Cities as parasitic burden? Malaria prevalence and diversity along an urbanization gradient in great tits

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ABSTRACT

Urbanization is a worldwide phenomenon tremendously modifying environmental features. By affecting the reservoirs of pathogens and the body and immune conditions of hosts, it is expected that urbanization alters the epidemiological dynamics and diversity of diseases. Cities could act as a shield against the parasite load or as a parasite burden, depending on whether urban features have positive or negative effects on vectors and hosts. In this study, we investigated the prevalence and diversity of avian malaria parasites (*Plasmodium/Haemoproteus sp*. and *Leucocytozoon sp.*) in great tits (*Parus major*) living across an urbanization gradient. We overall observed high prevalence in adult birds (from 95% to 100%), yet low prevalence in fledglings (from 0% to 38%). Malaria prevalence tended to increase with increasing urbanization in adults, and urban nestlings were more infected with *Plasmodium/Haemoproteus sp*. than forest ones. We found evidence for lower diversity in malaria lineages in the most natural site but higher diversity in the most natural urban park, while parasite diversity was overall similar across other urbanization levels. Parasite lineages were not habitat specific but for one *Plasmodium* sp. lineage (YWT4) associated with urban areas and some rare lineages (AFR065) present only in a zoo area perhaps because of the presence of African birds there. This study suggests that urbanization can lead to a “parasite burden" for urban dwelling species and can favour different avian malaria lineages. Such results open questions on the eco-evolutionary processes favouring certain disease lineages in urban environments.

KEYWORDS: urbanization, avian malaria, parasite, diversity, prevalence, epidemiology

INTRODUCTION

Urbanization is a worldwide phenomenon driving tremendous environmental change leading to the emergence of highly artificial habitats (Marzluff 2001; Gaston et al. 2015). Urban areas are a combination of remnant natural habitat patches as well as a complex assemblage of anthropogenic perturbations. They are characterised by new environmental conditions such as higher levels of chemical, light and sound pollution, more impervious surfaces and altered vegetal communities mainly dominated by exotic plants (Forman and Godron 1986). Such extensive modifications of habitats

affect biodiversity at multiple ecological levels, from individual phenotypes to community assemblages. Notably, if some species thrive in cities, others are not able to cope with urban conditions. Hence, urban communities are altered and mainly composed of fewer, often generalist species, with higher population densities compared to natural habitats (Shochat et al. 2006; Faeth et al. 2011).

If urbanization affects species singularly, it also affects the way species interact with each other (Faeth et al. 2011) ultimately impacting species evolution (Ots and Hõrak 1998; Marzal et al. 2005; Dyrcz et al. 2005). In particular, host-parasite interactions can be altered in urban habitats (Martin and Boruta 2013; Becker et al. 2015) for several reasons including (1) variation in both the occurrence and density of vector species (Reyes et al. 2013; Giraudeau et al. 2014; Neiderud 2015), (2) changes in vectors' feeding preferences in urban areas (Santiago-Alarcon et al. 2012; Abella-Medrano et al. 2018) and (3) shifts in body condition and immune system efficiency of host species in link with urban environmental conditions (Bailly et al. 2016; Capilla-Lasheras et al. 2017; Partecke et al. 2020). As such, depending on the positive and/or negative impact on the vector and host species, the effect of urbanization on disease prevalence can be twofold. In cases where urbanization disadvantages the vector species and/or favours more the host species (e.g. if environmental requirements for parasite development are not met, Calegaro-Marques and Amato 2014), urban areas may act as a parasitic shield, and urban animal populations should face lower risks of infections compared to their non-urban counterparts (Geue and Partecke 2008; Evans et al. 2009). On the contrary, if the host species is more disfavoured by the urban conditions (e.g. immune depression in the host species, Bailly et al. 2016) urban individuals might suffer a parasitic burden (e.g. Bichet et al. 2013).

Empirical evidence has up to now not favoured one or the other of these two scenarii, revealing case- and host-species dependence (Evans et al. 2009; Belo et al. 2011; Bichet et al. 2013b; Santiago-Alarcon et al. 2018). This might be because of the binary view of comparing urban *versus* non-urban habitats, with the postulate that the urban and rural environments stand as homogeneous and dichotomic environments. Yet, the reality is much else. At a finer scale, the urban matrix consists in a heterogeneous mosaic of local environments, some of which might be covered by impervious surfaces, while others highly vegetated. For example, parks offer great potential for multiple species to prosper

(Nielsen et al. 2014; Lepczyk et al. 2017), sometimes leading to more diverse and species-rich areas than in nearby wild habitats (McKinney 2008). It therefore seems necessary to move from a binary perspective (i.e. the comparison between natural and rural habitats) to the study of a continuous urbanization gradient (e.g. (French et al. 2008). Yet, despite the growing body of literature on host-parasite interactions in urban habitats, their variation along an urbanization gradient are still poorly understood (Bradley and Altizer 2007; Delgado-V. and French 2012).

In this study, we investigated the prevalence of avian malaria parasites in Great tits (*Parus major*) in and around the city of Montpellier, south of France. Avian malaria parasites belong to *Haemoproteus*, *Plasmodium* or *Leucocytozoon* genera and are widely studied in the context of host-parasite interactions (Rivero and Gandon 2018). Avian haemosporidians are vector-borne parasites infecting blood cells, and mainly transmitted by five families of Dipteras: *Culicidae*, *Hippoboscidae*, *Simuliidae*, *Ceratopogonidae* and *Psychodidae* (Valkiunas and Iezhova 2018). These vectors are frequently encountered both in rural and urban areas, although their diversity and richness varies with habitat (Coene 1993). Indeed, the presence of water sources (river or pond) in urban areas may be sufficiently propitious for reproduction, hence population survival (Asghar et al. 2011). Among these vectors, some are known to be generalists and to feed on several vertebrate groups, especially in urban habitats (Jansen et al. 2009). Great tits are common birds in Eurasia and are abundantly present in a wide range of habitats, from natural forests to heavily urbanised city centres (Fink et al. 2022). They stand as a good model species for ecologists and evolutionary biologists as they nest in human-provided nest boxes, and are easy to capture and manipulate. Infection by avian malaria in *Passeridae* is known to often induce an increase in immune response, a lower survival and a lower reproductive success (Ots and Hõrak 1998; Hõrak et al. 2001; Asghar et al. 2011; Lachish et al. 2011; Christe et al. 2012; Pigeault et al. 2018). Therefore, if host-parasite interactions are affected by urbanization levels, the outcome for bird populations could depend on their territory position along the urban gradient. Here, as a first step to unravel this issue, we aimed to understand how malaria prevalence and diversity varied along a gradient of urbanization. More precisely, we (1) compared the prevalence in nestlings and adult individuals across different urbanization levels, (2) characterised

lineage richness and diversity along the gradient of urbanization, and (3) assessed the role of urbanization levels on parasitic diversity.

METHODS

Study sites along an urbanization gradient

The study took place in two anthropogenically contrasted areas. First the city of Montpellier, in southern France (43°36′N 3°53′E) a metropolitan area hosting 480,000 inhabitants. Second, the Rouvière oak forest located 20 km northwest of Montpellier (Figure 1). In these city and forest contexts, long-term monitoring programmes of the breeding populations of great tits have been conducted since 2011 and 1991, respectively (Charmantier et al. 2017), with weekly visits between mid-March and mid-July to document great tit reproduction in artificial nest boxes scattered in eight sites across the city (Figure 1), and across the forest of La Rouvière. The climate is typically Mediterranean, with mild winters and dry summers. Spring is marked by a sudden rise in temperature, coinciding with the great tit breeding season. This region of France hosts high densities of avian malaria vectors such as *Culex pipiens*, for which massive insecticide-based control treatments have been deployed for more than 60 years (EID, 2020).

We characterised the level of urbanization and anthropogenic disturbance at each nest box, considering the area defined by a 50 m circular buffer around each nest-box where parents and nestlings were captured and sampled, an area typically considered representative of a breeding great tit foraging area (Perrins 1979). We quantified four environmental features: the extent of the vegetation cover, the motorised traffic disturbance, the pedestrian disturbance and the light pollution. We measured the surface of vegetation cover (canopy and grass) around each nest box based on satellite images from google maps. We quantified the motorised traffic perturbation by counting the number of motorised engines passing in the area during a 5 min count performed for each box in the early morning. This count showed a 0.85 correlation with traffic data provided by the city of Montpellier (opendata.montpelliernumerique.fr/) in a given area (Demeyrier et al. 2016). We similarly estimated pedestrian disturbance with counts of pedestrians, bikes and scooters. Finally, we defined local light pollution as the surface of artificial light from lamp posts covered, assuming that a lamp post would

illuminate a circular area of 50 m from its location. We summarised those four metrics using a principal component analysis as in Caizergues et al. (2021) to describe urbanization and disturbance at the nest level along two composite measures. In brief, we retained the two main axes explaining 67.8% of the variation in urban features, from which we use only the first axis in the present study. This first axis, explaining 42.4% of variance, is defined as the "naturalness" gradient, with positive values associated with larger vegetation cover, lower traffic disturbance and lower light pollution. The second axis, defined as the "pedestrian frequency" gradient (25.4% of variance explained), is not used in the current study since it is not correlated with the habitat artificialization of an area but rather to the number of pedestrians passing by each nest box. We obtained site-level measures of "naturalness" for the eight urban sites and La Rouvière (sites hereafter referred to by acronyms made of their first three letters, see Table S1) by averaging these composite measures considering all nest boxes within a given site. This ranged from the most "natural" site, La Rouvière (ROU), to the most urbanised one, Mas Nouguier (MAS).

