B), using three independent statistical tests (bold characters*
327 *indicate the strongest effect).*

328 (A)

Community dissimilarity test	Local urbanisation levels		Landscape urbanisation levels	
ADONIS	**SSq=0.34**	***p=0.001***	SSq=0.23	*p=0.006*
EnvFit	**R^2=0.06**	***p=0.002***	R^2=0.03	*p=0.001*
Mantel	**r=0.18**	***p=0.002***	r=0.09	*p=0.002*

329 (B)

Community dissimilarity test	Local urbanisation levels		Landscape urbanisation levels	
ADONIS	SSq=0,21	*p=0,05*	**SSq=0,33**	***p=0,004***
EnvFit	R^2=0,06	*p=0,009*	**R^2=0,09**	***p=0,001***
Mantel	r=0,15	*p=0,001*	r=0,16	*p=0,001*

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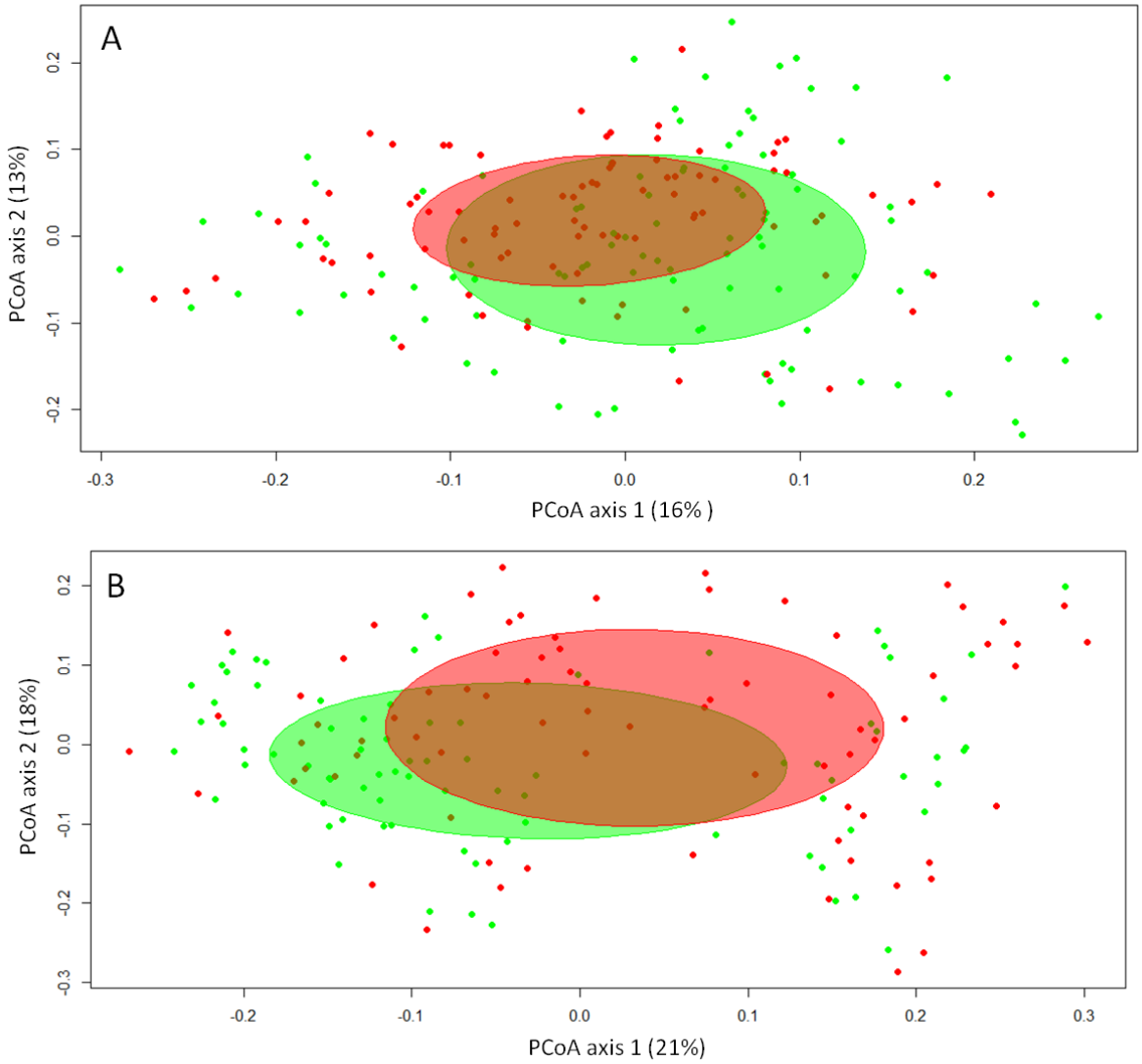

Figure 4: Ordination of the gut bacterial communities according to the first two PCoA axes in autumn (A) and Winter (B). Samples are coloured according to local urbaniaation categories (green: low levels of urbanisation and red: high levels of urbanisation). Ellipses represent standard deviations around the centroids of the two groups. Numbers in parentheses refer to the variance explained by the PCoA axes.