Serologic sampling and molecular analyses

Blood sample collection

Between 2014 and 2019, we collected serologic samples between mid-March and mid-July from 15 days old nestling and adult great tits across the nine city and forest sites. We captured the parents when nestlings were 10 to 15 days old. All nestlings and adults were uniquely identified with rings provided by the Centre de Recherches sur la Biologie des Populations d'Oiseaux (CRBPO, Paris, France). A total of 296 adults (154 females 142 males) and 90 nestlings (not sexed and all sampled in 2014).

We collected 10 µL of blood by performing a venipuncture in either the ulnar (i.e. wing) vein or a small neck vein. We transferred blood samples using a capillary into an Eppendorf filled with 1 mL of Queen's lysis buffer, then stored in 4°C refrigerators at the end of the field day until DNA extraction.

DNA extraction

We extracted total genomic DNA from blood samples using Dneasy Blood and Tissue kit (Qiagen) standard protocol. We adapted the protocol by mixing 500 µL of solution of blood and Queen's buffer (~1/100 of blood) with 40 µL of proteinase K and 250 µL AL buffer and then incubating the mixture at 56°C for one hour and half. Afterwards, we added 8 µL of RNase A (100 mg/ml). We then performed DNA precipitation, adding 400 µL of ethanol.

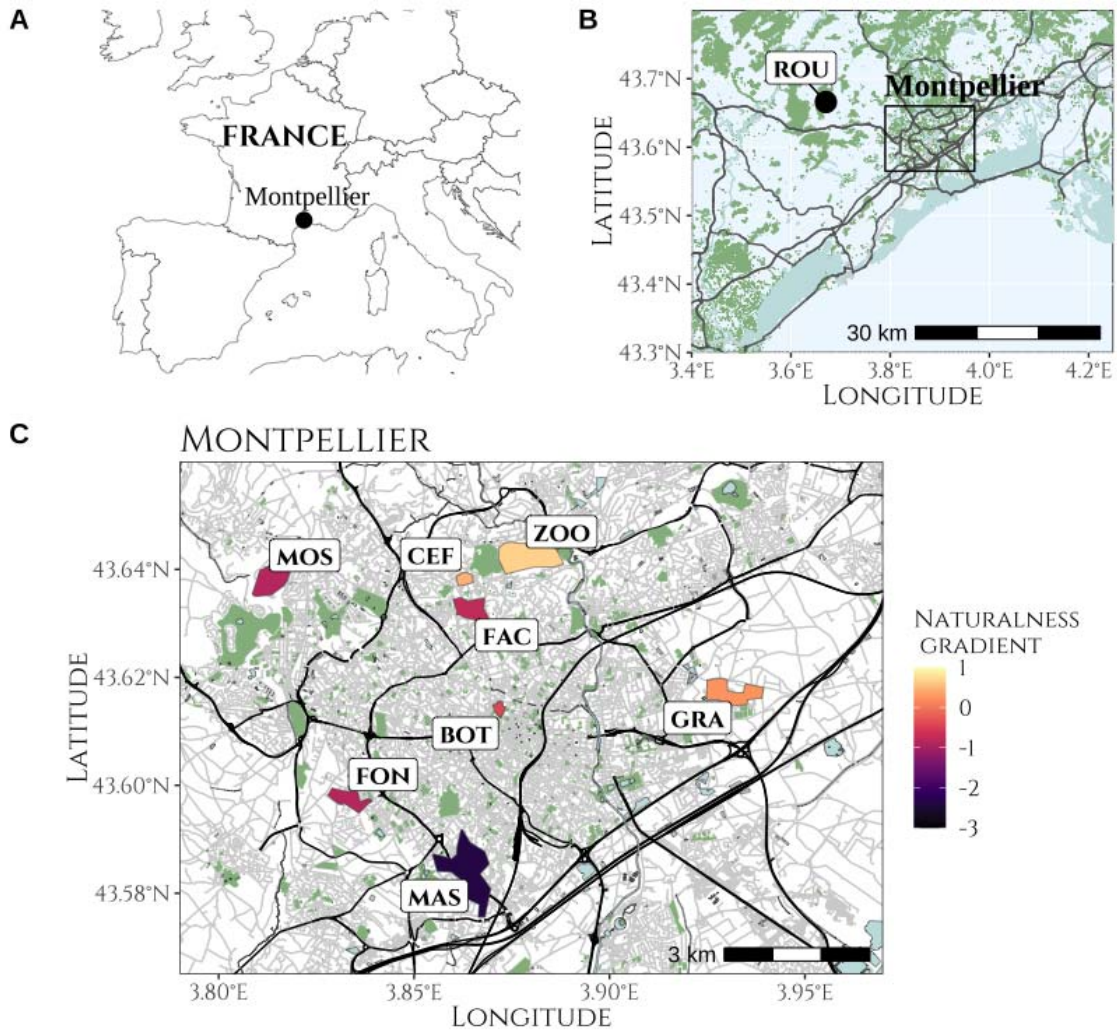

Figure 1: Maps of the sampling locations A) at European scale, B) at regional scale and C) at the city scale, where each polygon represents the limits of an urban sampling site and the colour represents the naturalness score of the site.

Infection detection

We detected and identified parasites adapting Hellgren et al. (2004) protocol. We first amplified possible large fragments of mtDNA from *Plasmodium* sp., *Haemoproteus* sp. and *Leucocytozoon* sp. using a first polymerase chain reaction (PCR) relying on the HaemNF, HaemNR2 and HaemNR3 primers let 15 min at 94°C, followed by 25 cycles of 30 sec at 94°C, 40 sec at 50°C, 1 min at 72°C, and a last cycle of 10 min at 60°C. Using 1 µL from the first amplified solutions, we then performed a secondary and more specific PCR to separately identify *Leucocytozoons* sp. and *Plasmodium-Haemoproteus* sp. presence relying on two different sets of primers: (i) we used the HaemF/ HaemR2 primers to amplifly *Plasmodium* ssp. and *Haemoproteus* ssp. (test PH); (ii) and HaemFL / HaemRL primers to amplify *Leucocytozoon* sp. (test L). We performed this second PCR using Multiplex PCR kit Qiagen in a final volume of 10 µL following one cycle of 15 min at 94°C, 35 cycles of 30 sec at 94°C, 40 sec at 51°C/52°C (for *Leucocytozoon* sp./*Plasmodium* sp. or *Haemoproteus* sp., respectively), 1 min at 72°C and one last cycle of 10 min at 60°C. We finally assessed amplification success on agarose gel 2% leading to four possible infection status: (1) uninfected (negative test PH and L), (2) infected by *Plasmodium* sp. and/or *Haemoproteus* sp. (positive test PH, negative test L), (3) infected by *Leucocytozoon* sp. (positive test L, negative test PH), (4) coinfected by *Plasmodium* sp. and/or *Haemoproteus* sp. and *Leucocytozoon* sp. (positive test PH and L).

Lineage identification

We sent positive samples to Eurofins Genomics Company for Sanger sequencing. We then blasted sequences against the *MalAvi* database for the lineage identification (Bensch et al. 2009). Due to the high quality of the sequencing, we robustly identified single and multiple infections of *Plasmodium* sp. and *Haemoproteus* sp. By contrast, we could only identify five equally most likely lineages for *Leucocytozoon* sp., the quality of sequencing being lower.

Statistical analyses

We performed all analyses with *R* software (version 4.2.1, R Core Team 2022). A complete list of the packages, associate versions and reference used for data processing, analyses and plotting is further provided in Supplementary Material Table S32.

Quantifying parasitic prevalence at the different sites

We estimated the prevalence at the site level in nestlings and adults of *Plasmodium/Haemoproteus* sp. and *Leucocytozoon* sp. as the proportion of infected individuals as well as their 95% confidence based on the Wilson score interval using the "propCI" function of the *prevalence* package.

To further assess the role of urbanization in shaping prevalence patterns, we ran linear models separately on nestlings and adult individuals, and for the two different parasite types (*Plasmodium/Haemoproteus* sp. and *Leucocytozoon* sp., respectively*)*, to link infection probability to the urban context across different spatial scales. Specifically, we fitted three logistic regressions ("glm" function of the *stat* R package, and log-link function, Bates et al. 2015) with infection (binary response, 0 as not infected, 1 as infected) as a function of either i) habitat type (binary variable, 0 as forest, 1 as urban), ii) the site-level naturalness (first axis of the PCA, see above) or iii) the local nest-level naturalness. For models ran on data from adult individuals, we further controlled for sex, age (in years), as well as year of sampling. Because of the low number of samples in years 2017 (N = 6) and 2018 (N = 18) creating complete separation issues, we removed these data from this analysis. We assessed the significance of each predictor using likelihood ratio tests ("drop1" function of the *stats* package) while dropping one predictor at a time.