3.3. Gut microbiota taxonomic composition

We found no significant effect of the local or landscape urbanisation on Firmicutes (GLMM, local_urb: $t_{1,38}$=-0.3, *p=0.76*; landscape_urb: $t_{1,38}$=-0.17, *p=0.86*) or Proteobacteria (GLMM,

local_urb: $t_{1,38}$=-0.17, *p=0.86*; landscape_urb: $t_{1,38}$=-0.03, *p=0.97*) relative abundance. Actinobacteria abundance differed between local-urbanisation levels according to the season (GLMM, season*local_urb: Actinobacteria: $t_{1,325}$=2.28, *p=0.02*, figure 5) whereby urban birds had significantly fewer Actinobacteria in autumn but not in winter. When taking into account the landscape scale, we found a significant interaction between local and landscape urbanisation (GLMM, local-urb*landscape_urb, $t_{1,36}$=2.64, *p=0.03*). More specifically, the low abundance of Actinobacteria found in local urban patches was even lower when these were embedded in less urbanised landscapes but not in highly urbanised landscapes.

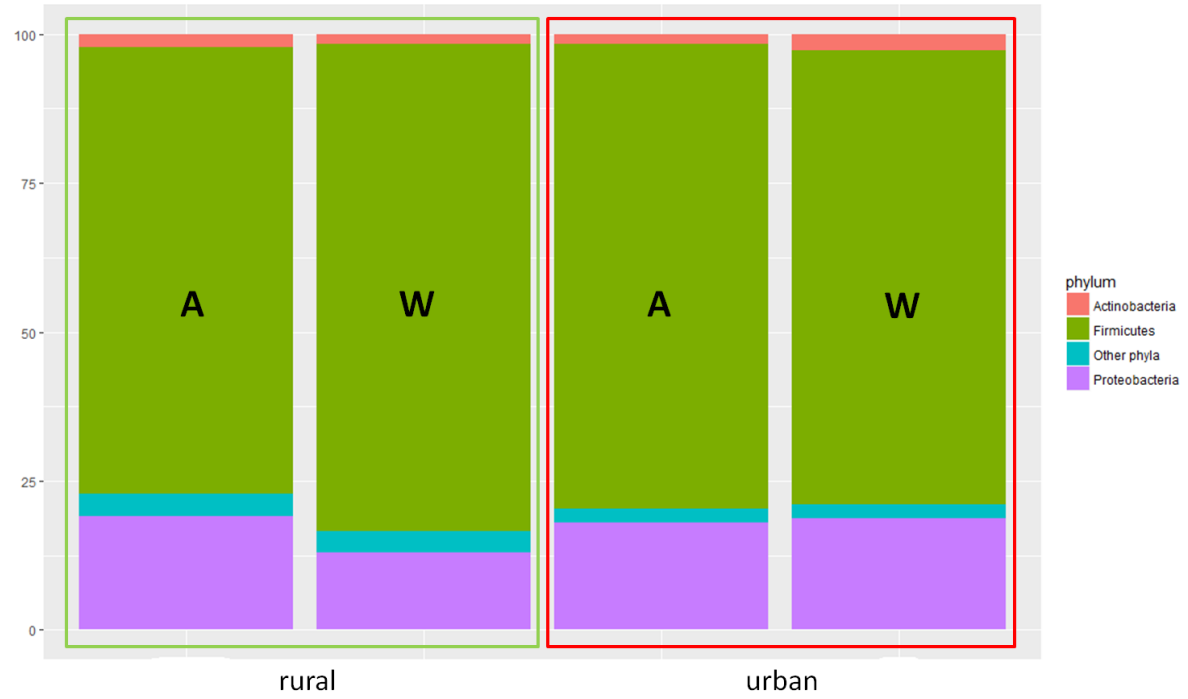

Figure 5: Relative abundance of main bacterial phyla in rural and urban local patches according to season (A: autumn, W: winter)

3.4. Gut microbiota functional composition

As for the other metrics of microbiota structure, we found a strong effect of season on the functional profiles of the gut microbiota (GLMM: season: $t_{1,327}$=-2.58, *p=0.01*), with

functional differences according to local and landscape urbanisation levels in autumn but not in winter. In autumn, functional features related to metabolism were relative less abundant in urban environments than in rural environments, both a local and landscape scales, (ANOVA: local: diff.mean=0.697, *p=0.025*, figure 6A; landscape: diff.mean=0.502, *p=0.015*, figure 6B).

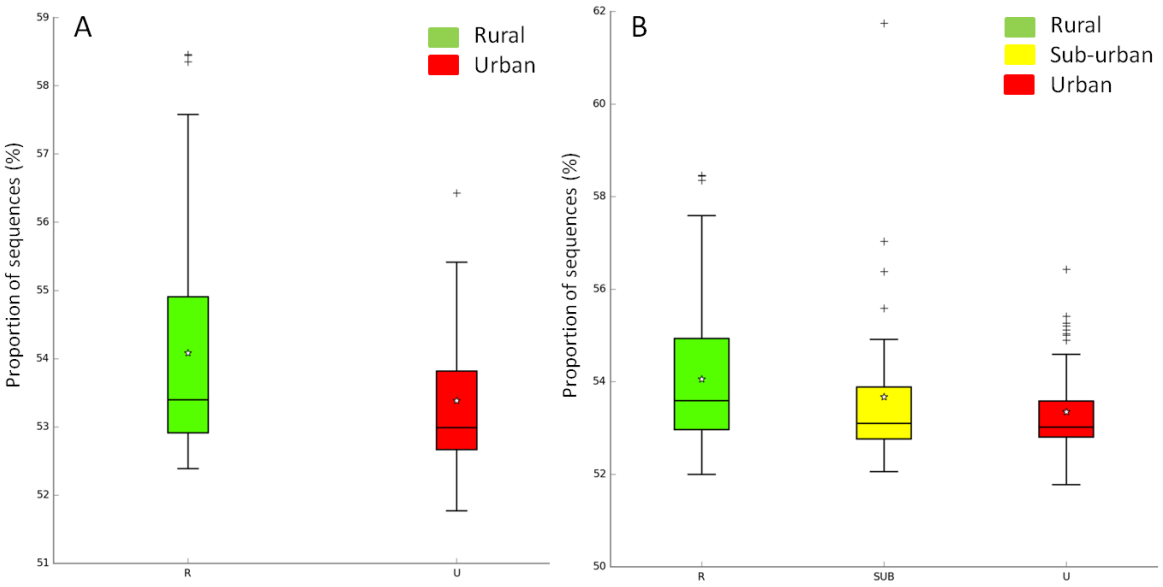

Figure 6: Relative abundance of genes (proportion of sequences) involved in metabolic functions (KEGG1 Metabolism) according to the urbanisation level of the local scale (A) and the landscape scale (B) in autumn. Boxes represent the first and third quartile, lines the median, stars the mean, and whiskers the highest and the lowest values within the 1.5 interquartile range of the third and first quartile.

We then examined the 12 specific metabolic functions (KEGG2 pathways) found within the Metabolism (KEGG1) pathway by extracting the first three principal components from a principal component analysis (see table S4 for details). We found no significant correlation between PC1 or PC2 and any environmental parameters. We did however find that PC3, mostly driven by nucleotide metabolism (negatively), amino acid metabolism and xenobiotics biodegradation and metabolism (positively), were significantly correlated to urbanisation

levels at both landscape (GLMM: $t_{1,38}$=-9.61, *p=0.05*) and local scale (GLMM: $t_{1,38}$=2.28, *p=0.03*). In other words, functions related to xenobiotics biodegradation for instance were more abundant in the gut microbiota of urban birds than that of rural birds.

3.5. Microbiota structure and host characteristics

We found no significant correlation between OTU richness or Shannon index and host sex (GLMM: OtuRichness: $t_{1,326}$=-0.08, *p=0.93*; Shannon: $t_{1,326}$=-1.58, *p=0.12*) or host body condition (GLMM: OtuRichness: $t_{1,299}$=0.72, *p=0.47*; Shannon: $t_{1,299}$=-050, *p=0.61*).

We further examined microbiota β-diversity and found that host body condition (envfit: R^2=0.007, *p=0.33*, Adonis: F=1.37, *p=0.09*, Mantel: R=-0.05, *p=0.98*) did not significantly explain the variance in gut community dissimilarity.

Regarding taxonomic composition, we found that the relative abundance of Firmicutes was significantly related with host body condition with sparrows harbouring more Firmicutes being in better body condition (GLMM: $t_{1,298}$=2.62, *p=0.01*) whereas the relative abundance of the two other main phyla was not associated to host condition (GLMM: Actinobacteria: $t_{1,298}$=-0.17, *p=0.86*; Proteobacteria: $t_{1,298}$=-1.6, *p=0.1*).

Lastly, we considered the functional features of the gut microbiota and found no effect of sex (GLMM: PC1: $t_{1.298}$=0.36, *p=0.7*; PC2: $t_{1.298}$=-0.49, *p=0.6*; PC3: $t_{1.298}$=-0.01, *p=0.9*) but a significant negative correlation of PC3 with host body condition (GLMM: $t_{1,298}$=-2.09, *p=0.03*).