We verified that linear models' assumptions were not violated using various visual controls of residual distributions and associated statistical tests (histogram of residuals, Q-Q plot of expected residuals vs observed residuals, scattered plot of residuals vs estimates) using the *DHARMa* package as well as the *performance* package (see Supplementary Material: Supplementary text 1, Tables S2 to S31 and Figures S1 to S23). This raised no problem of collinearity, singular fit, convergence or influential points.

Characterising lineage diversity and habitat specificity at the different sites

For subsequent analyses, we focused on adult individuals, as the quality of nestling malaria sequences was low, preventing us from correctly identifying lineages. Given the uncertainty in the *Leucocytozoon* sp. lineage identification, we repeated the analyses (below) 1000 times for this parasite type, each time randomly considering that individuals were infected by only one lineage among the possibly identified ones. Thus, for *Leucocytozoon* sp., estimates and associated 95% confidence intervals represent the median estimate and its associated confidence interval.

Lineage diversity

Haemosporidian lineages richness and abundance were analysed with the *vegan* and *BiodiversityR* packages. To analyse patterns of lineage diversity per site we estimated the Shannon and inverse-Simpson diversity indices for each study site, that highlight more specifically the richness or the evenness of diversity (Nagendra 2002).

We estimated dissimilarities in lineage composition between sites using the Bray-Curtis dissimilarity index ("vegdist" function of the *vegan* package). We computed this index on the binary sequence (i.e. indicating whether a given lineage was present or absent), and on the sequence of individual prevalence for each lineage (i.e. percent of infected individuals having the lineage). The former would thus give insight into parasite composition resemblance, and the latter into prevalence resemblance. Hereafter, we refer to them by Bray-Curtis composition and Bray-Curtis prevalence, respectively.

Habitat specificity

To investigate whether some lineages occurred more frequently than randomly expected in urban vs forest environment, we compared the proportion of urban nest boxes at which each lineage was present to the overall number of nest boxes sampled using a binomial test ("binom.test" function). We computed the test only for lineages for which the type II error was below 0.20 and that occurred at least 10 times overall, to ensure statistical robustness.

In addition, we investigated if parasitic community similarity was linked to urbanization at two scales: sampling site and nest box. We analysed the correlation between naturalness distance (absolute difference in "naturalness" level) and (Bray-Curtis composition) parasite-dissimilarity matrices using a mantel test with 999 permutations ("mantel.test" function of the *ape* package). We also controlled for spatial autocorrelation by testing whether parasitic community similarity was related to geographic proximity, repeating those analyses comparing the Euclidean distance between pairs of sites or nest boxes ("st_distance" function of the *sf* package) to parasite dissimilarity.

RESULTS

Plasmodium, *Haemoproteus* and *Leucocytozoon* prevalences

Low parasitic prevalence in nestlings

In 15 day-old nestlings, avian haemosporidian prevalence was low in both habitats (< 40%, Figure 2A) with some heterogeneity among the sites. No nestling was simultaneously infected by *Plasmodium/Haemoproteus* and *Leucocytozoon* parasites.

The prevalence in *Plasmodium/Haemoproteus* parasites ranged from 0% to 38%, with an average of 16.33% (Figure 2A). It was significantly higher in the city nestlings compared to forest ones (16.67% averaged on all city sites vs. 0% in the forest; $\chi^2_1 = 9.854$, P = 0.002,), yet unrelated to the nest- and site-level naturalness gradient ($\chi^2_1 = 0.012$, P = 0.908; $\chi^2_1 = 1.186$, P = 0.276, respectively).

The prevalence in *Leucocytozoon* ranged from 0% to 40%, with an average of 9.90% and did not differ consistently between city and forest nestlings (11.11% averaged on all city sites vs. 2.78% in the forest; $\chi^2_1 = 2.383$, P = 0.123) and was unrelated to the nest- or site-level naturalness gradient (χ^2_1= 1.837, P = 0.175; $\chi^2_1 = 1.291$, P = 0.256, respectively).

High parasitic prevalence in breeding individuals

Avian haemosporidian prevalence ranged from 95% to 100% for *Plasmodium/Haemoproteus sp.*, and 80% to 100% for *Leucocytozzon sp.* in breeding great tits, with an average of 97.04% and 92.93%

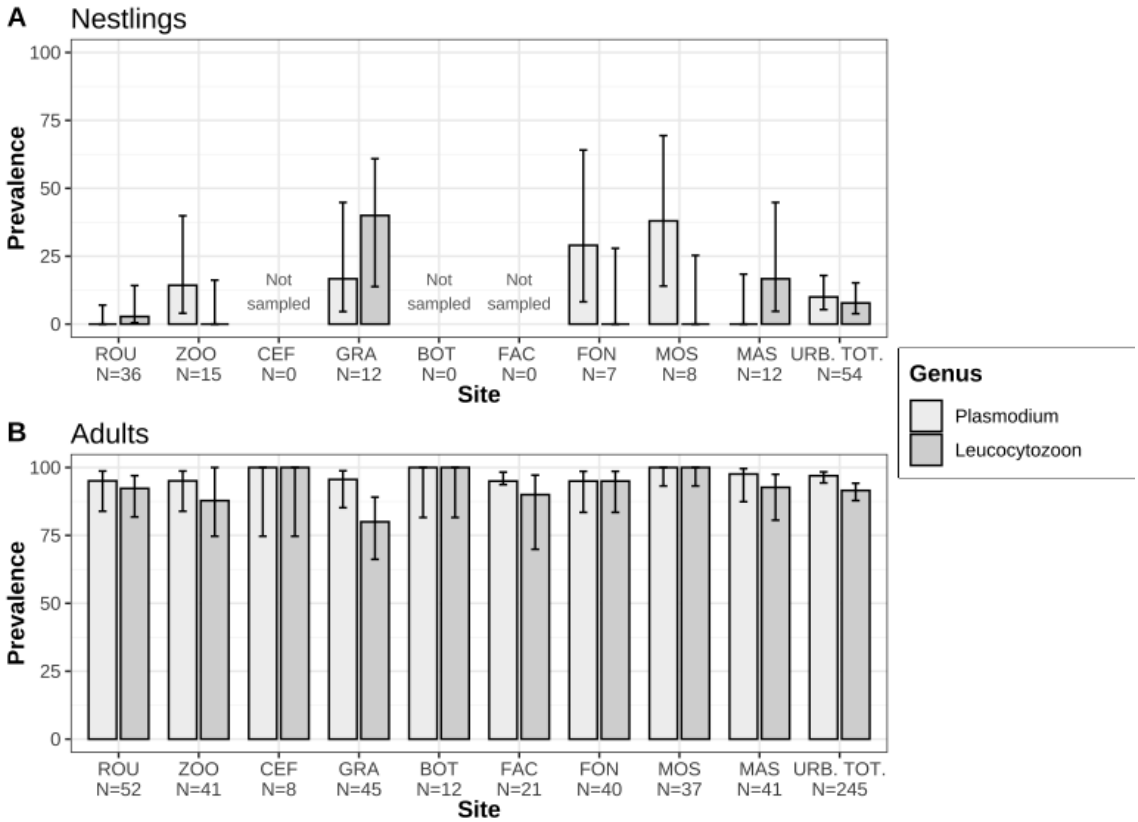

Figure 2: Mean avian *Plasmodium* sp. (dark grey) and *Leucocytozoon* sp. (light grey) prevalences per site in great tit (A) nestlings and (B) adults. Error bars represent 95% confidence intervals. Sites are ordered by increasing urbanization level.

respectively (Figure 2B). Double infection was frequent (91.9% of individuals). In particular, all individuals infected with *Leucocytozoon* were systematically infected with *Plasmodium*/*Haemoproteus.*

Prevalence of *Plasmodium/Haemoproteus* and *Leucocytozoon* did not vary significantly between city and forest (χ^2_1 = 0.003, P = 0.955; χ^2_1 = 1.71, P = 0.191, respectively) nor with the site-level naturalness (χ^2_1 = 0.360, P = 0.548; χ^2_1 = 0.012, P = 0.911, respectively). However, naturalness gradient (around each nest-box) had a marginally significant effect on *Plasmodium*/*Heamoproteus* prevalence (glm: est. ± S.E. = -0.615 ± 0.397, χ^2_1 = 2.937, P = 0.087) with a tendency for lower prevalence in less urbanized areas, which was not the case for *Leucocytozoon* prevalence (χ^2_1 = 0.567, P = 0.452). In addition, prevalence of both parasites did not vary between males and females (all P > 0.05) or with age (all P >0.05), *Leucocytozoon* prevalence models showed a significant year effect in the habitat model (glm: est. ± S.E. = 1.051 ± 0.484 χ^2_1 = 5.075, P = 0.024), which was not the case for *Plasmodium*/*Heamoproteus* prevalence (all P >0.05).

Prevalence in nestlings versus breeding individuals

Plasmodium/*Heamoproteus* and *Leucocytozoon* prevalences at each site were not correlated between nestling and adult stages (Spearman correlation test, ρ= 0.133, P = 0.803 and ρ = -0.577, P = 0.231).

Diversity and richness of *Plasmodium* lineages in breeders

A combination of 47 lineages of *Plasmodium* and *Leucocytozoon* species were recorded across all study sites (Figure 3), including 5 *Plasmodium* and 42 *Leucocytozoon* (total number of lineages identified by BLAST, not accounting for uncertainty in lineage identification). The well-known SGS1 lineage was the most represented of all lineages, with 272 infected birds out of 296 individuals sampled. Whereas the least urbanised urban site (namely 'ZOO', a large urban park of around 80 ha) showed the highest richness and diversity (Shannon index, Table 1), the forest site, was on the contrary the site with the lowest diversity (Inverse Simpson index, Table1).

Table 1: Haemosporidian lineages richness and diversity indices (Shannon and Inverse Simpson) across the eight city sites and the forest site (ROU).

Site	Naturalness index	Richness	Shannon	Inverse Simpson
MAS	-2.383	18 (15 - 20)	1.98 (1.88 - 2.06)	3.65 (3.56 - 3.72)
MOS	-0.865	16 (14 - 19)	2.09 (1.99- 2.17)	4.53 (4.41 - 4.61)
FONT	-0.854	18 (16 - 21)	2.09 (1.99 - 2.17)	4.11 (4.02 - 4.17)
FAC	-0.750	15 (13 - 17)	2.14 (2.01 - 2.23)	4.98 (4.77 - 5.13)
BOT	-0.406	9 (7 - 11)	1.71 (1.54 - 1.84)	3.51 (3.33-3.64)
GRAM	0.254	16 (13 - 18)	1.86 (1.76 - 1.94)	3.33 (3.25 - 3.38)
CEF	0.458	8 (6 - 9)	1.71 (1.49 - 1.80)	3.81 (3.46 - 3.95)
ZOO	0.687	22 (19 - 25)	2.20 (2.10 - 2.28)	4.11 (4 - 4.17)
ROU	1.221	16 (14 - 19)	1.79 (1.69 - 1.88)	3.08 (3.02 - 3.13)

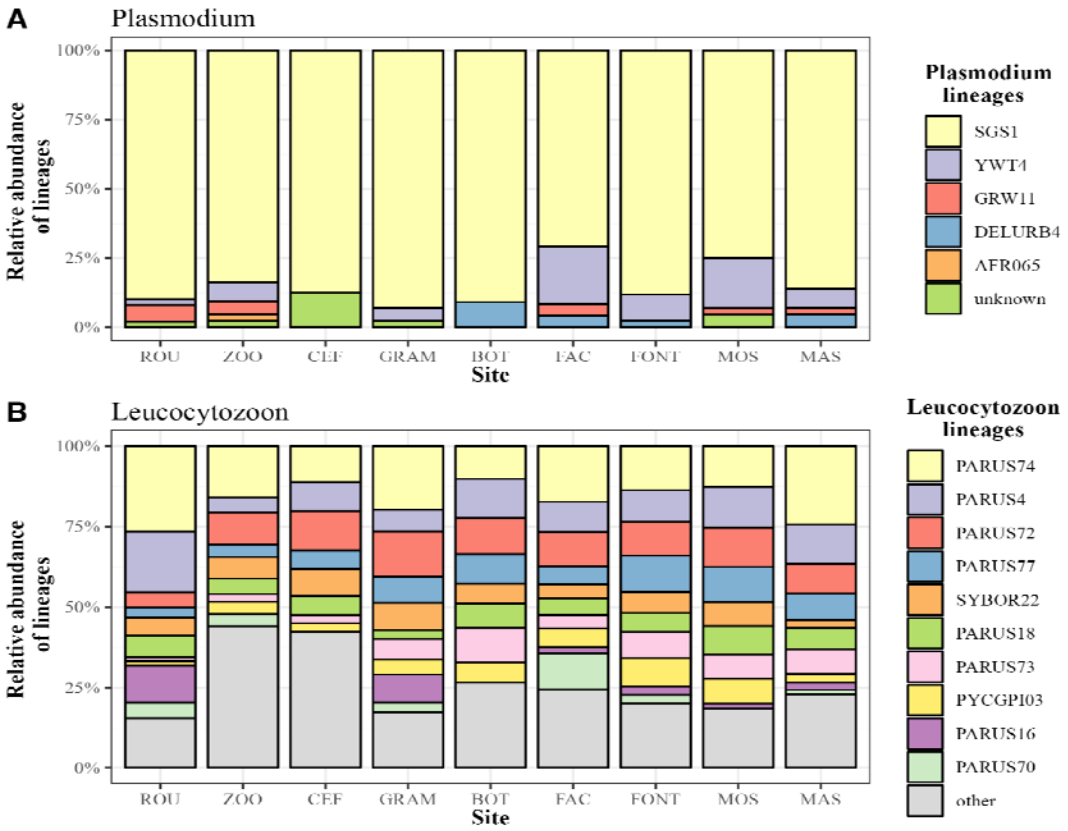

Figure 3: Proportions of (A) *Plasmodium/Haemoproteus* and (B) *Leucocytozoon* lineages found in each study site. For *Leucocytozoon* sp., only the most abundant lineages are shown in detail and lineages with less than 15 total occurrences were grouped as "other".

Habitat specificity of lineages in breeders

Regarding lineage habitat specificity, we found one lineage, YWT4 (*Plasmodium sp.*), that occurred more in urban habitats than expected by chance (Figure 4). None of the other *Plasmodium/Haemoproteus* or *Leucocytozoon* lineages were statistically more associated with one habitat than the other.

Resemblances between sites were high, both from a compositional (i.e., in terms of lineage diversity) or a prevalence (i.e., in terms of infection rate for a given lineage) point of view (Bray-Curtis index range for composition and prevalence: 0.16-0.50 ; 0.15-0.75, respectively ; Figure 5), with the sites the least resembling being the least sampled ones (BOT and CEF) .

We found no statistical link between parasitic community similarity and naturalness gradient or geographical proximity at both the site or the nest box levels (Mantel test: $P > 0.05$ for all the 1000 subsampled datasets; p-values were adjusted to maintain the false discovery rate to 5%).

DISCUSSION

In this study, we investigated the link between urbanization and avian malaria prevalence and lineage diversity at different scales across wild populations of great tits in and around a metropolis of almost half a million inhabitants. We found marked differences in prevalence between life stages, with 15 day-old nestlings showing substantially lower prevalence than adult birds. Malaria prevalence also varied depending on the environment, with urban nestlings significantly more infected that forest nestlings and a tendency for higher prevalence in adults from more urbanized areas, suggesting the existence of a parasitic burden in the city. Interestingly, the forest habitat was the least diverse site, yet, diversity did not decrease with urbanization level in the city and the most natural-like urban park was even the most diverse. In Haemosporidian lineages, some lineages occurred only or more often in urban areas, suggesting the existence of habitat specificity.

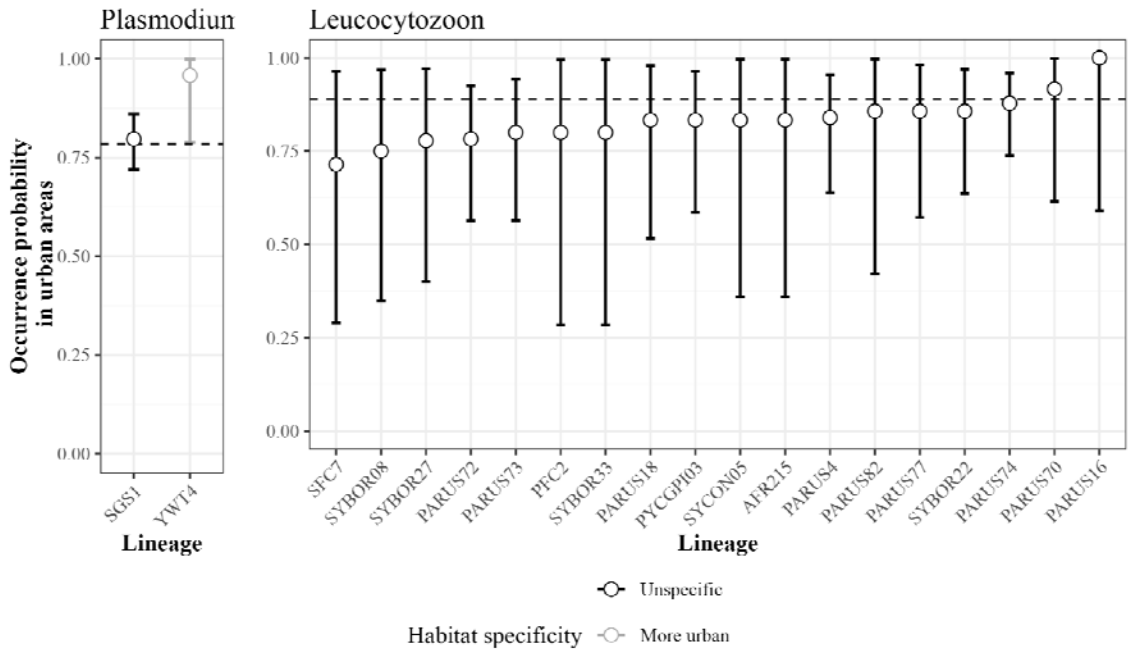

Figure 4: Occurrence probability of avian haemosporidian lineages in the urban habitat for (A) *Plasmodium/Haemoproteus* sp. and (B) *Leucocytozoon sp*. Error bars represent 95% confidence intervals. The dashed line represents the expected probability of occurrence of a lineage in the urban habitat under random distribution. Grey dots and error bars represent lineages that are found statistically more in the urban habitat, and black, lineages that are not habitat-specific.