4. DISCUSSION

By sampling sparrow gut microbiota in populations from contrasting environments, we show that urbanisation is associated to lower microbiota species diversity, to modifications in taxonomic composition and community structure as well as to changes in functional composition. These results are in line with the growing body of evidence that perturbations associated with urbanisation, such as increased water, soil and atmospheric pollution, changes in ambient temperature and habitat fragmentation, may alter host-associated microbiota in natural populations (Barelli et al., 2015; Bestion et al., 2017; Costa et al., 2016). We additionally show that urbanisation effects may be dependent on the season and spatial scale at which they are considered.

4.1. Role of environmental and host changes in microbial shifts

Our results show a general pattern linking urbanisation with gut microbiota structure in sparrows, and thus ask the question of the mechanisms by which this pattern arises. Is the microbiota modified directly via environmental changes including dietary shifts or indirectly via changes incurred by the hosts? The environmental changes described above modify the bacterial communities present in the soil, water and vegetation (Bartlewicz et al., 2016; Medeiros et al., 2016; Wang et al., 2017) surrounding the host, and therefore the pool of bacterial species likely to colonize the gut. The importance of environmental bacteria in shaping the gut microbiota is supported by evidence showing similarities between environmental and gut bacterial assemblages (Sullam et al., 2012). The environmental influence on the gut community is further shown by local microbial signatures exhibited by hosts from the same locality (Bletz et al., 2016; Sullam et al., 2012) which has also been shown in birds (e.g. Hird et al., 2014). Although environmental bacteria may be acquired by

direct contact with the surrounding substrate, diet comprises one of the major forces shaping gut communities as it is the main interface between the gut and the host's environment (Wu et al., 2011). Urban environments provide very different trophic resources compared to natural or rural habitats. As a consequence, dietary shifts in food type and abundance have been observed in a number of bird species along urbanisation gradients (Auman et al., 2008; Kristan et al., 2004). In house sparrows particularly, the diet of urban birds is mainly composed of human refuse (bread, peanuts, etc.), commercial birdseeds (sunflower seeds, millet, milo) and a lower diversity in arthropod prey whereas the rural diet generally contains more cereal grains (corn, wheat, oat), plant fragments and higher diversity of invertebrate prey (e.g. Gavett and Wakeley, 1986). Given these dietary shifts, it is therefore likely that differences in gut microbiota we observe along the urban gradient may be largely mediated by diet. Whether it is through diet or other means, external environmental perturbations may thus directly induce alterations in the gut microbiota. However, such perturbations may also impact the microbiota indirectly via changes incurred by the hosts themselves. Urbanisation levels has been shown to impact several individual characteristics such as morphological traits (Liker et al., 2008), physiological traits (Chávez-Zichinelli et al., 2010) and immunity (Bailly et al., 2016). Because such host factors have been shown to contribute to shaping the gut communities, the changes in microbiota we observed could thus be explained by various host responses to urban environments. Our analyses revealed no consistent between sparrow characteristics such as sex, body condition and gut microbiota structure. This could indicate that the host traits we have considered have little influence on gut bacterial communities in sparrows and that physiological or immune measures may comprise more relevant parameters given their importance in modulating the microbiota (Hansen et al., 2010). Alternatively, the lack of significant relation in our study may indicate that environmental factors are relatively more important than host-associated factors in the context of urbanisation. The fact that

445 urbanisation, and not the examined host factors, explained most of the microbiota variation in
446 our models highlight the dominant role of environmental drivers in the context of
447 anthropogenic perturbations. Further studies, including common garden experiments or
448 manipulation of host traits, are needed to fully understand the relative role of the external
449 environment and the host in mediating the gut microbiota. Beyond these considerations,
450 because the gut bacterial communities are shaped by both environmental and host condition
451 indices, it may be more sensitive to environmental modifications than hosts themselves. The
452 gut microbiota may thus comprise a reliable snapshot of present and past environmental
453 conditions experienced by the host and constitute a relevant bioindicator of anthropogenic
454 environmental perturbations.

455 *4.2. Seasonal effect*

456 Another finding of our study was a consistent effect of season on microbiota diversity and
457 composition measures. Marked differences in microbiota according to season have been
458 found in a number of wild species (Janiga et al., 2007; Maurice et al., 2015) showing the
459 importance of this temporal environmental factor. More interestingly, we found differential
460 effects of urbanisation on sparrow microbiota according to the season. Specifically, rural
461 sparrows underwent a loss of bacterial diversity between autumn and winter whereas diversity
462 remains constant through the seasons in cities. Similarly, rural sparrows sustained a shift in
463 taxonomic composition between autumn and winter whereas it remained constant in urban
464 sparrows. These results make sense when one considers the temporal characteristics of urban
465 environments. Indeed, there is evidence that cities have a buffering effect on the seasonal
466 fluctuations of the environment (Shochat et al., 2006). Temperature ranges, for instance, are
467 reduced due to the heat island effect (Rizwan et al., 2008). Vegetation productivity is also
468 maintained because of the abundance of evergreen exotic plants in parks and gardens (Imhoff
469 et al., 2000). Most importantly for urban sparrows, the quality and quantity of food resources

remain relatively constant throughout the year because they rely mostly on human scraps and birds feeders (e.g. Anderies et al., 2007). Such stability in food availability as well as other environmental conditions could explain why gut microbiota diversity and composition remain constant in urban sparrows but not in rural sparrows, which are subjected to increased seasonal fluctuations. Our results are corroborated by similar findings in another sparrow population (White, unpublished data) sampled along an urban gradient. Overall, our results highlight the fact that urbanisation dampens the natural seasonal variation of the gut microbiota observed in undisturbed habitats, with potential fitness implications for urban organisms.