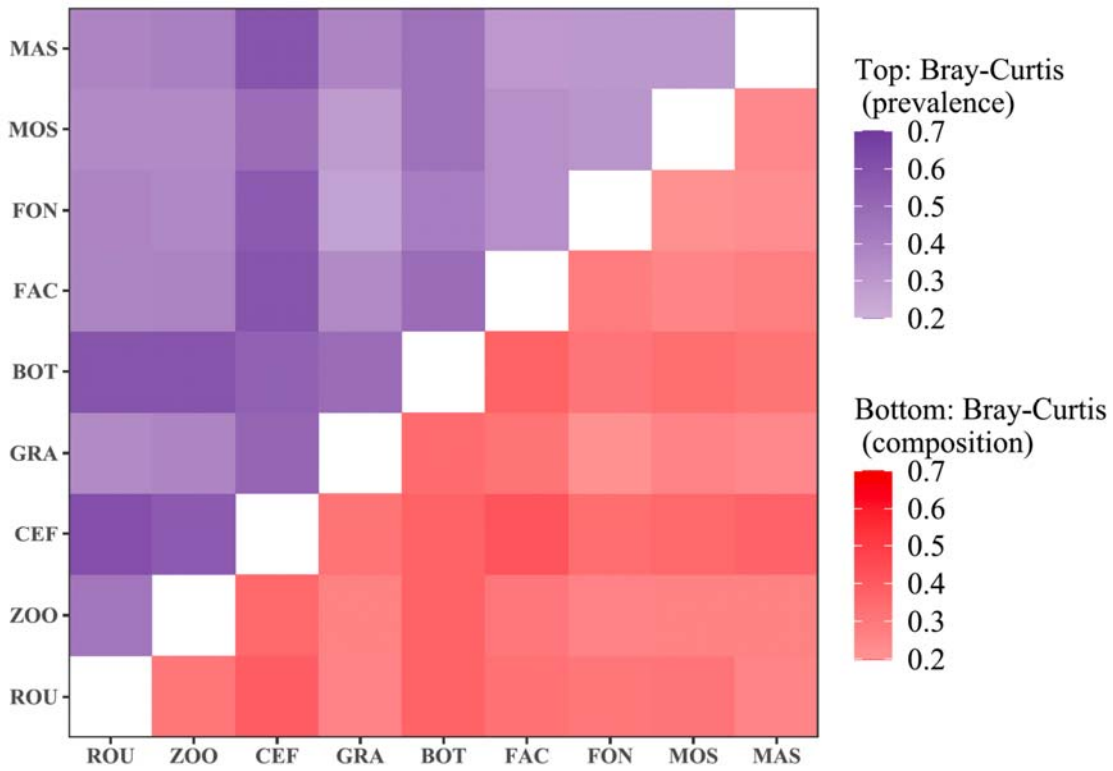

Figure 5: Heatmap of Bray-Curtis dissimilarity between each site considering binary sequences of lineage presence (composition) (bottom) or the prevalence of each lineage among infected individuals (prevalence) (top). Darker colors represent higher values of Bray Curtis index and stronger differences in lineage composition/prevalence between a pair of sites.

Life stage but not habitat-dependent prevalence

Overall, infection by *Plasmodium* genus was greater than infection by *Leucocytozoon* genus, which is a common pattern observed across bird species (Valkiunas and Iezhova 2018). Heamosporidian prevalence was overall low in nestlings (from 0% to 38%) but high in adults (from 95% to 100%), both in urban and forest areas. Such prevalence levels are comparable to previous studies for adult great tits (Glaizot et al. 2012; Rooyen et al. 2013). To our knowledge, this was however never tested in 15 days old great tit nestlings. In fact, only lower prevalence in young juvenile (one year old) birds compared to adults was previously described in great tits and other passerine species (Wood et al. 2007; Santiago-Alarcon et al. 2016). The higher infection detection in adults than in nestlings frames coherently with the vector (e.g. *Culex pipiens*) life cycle, with a progressive increase in adult mosquitos, hence of infection risk, from spring to summer (Zélé et al. 2014). As a consequence, the risk for 15 days old nestlings of being infected is expected to be low as they were sampled during spring. Similarly, Valkiunas and Iezhova (2018) found that young adults presented lower prevalence than older ones, which seems in line with the fact that Haemosporidian infections yield an acute infection followed by a life-long chronic infection. Hence, the longer the exposure to the parasites, the higher the probability of eventually being infected.

Interestingly, while overall malaria prevalence was similar in urban and forest adults, we found significantly higher *Plasmodium/Haemoproteus* prevalence in urban areas compared to forest nestlings and also a somewhat higher *Plasmodium/Haemoproteus* prevalence in adults in highly urbanised areas. These results support the hypothesis of the existence of a parasitic burden in more urbanized areas. This finding contrats frequent reports of lower parasitic prevalence in urban areas including in our focal species (Bailly et al. 2016). Whether avian malaria is more or less prevalent in cities thus appears strongly case-specific (Evans et al. 2009). These differences in parasite prevalence between habitats may be directly induced by variations in the presence and/or density of vectors (e.g. Martínez-De La Puente et al. 2013). These variations should be the consequence of presence or absence of their suitable ecological niches. For instance, among the 11 paired populations of blackbirds *Turdus merula* studied in Evans et al. (2009), in 3 cases avian malaria prevalence was

found to be higher in urban areas as a consequence of underwater area presence, a necessary component for vectors to prosper. While fine scale densities of vectors are not yet known for the city of Montpellier and its surrounding, a tendency towards higher malaria prevalence in more urbanized areas could indicate higher population size or densities of vectors in such areas, which remains to be empirically demonstrated.

In addition, urban nestlings showed higher prevalence than forest ones. While reasons for increased early infections in urban nestlings remains to be addressed specifically, one reason explanation may stem from the urban heat island effect. Indeed, Paz and Albersheim (2008) showed that higher temperatures in urban areas proved beneficial to *Culex pipiens* mosquitoes growth and that some diseases (i.e. the human West Nile Fever) transmitted by this vector appeared earlier in the season in the city compared with surrounding countryside areas. Hence, environmental shifts observed in urban areas can be directly linked to spatial and temporal parasite infections. In addition, malaria infections are known to vary in time, with seasonal fluctuations (Zélé et al. 2014). Given the role of the urban area in buffering on climatic variations, urbanization could be responsible for major changes in seasonality of parasitic infection. As evidenced here, this could cascade on the emergence of earlier disease outbreak, with earlier nestling contamination. The link between urban specific climatic features and seasonality of vectors and disease outbreaks in urban areas remains however overlooked and should be the focus of further research avenues.

Spatial heterogeneity in lineage diversity

When exploring diversity of haemosporidian lineages across sites, we found similar levels of diversity along the urbanization gradient and no strong “cluster” of similar lineages in similarly urbanised or closer sites. Despite the fact that no clear pattern of diversity emerged along the urbanization gradient, we found that the forest sites had the lowest Haemosporidian lineage diversity, whereas the large zoo urban park had the highest. Interestingly, previous studies reported that urban parks with higher diversity of plant and bird species were also the most diverse in terms of Haemosporidian lineages (in multiple species: Carbó-Ramírez et al. 2017 ; in the House Sparrow: Jiménez-Peñuela et al. 2021). In our case, the Zoo du Lunaret of Montpellier consists of a 80-ha natural area where a large diversity of

both native and exotic plant and bird species coexist. Interestingly, the only occurrence of *Haemoproteus* sp. AFR065 lineage was in this zoo. According to the *MalAvi* database (Bensch et al. 2009), this lineage was found previously only on the African continent, in two bird genus in Malawi (*Cercotrichas* and *Andropadus*, Lutz et al. 2015). Hence, the presence of such lineages in this particular area of the city is most probably linked to the presence of captive African birds in the zoo (see next section for details on these birds).

Surprisingly however, our forest site of La Rouvière, 20 km away from the city of Montpellier, showed the lowest level of Haemosporidian lineage diversity, which contrasts with previous results found showing opposite trends (e.g. in the House Sparrow : Jiménez-Peñuela et al. 2021). In our study site, the overall urban habitat presents numerous ornamental plant species, whereas the forest habitat, which is a Mediterranean forest, is mainly dominated by oak trees. Hence, even with lower density of vegetation, the urban areas might be prone to a maintain high diversity of pathogens (Carbó-Ramírez et al. 2017). However, such hypothesis remains to be further tested. The replication of similar studies on multiple urbanization gradients throughout Europe, including several mediterranean areas, appears to be of major importance to have a holistic view on how artificial biomes could play a role in parasite diversity.

Habitat specific lineages

While none of the sampled sites revealed a particularly divergent composition in Haemosporidian lineages, we still observed some heterogeneity in lineage occurrence. Because of the uncertainty in the identification of *Leucocytozoon* lineages, we only discuss *Plasmodium sp.* lineages here (none of the lineages identified here belonged to *Haemoproteus sp.*). Overall, the *Plasmodium sp.* infections were mainly dominated by SGS1 lineage (*Plasmodium relictum*). SGS1 is known to be a generalist lineage, present in multiple avian species and environments (Rooyen et al. 2013) and transmitted by *Culex pipiens* (Ventim et al. 2012; Inci et al. 2012) which is widely present in the south of France in both habitats. Aside from SGS1, some lineages were found (in low occurrence)

exclusively in the urban habitat: AFR065 occurred once in the zoo and DELURB4 occurred in urban sites only. However, habitat specificity analyses controlling for unequal sampling across the sites, revealed that only one Haemosporidian lineage was occurring more than randomly expected in urban habitat: YWT4 (*Plasmodium* sp.), the other urban exclusive lineages occurring too rarely to robustly conclude on their habitat-specificity. When investigating the previous occurrences of these 3 specific lineages (i.e. AFR065, DELURB4 and YWT4) in the MalAvi database, we found that they were relatively rarely encountered, at least in great tits.

AFR065 was reported only twice, once in the Miombo scrub robin (*Cercotrichas barbata*, Muscicapidae) and once in the western greenbul (*Andropadus tephrolaemus*, Pycnonotidae) in Malawi and never on the European continent nor in the great tits (Lutz et al. 2015). As mentioned before, the individual infected by AFR065 was captured in the Zoo du Lunaret (most natural urban site). At the time of the sampling for this study, the zoo hosted 65 African birds from 14 different species. While malaria infection status of these captive birds held in the zoo are low (<5%, unpublished data), we can easily hypothesise that they were the initial carriers of AFR065 that was then transferred to a great tit via the contaminated vector. This result raises concern regarding local wildlife epidemiology when introducing or keeping exotic wildlife captive in contact with for native species.

Regarding DELURB4, we found no previous occurrence of this lineage in great tits in the *MalAvi* database, even if this lineage was previously shown to be the second most common lineage present in the vector *C. pipiens* in the area (Zélé et al. 2014) and numerously recorded in the close sister species the Blue tit *Cyanistes caeruleus* (Ferrer et al. 2012) and in other bird families (e.g. *Passeridae*, *Turdidae* and *Muscicapidae*) in several European countries (Spain, Italy, Bulgaria, Russia according to the *MalAvi* database). Similarly, YWT4 is a rare lineage with only 7 occurrences in the whole *MalAvi* database, mainly in the Western yellow wagtail (*Motacilla flava*), but was found 25 times in the studied urban great tits, and once in a forest bird. Reasons why these lineages were more common in urban areas than in forest habitat remain to be explored. A possible explanation could be the difference in bird community compositions between habitats, leading to contact with different bird species, each with their own body of specific Haemosporidian parasite lineages. Testing this

hypothesis would require a thorough scan of Haemosporidian infections in multiple species from both urban and forest habitats in replicated cities.

Conclusion

While we found no striking difference in malaria prevalence between urban and forest great tits, urbanization was associated with earlier infections in nestlings. In addition, *Plasmodium/Haemoproteus* sp. prevalence tended to be higher in the more urbanized parts of the city. Taken together these results suggest that urbanization may lead to a parasitic burden for urban dwelling species. Interestingly, although sites displayed no major differences in Haemosporidian lineage community composition, the forest habitat showed the lowest diversity in Haemosporidian lineages and some lineages, that turned out to rarely occur in malaria databases, were preferentially occurring in urban areas This suggests that urbanization could play a role in the emergence and spread of previously rare disease strains.

STATEMENTS AND DECLARATIONS

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Conflict of interest

The authors declare no conflict of interest.

Ethical statement

Captures were performed under personal ringing permits delivered by the CRBPO (Centre de Recherches par le Baguage des Populations d’Oiseaux, e.g. ringing permit for Anne Charmantier number 1907) for the Research Ringing Programme number 369. All experimental protocols were approved by the ethics committee for animal experimentation of Languedoc Roussillon (CEEA-LR,

most recent approval in 2018 for APAFIS#8608-2017012011062214) as well as by Regional Institutions (most recent bylaw issued on 07/04/2022 by the Prefecture n° 2B-2022-04-07-00002).

Data and code sharing

Data and code used for this study are freely available from Aude E. Caizergues' github repository (https://github.com/AudeCaizergues/Malaria_Great_Tits).

Authors contribution

A.E.C., S.P & A.C. collected the samples along with field collaborators. M.J. & A.B. performed the molecular analyses. A.E.C. & B.R. conducted the statistical analyses and wrote the manuscript. C.P., S.G. & A.C. conceptualised the research. S.G. & A.C. financed the project. All authors contributed to writing the manuscript.