4.3. Spatial scales involved

Because species and community responses to urbanisation can differ according to spatial scales (Concepción et al., 2015; Lindström and Langenheder, 2012), we considered the environmental characteristics of sparrow habitat at two biologically relevant scales: a local scale (400 m radius: maximum daily mobility range) and a landscape scale (1600 m radius: maximum dispersal distance). Our results show that although the microbiota is affected by urbanisation at both local and landscape scales, changes in microbiota diversity and composition are much more strongly associated to local levels of urbanisation. This major role of the local environment on organisms has been found in several studies (see for example Dauber et al., 2005) and highlight the importance of the direct environment in which organisms live. However, we found that local urbanisation did not always impacts microbiota diversity in the same way according to the landscape urbanisation levels. First, we observed that gut diversity was generally higher in homogeneous habitats (same levels of urbanisation at local and landscape scales) with homogeneous rural habitats hosting the highest gut diversity. Second, when we considered contrasting habitats, we observed that the gut diversity of sparrows living in “green” areas within urbanised landscapes is much higher than that of

495 sparrows from very urbanised local areas surrounded by rural landscapes. The former
496 basically corresponds to sparrow populations living in city parks. City parks are known to be
497 refuges for organisms living in an urban environment and could provide a number of crucial
498 resources to their inhabitants (Lin et al., 2009). Further investigations are needed to
499 understand why sparrows from urbanised local patches within rural landscapes host such low
500 gut diversity. However, if one considers that low diversity is associated to lower host health,
501 one interpretation could be that populations from such habitats suffer from the disadvantages
502 of local urbanisation (suboptimal environment) without the advantages of a larger urban
503 landscape (heat island effect, less fluctuating food resources, etc). Overall, as well as
504 showing a general effect of urbanisation, our study also reveals the influence of the wider
505 landscape on the gut microbiota of this bird species and its sensitivity to environmental
506 characteristics at different scales.

507 *4.4. Possible consequences for urban individuals and populations*

508 Here we show an impact of urbanisation on diversity, taxonomic and functional composition
509 of the gut microbiota of house sparrows. Although it was not the main objective of our study,
510 one may ask whether such changes in microbiota bare any consequences for the hosts. We
511 found no relationship between microbiota diversity and host condition, but do provide
512 evidence that taxonomic composition, and more specifically the proportion of Firmicutes
513 within the gut, is related to host condition. Although diversity was not specifically related to
514 host condition in our study, the decrease in gut diversity observed in more urbanised areas
515 could have potential consequences for the sparrows. A lower diversity is usually considered to
516 be detrimental to hosts (Le Chatelier et al., 2013) because it entails a loss of essential
517 functions leading to reduced nutrient assimilation or immunodeficiency, for instance.
518 Furthermore, diverse gut communities are more resistant to pathogens invasions, are generally
519 more stable and have higher resilience following perturbations (Buffie and Pamer, 2013). In

lizards, climate warming was found to induce a strong reduction in gut microbiota diversity, leading to subsequent higher mortality (Bestion et al., 2017). If a similar process is occurring with urbanisation, this could suggest that the loss of bacterial species caused by urbanisation may have deleterious effect on the house sparrows. In a similar way, the shifts in gut taxonomic composition we observe along the urbanisation gradient could potentially affect host health, especially if certain phyla tend to comprise more beneficial or pathogenic bacteria. Firmicutes for instance, whose relative abundance is correlated to sparrow body condition in this study, are the largest portion of the gut microbiome of most organism and play an important role in energy resorption (Semova et al., 2012). Shifts in functional composition and diversity are expected to be more strongly related to host condition than shifts in taxonomic composition especially because taxonomically varied gut communities can be functionally redundant (Lozupone et al., 2012). By inferring functional features from the 16S sequences amplified in the gut microbiota of sparrows, we were able to show that microbiota of urban birds contained relative fewer genes involved in metabolic functions than rural birds. This result is interesting in view of our results on gut microbiota diversity, because it suggests that the loss of bacterial species induced by urbanisation also leads to a loss in metabolic functions, thus hampering the microbiota's capacity to metabolise and degrade a wide range of nutrients, with potential adverse effects on the host. When we examined more specific metabolic functions, we found that several functions such as nucleotide and amino acid metabolism, which varied along the urbanisation gradient, were significantly related to host body condition, confirming that shifts in functional composition induced by urbanisation may have negative fitness consequences for the hosts.