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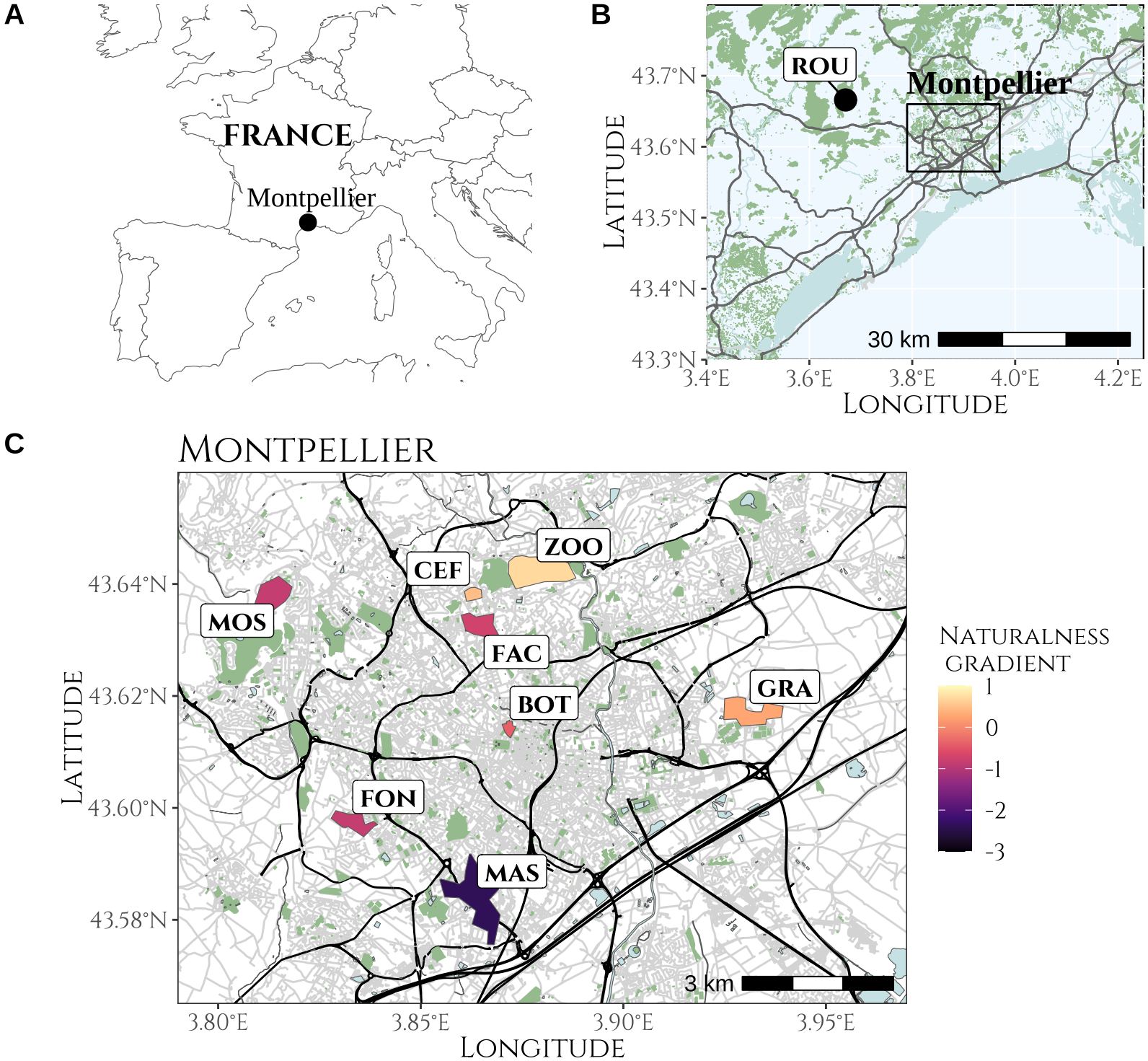
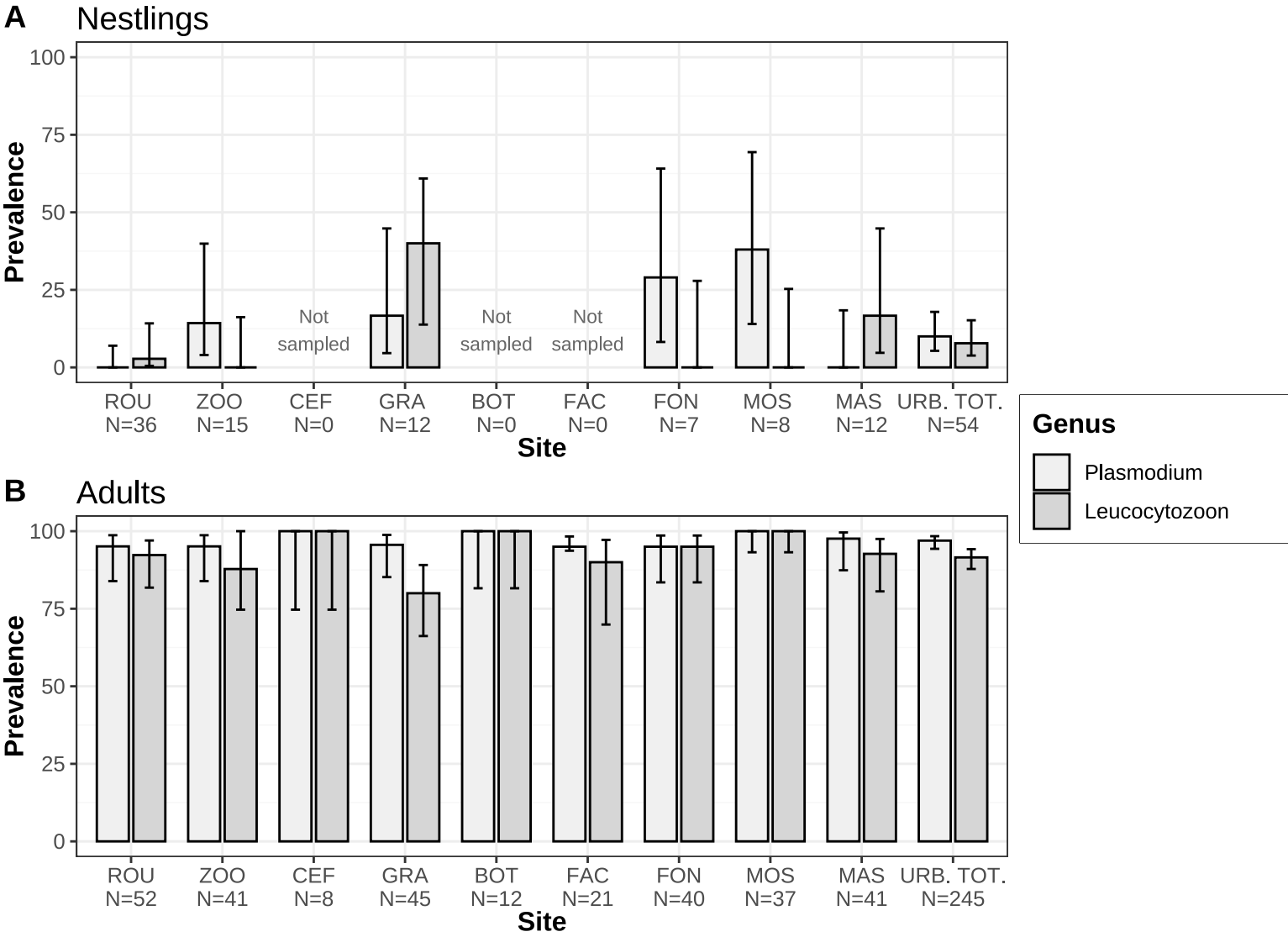
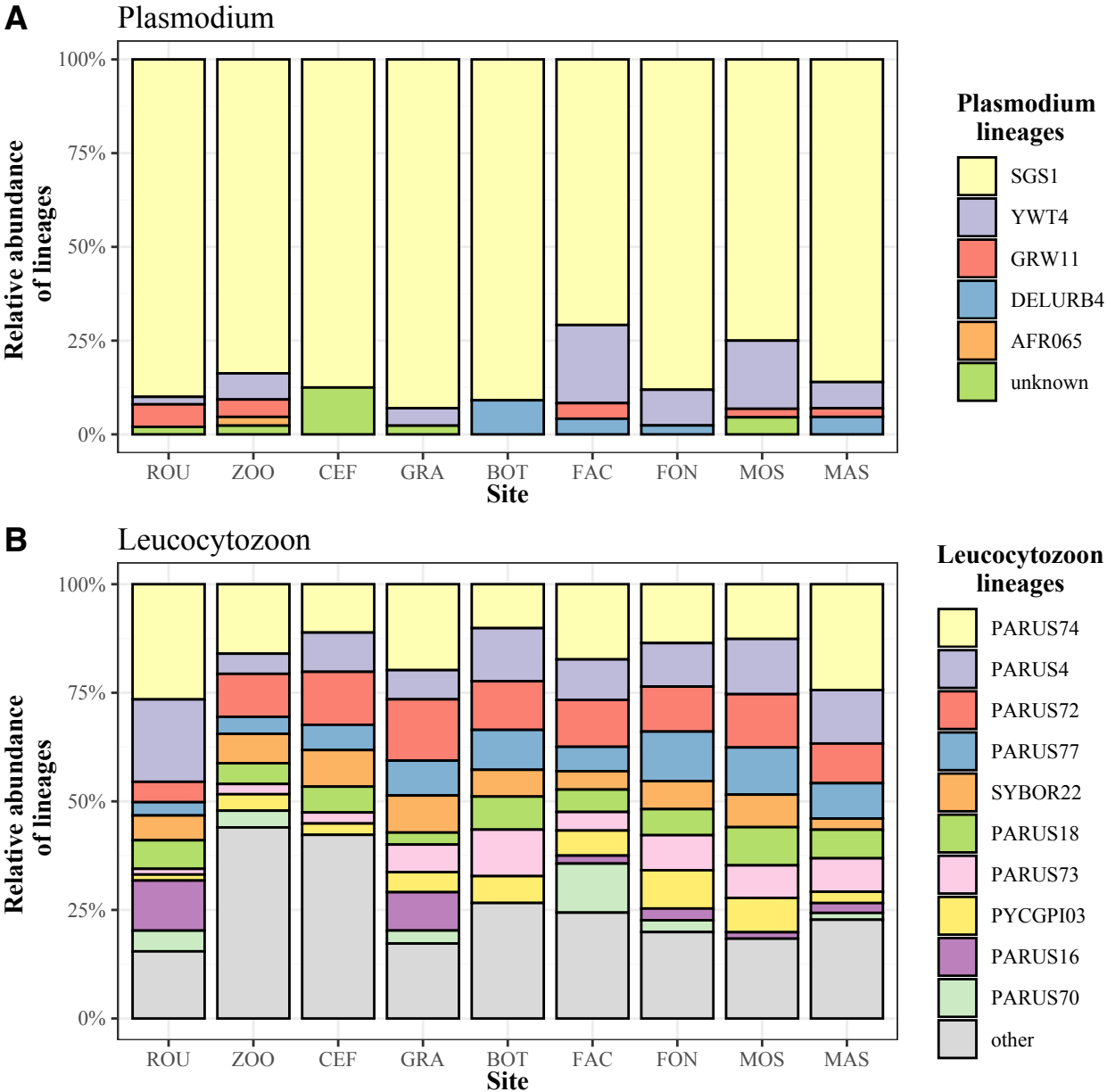
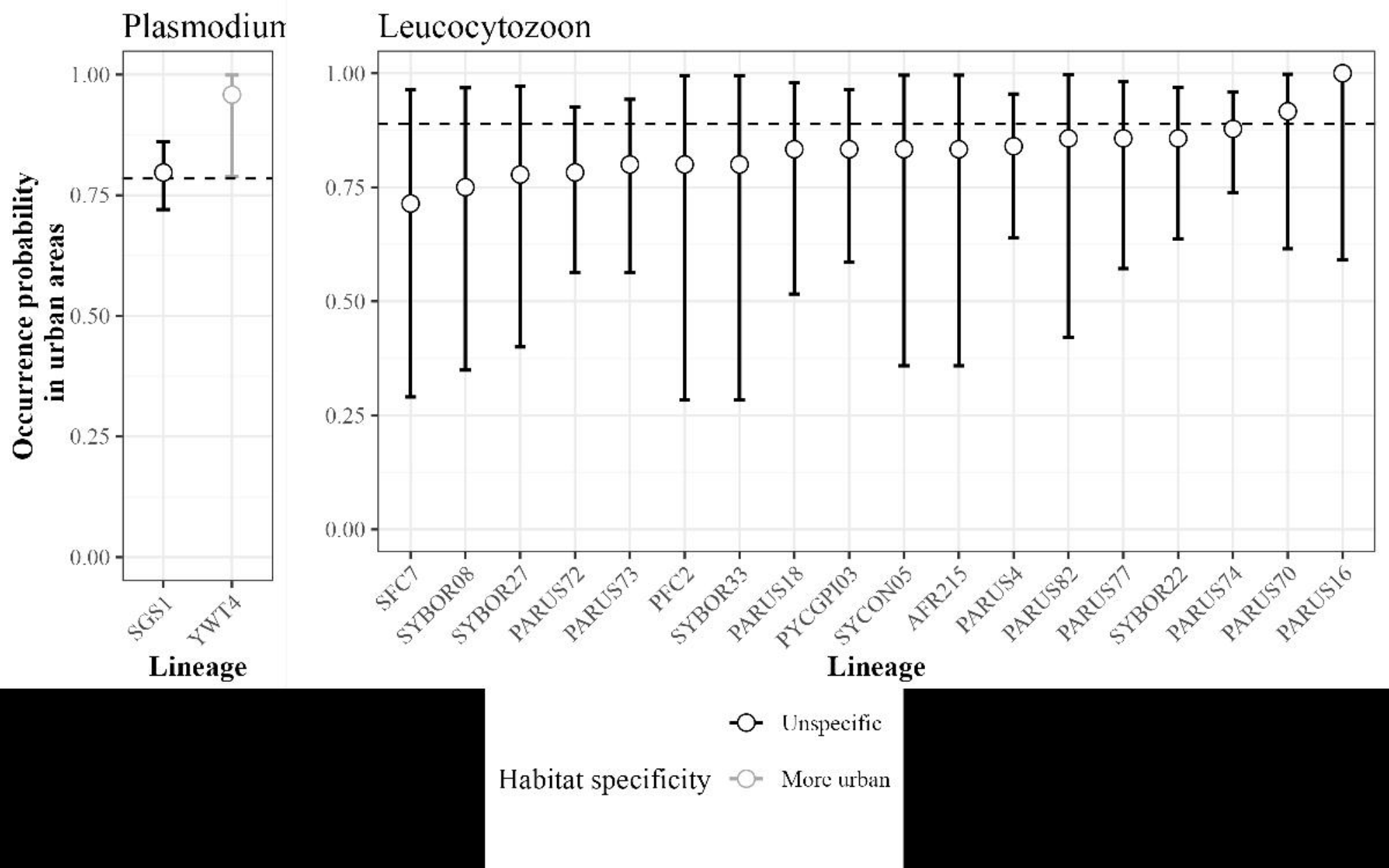
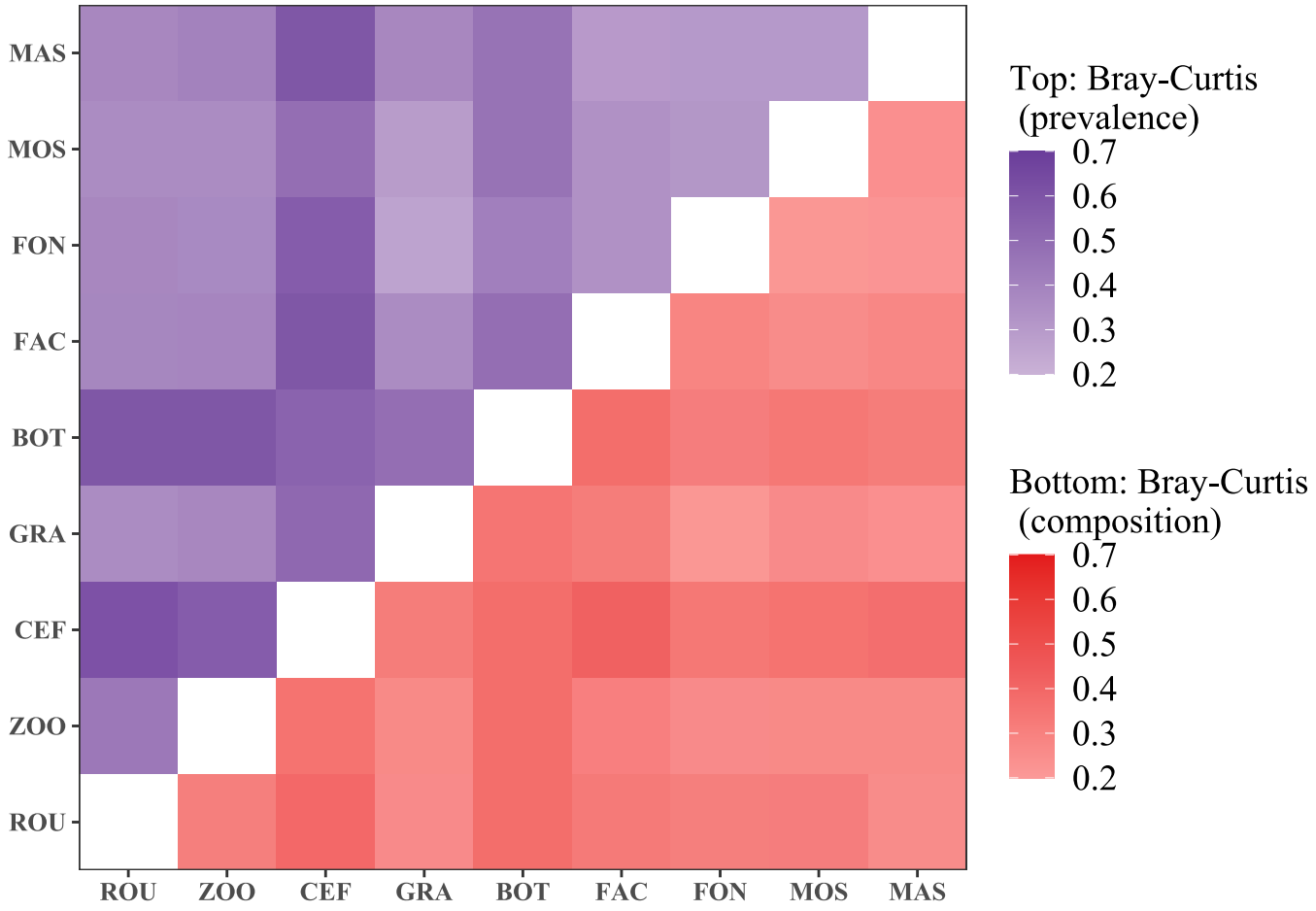

A
FRANCE
Montpellier
B
ROU
Montpellier
43.7°N
43.6°N
43.5°N
43.4°N
43.3°N
Latitude
30 km
3.4°E
3.6°E
3.8°E
4.0°E
4.2°E
Longitude
C
Montpellier
ZOO
CEF
MOS
FAC
BOT
GRA
FON
MAS
43.64°N
43.62°N
43.60°N
43.58°N
Latitude
3 km
3.80°E
3.85°E
3.90°E
3.95°E
Longitude
Naturalness gradient
1
0
-1
-2
-3


A
Nestlings
Prevalence
100
75
50
25
0
Not sampled
Not sampled
Not sampled
ROU N=36
ZOO N=15
CEF N=0
GRA N=12
BOT N=0
FAC N=0
FON N=7
MOS N=8
MAS N=12
URB. TOT. N=54
Site
B
Adults
Prevalence
100
75
50
25
0
ROU N=52
ZOO N=41
CEF N=8
GRA N=45
BOT N=12
FAC N=21
FON N=40
MOS N=37
MAS N=41
URB. TOT. N=245
Site
Genus
Plasmodium
Leucocytozoon


A
Plasmodium
100%
75%
50%
25%
0%
Relative abundance of lineages
ROU
ZOO
CEF
GRA
BOT
FAC
FON
MOS
MAS
Site
Plasmodium lineages
SGS1
YWT4
GRW11
DELURB4
AFR065
unknown
B
Leucocytozoon
100%
75%
50%
25%
0%
Relative abundance of lineages
ROU
ZOO
CEF
GRA
BOT
FAC
FON
MOS
MAS
Site
Leucocytozoon lineages
PARUS74
PARUS4
PARUS72
PARUS77
SYBOR22
PARUS18
PARUS73
PYCGPI03
PARUS16
PARUS70
other


Plasmodium
Leucocytozoon
Occurrence probability in urban areas
1.00
0.75
0.50
0.25
0.00
SGS1
YWT4
Lineage
SFC7
SYBOR08
SYBOR27
PARUS72
PARUS73
PFC2
SYBOR33
PARUS18
PYCGPI03
SYCON05
AFR215
PARUS4
PARUS82
PARUS77
SYBOR22
PARUS74
PARUS70
PARUS16
Lineage
Habitat specificity
Unspecific
More urban


MAS
MOS
FON
FAC
BOT
GRA
CEF
ZOO
ROU
ROU
ZOO
CEF
GRA
BOT
FAC
FON
MOS
MAS
Top: Bray-Curtis (prevalence)
0.7
0.6
0.5
0.4
0.3
0.2
Bottom: Bray-Curtis (composition)
0.7
0.6
0.5
0.4
0.3
0.2