In the light of these considerations, one may ask to what extent alterations in gut microbiota diversity, structure and function linked to urbanisation and the possible consequences for hosts may affect the population dynamics of urban sparrows in the long term. In the last

545 decades, urban house sparrows populations have experienced an important decline in several
546 cities all around the world (Ghosh et al., 2010; Laet and Summers-Smith, 2007; Shaw et al.,
547 2008), whereas the decline in rural areas seems to be stabilized. Many potential factors have
548 been proposed in order to explain this decline, such a reduction in the quality and quantity of
549 food resource, especially during nestlings development (Meillère et al., 2017), exposure to air
550 pollution (Herrera-Dueñas et al., 2014), or higher predation risk (Bell et al., 2010) for
551 instance. While the difference in the timing and the extent of the decrease between rural and
552 urban populations suggests that the explanation to this negative population trend is likely
553 multifactorial, gut microbiota alterations induced by urbanisation and potential associated
554 dysbioses may comprise one the mechanisms contributing to the current house sparrow
555 population decline in cities.

556

557 *4.5. Broader implications*

558 One possible process explaining why urbanisation causes shifts in gut diversity and
559 composition that do not necessarily translate into significant changes in host body condition is
560 the recent idea that the plasticity of the gut microbiota might be an essential factor
561 determining phenotypic plasticity of hosts, and that it might play a pivotal role in the
562 acclimation and/or adaptation of hosts to fast environmental variation, such as urbanisation
563 (Alberdi et al., 2016). Our finding that the gut microbiota of urban birds had a higher
564 metabolic potential to detoxify xenobiotics, supposedly more present in urban environments,
565 may corroborate this idea. Going beyond the specific case of the house sparrow, our results
566 shed light on a hitherto little considered perspective, i.e. that the negative effects of
567 urbanisation on city-dwelling organisms may extend to their microbiomes causing potential
568 dysbioses and may thus produce additive or synergic negative effects to host organisms and
569 populations, thereby contributing to new forms of extinction vortices. In sum, our study

provides the first evidence, to our knowledge, of an impact of urbanisation on the gut microbiota of natural populations, and together with recent work examining the effect of climate change (Bestion et al., 2017) and habitat fragmentation (Barelli et al., 2015), highlights the importance of going beyond the individual *per se* and accounting for host-microbiome interactions, when examining the response of organisms to global change.

ACKNOWLEDGES

We are grateful to H. Matheve and P. Vantieghem for field assistance. We also thank S. Manzi, A. Iribar-Pelozuelo, N. Parthuisot for laboratory assistance and L. Zinger, S.Leclaire and S. Jacob for their help with bioinformatics and microbiota analysis. We are grateful to the genotoul bioinformatics platform Toulouse Midi-Pyrenees (Bioinfo Genotoul) for providing computing and storage resources This research has been funded by the Interuniversity Attraction Poles Programme Phase VII (P07/4) initiated by the Belgian Science Policy Office. This work was also supported by the French Laboratory of Excellence project "TULIP" (ANR-10-LABX-41:ANR-11-IDEX-0002-02)

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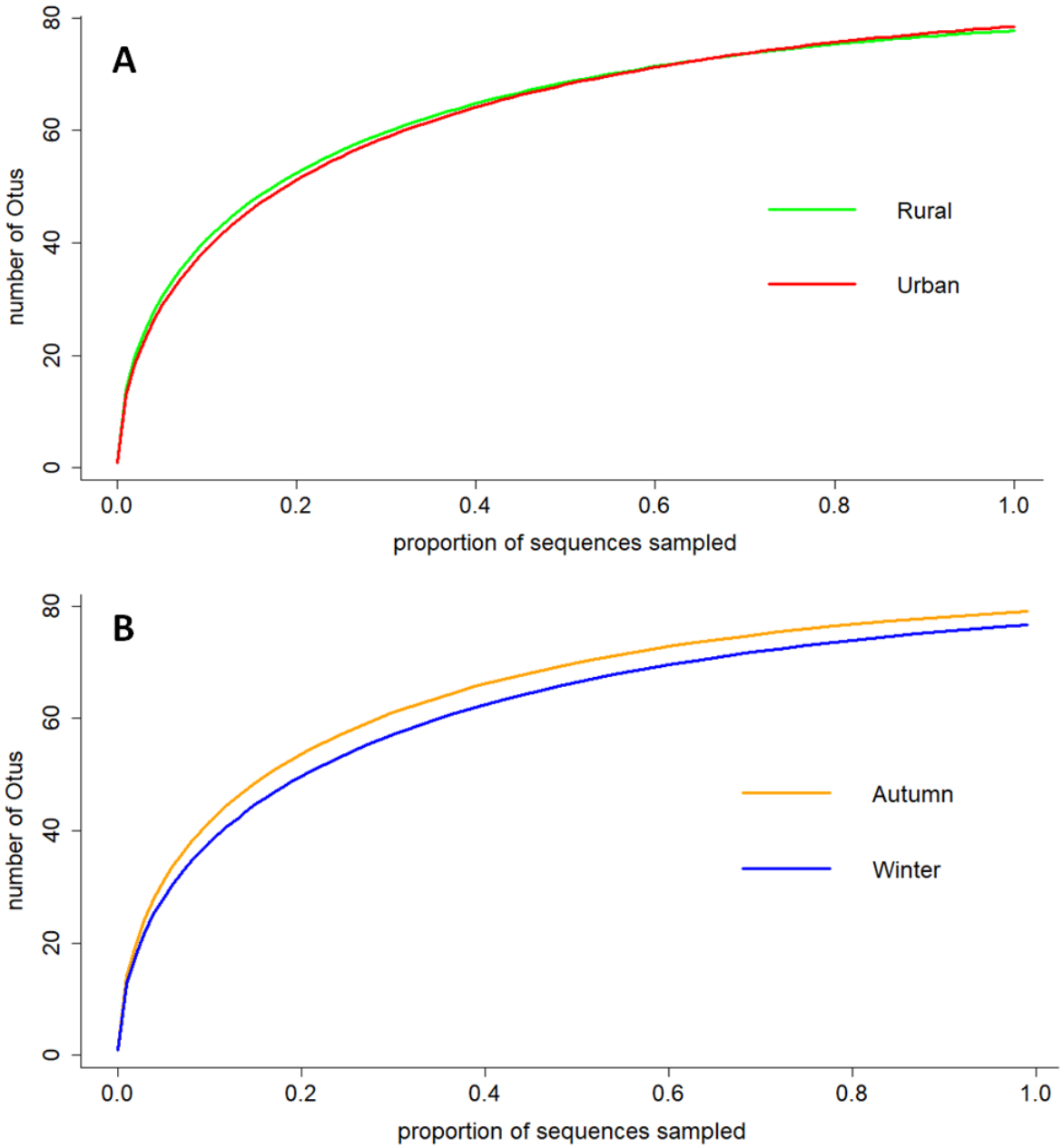
SUPPLEMENTARY INFORMATION



FigureS1: Rarefaction curve of the number of OTUs with increasing number of sequences sampled depending of the level of urbanisation at the local scale (A) and the season (B).

Region	landscape	local	city	autumn	winter	recapture
Antwerpen	U	R	Antwerpen	6	2	
Antwerpen	U	U	Antwerpen	5	9	
Antwerpen	R	R	Herenthout	5		
Antwerpen	R	U	Herenthout	7	4	
Antwerpen	SUB	R	Lint	5	3	1
Antwerpen	SUB	U	Lint	5	2	
Antwerpen	U	R	Mechelen	7	4	1
Antwerpen	U	U	Mechelen	7	4	
Antwerpen	R	R	Pulderbos	6	5	1
Antwerpen	R	U	Pulderbos	9	8	1
Antwerpen	SUB	R	Ruisbroek	5	5	1
Antwerpen	SUB	U	Ruisbroek	9	3	2
Gent	SUB	R	Beervelde	4	11	1
Gent	SUB	U	Beervelde	4	2	
Gent	U	R	Gent	7	5	
Gent	U	U	Gent	7	13	2
Gent	SUB	R	Hillegem	6	6	
Gent	SUB	U	Hillegem	8	4	3
Gent	R	R	Kalken	6	8	
Gent	R	U	Kalken	5	3	
Gent	R	R	Melsen	8	2	
Gent	R	U	Melsen	8		
Gent	U	R	oudenaarde	6	5	1
Gent	U	U	oudenaarde	5	5	
Leuven	R	R	Houwaart	11	4	
Leuven	R	U	Houwaart	11	7	3
Leuven	R	R	Kerkom	7	5	1
Leuven	R	U	Kerkom	5	6	
Leuven	U	R	Leuven	7	8	
Leuven	U	U	Leuven	5	6	1
Leuven	SUB	R	Overijse	5	3	
Leuven	SUB	U	Overijse	4	6	
Leuven	U	R	Tienen	6	7	1
Leuven	U	U	Tienen	13	2	
Leuven	SUB	R	Wezemaal	3	4	
Leuven	SUB	U	Wezemaal	4	6	1

Table S1: summary of the different house sparrows population samples during sept-2013 to march-2014

KEGG1 functions	Total abundance (%)
Metabolism	53,6323237
Genetic Information Processing	22,5846526
Environmental Information Processing	18,9847486
Cellular Processes	3,29226549
Human Diseases	1,03874471
Organismal Systems	0,46726502

Table S2: Summary of the main function identified within the KEGG1 pathway.

Metabolic functions	PC1	PC2	PC3
Explained variance	*44,712*	*18,96*	*18,014*
Carbohydrate Metabolism	**0,38762**	-0,20731	-0,18224
Amino Acid Metabolism	-0,34225	0,02547	**0,39548**
Energy Metabolism	-0,33332	0,24474	-0,25826
Nucleotide Metabolism	0,17006	-0,05514	**-0,57436**
Lipid Metabolism	0,24194	**0,35064**	0,2633
Metabolism of Cofactors and Vitamins	**-0,40788**	0,08293	0,14361
Xenobiotics Biodegradation and Metabolism	0,31135	0,19431	**0,35055**
Enzyme Families	0,19499	**0,51655**	-0,13531
Glycan Biosynthesis and Metabolism	**-0,39104**	-0,13711	-0,05544
Metabolism of Terpenoids and Polyketides	0,24529	**-0,35545**	0,24895
Metabolism of Other Amino Acids	0,05997	**0,51049**	0,0769
Biosynthesis of Other Secondary Metabolites	0,1267	-0,23083	0,3342

Table S3: PCA axis description based on the 12 metabolic pathways (KEGG2 pathways) found within the Metabolism (KEGG1) pathway.

Urbanisation Contrast Index:

We measured the discrepancy between urbanisation levels at local and landscape scale by calculating a urbanisation contrast index for each sampled population. This index was calculated as: log(% build-up area at local scale / % build-up area at landscape scale).

We found that this urbanisation constrast index significantly influenced OTU richness (GLMM, $t_{1,38}$=-2.28, *p=0.03*, Figure S2). More specifically, the gut microbiota of birds living in very urbanised patches within rural landscapes was less diverse than in green patches within urbanised landscapes. We did not find any such contrast effect on the Shannon index (GLMM, $t_{1,38}$=-0.82, *p=0.41*).

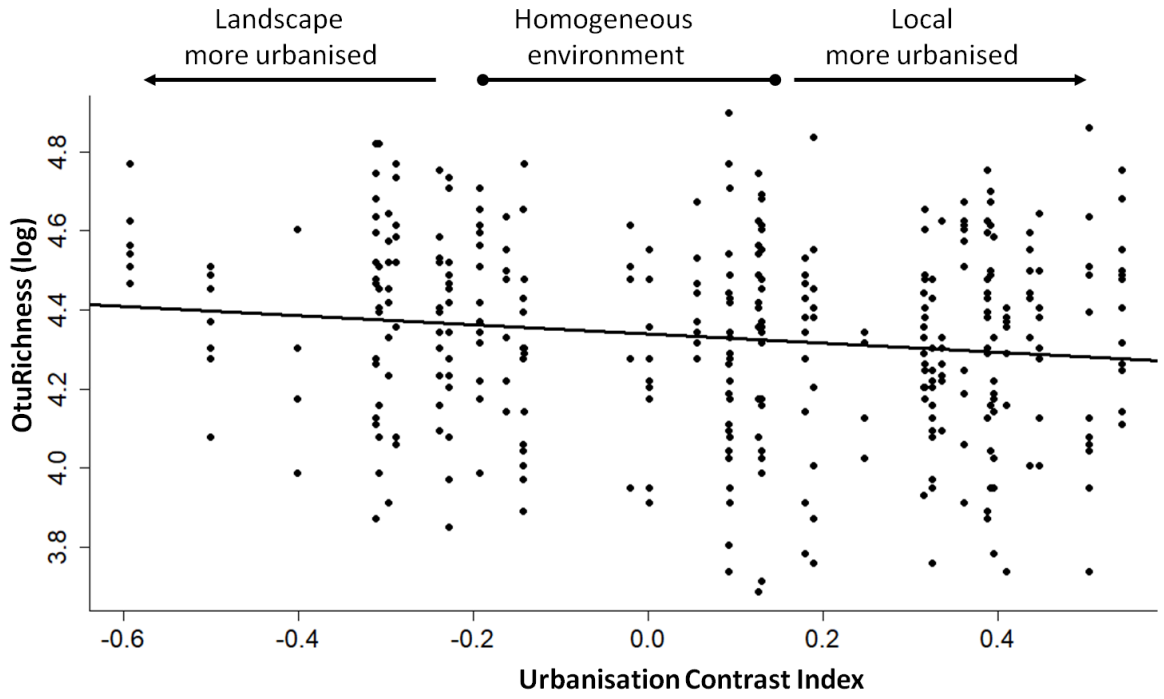


Figure S2: Gut species richness (Log of OTU Richness) according to the urbanisation contrast index (log(local_urb/landscape_urb).. Values around 0 refer to homogeneous environments whereas negative values indicate local patches that are relatively less urbanised than the surrounding landscape and positive values local patches that relatively more urbanised than the surrounding landscape.