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Fine scale genetics reveals the subtle negative effects of roads on an endangered bat

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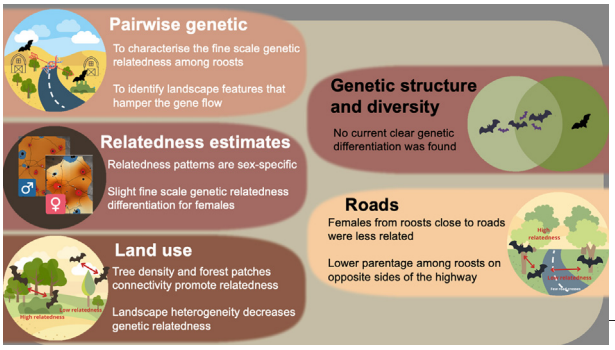
HIGHLIGHTS

- Landscape resistance is the major driver of gene flow for lesser horseshoe bats.
- Effect of landscape features on gene flow is sex-specific, more noticed in females.
- Roads may be acting as semi-permeable filters, slightly reducing gene flow.
- Tree cover and landscape homogeneity promote genetic relatedness.
- No current clear genetic differentiation, but long-term structuring may be ongoing.

GRAPHICAL ABSTRACT



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ABSTRACT

The effective management of species with small and fragmented populations requires an in-depth understanding of how the effects of human-induced habitat disturbance shape the structure and gene flow at fine spatial scales. Identification of putative environmental barriers that affect individual exchange among subpopulations is imperative to prevent extinction risks. Here, we investigated how landscape affects the gene flow and relatedness structure of a population of the endangered lesser horseshoe bat (*Rhinolophus hipposideros*). We also assessed the effects of sexbiased dispersal on genetic relatedness. We genotyped 287 bat samples collected across southern Portugal and developed resistance surfaces for landscape variables hypothesized to affect gene flow. Then, we used spatially explicit models to fit relatedness distance through the resistance surfaces. We found genetic evidence of sex-biased dispersal and identified a significant fine scale structuring in the relatedness regarding females, the philopatric sex. Males displayed uniform levels of relatedness throughout the landscape. The results indicated less relatedness between the female´ from roosts located on proximity of roads than in roosts away from roads. Also, when analysing the sexes together the relatedness on roosts separated by highway were subtly less related in comparison to those occurring on the same side. Roads seem to be major shapers of the contemporary population structure of females, regardless of being relatively recent structures in the landscape. Furthermore, the relatedness patterns detected suggested that high tree density among roosts and continuity of forest patches in broader surrounding areas, promotes the relatedness among individuals. Landscape

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heterogeneity among roosts slightly decreases genetic relatedness. Nevertheless, those relationships are still weak, suggesting that population structuring driven by those factors is slowly ongoing. Thus, effective management measures should focus on issues for promoting safe road passages and suitable habitat corridors, allowing for the exchange of individuals and gene flow among lesser horseshoe bat roosts.

1. Introduction

Over the last century, many bat populations experienced dramatic declines worldwide, mainly due to the destruction of foraging habitats (e.g., deforestation), disturbance of roost sites (e.g., enlargement of urban areas) and the disruption of landscape connectivity throughout human-induced barriers (e.g., construction of linear infrastructures) (Voigt and Kingston, 2016). Despite the loss and radical transformation of natural habitats and unrestrained increase in human disturbance around roosts, the degradation in landscape connectivity is increasingly recognized as a major driver on genetic diversity loss within populations which hamper the gene flow among bat populations. Particularly, the loss of landscape connectivity associated with the presence of linear infrastructures, such as roads, is a well-known phenomenon that plays a major role in shaping the genetic structure of many wildlife populations (Westekemper et al., 2021; Frankham et al., 2002). Road expansion is also a complex phenomenon with multiple deleterious effects on wildlife that are often cumulative with impacts coming from other sources (e.g., diseases and changes in agricultural practices) (van der Ree et al., 2015). Thus, the rapid growth of transportation infrastructures and their ubiquity worldwide, threatens long-term population persistence, increasing the risk of local extinctions, especially for species that are already declining due to other threats (Holderegger and Di Giulio, 2010; Reed et al., 2007).

The lesser horseshoe bat (*Rhinolophus hipposideros*) is a clutter-foraging bat (Abbott et al., 2012; Bontadina et al., 2002) that has suffered one of the most spectacular population decline across most of its distribution range. Such a decline has been attributed to a combination of several factors, including widespread pesticide use, habitat degradation, climate change and genetic inbreeding (Bontadina et al., 2008). Although, since 1990 the lesser horseshoe populations have partly recovered in Europe, as a consequence of stricter environmental regulation policies that protect bat roost sites (e.g., EUROBATS EU, Habitats Directive) (van der Meij et al., 2015; Warren and Witter, 2002), the species is still declining worldwide (Taylor, 2016). Several studies suggest that the lesser horseshoe bat is particularly vulnerable to the presence of linear infrastructures, such as roads (Afonso et al., 2016; Stone et al., 2012; Dietz et al., 2009; Motte and Libois, 2002). Le Roux et al. (2017) reported that the lesser horseshoe bat avoids foraging in areas with a dense road network and Stone et al. (2012) stated that road avoidance is associated to vehicle or roadside lighting disturbance. In the UK, Knight (2006) suggested that large roads with high traffic intensity (e.g., double-carriageway, motorways) were likely to form a significant barrier to the movement of bats. Notwithstanding, Knight (2006) also reported that movements around the roost were not restricted by long-established single-carriageway roads with low traffic. Although not posing a significant resistance to movement, lesser horseshoe bat have a high mortality rate on low traffic roads of (Fensome and Mathews, 2016; Iković et al., 2014; Medinas et al., 2013). Lesser horseshoe bats often fly lower than 4 m above the ground at a low speed, particularly when crossing open spaces (Denzinger and Schnitzler, 2013; Ramalho et al., 2021), hence they are particularly susceptible to roadkill. Thereby, regardless of most roads being considered as recent barriers to gene flow, especially for long-lived species, the combination of direct mortality and road avoidance can rapidly reduce connectivity among subpopulations on either side of the road (Ascensão et al., 2016; Ascensão et al., 2017; Holderegger and Di Giulio, 2010; Fahrig and Rytwinski, 2009; Balkenhol and Waits, 2009).

Previous studies based on radio-tracking have also stressed the importance of woodland patches, well-structured hedgerows, and other linear elements such as treelines in the dispersal movement of lesser horseshoe bats (Tournant et al., 2013; Ramovš et al., 2010; Zahn et al., 2008; Bontadina et al., 2002; Motte and Libois, 2002; Schofield, 1996). However, the importance of these features on the species´ occurrence and connectivity depends on the availability of roosts and on the quality of their surrounding hunting habitats (Boughey et al., 2011; Bontadina et al., 2002; Holzhaider et al., 2002). The lesser horseshoe bat is a particularly sedentary species, usually foraging within 2 km of their summer roosts (Farcy et al., 2009; Bontadina et al., 2002; Holzhaider et al., 2002) and travelling short distances (between 5 and 10 km) from winter to maternity roosts (Hutterer et al., 2005; Crucitti and Cavalletti, 2002; Weiner and Zahn, 2001). Females exhibit high fidelity to their natal colony and live together in crowded maternity roosts, while males generally live as isolated individuals or gathered in small groups (Gaisler, 1966). Tournant (2013) showed that although the genetic variability was evenly distributed throughout the lesser horseshoe bat populations in Eastern France, the weak genetic structure identified among maternity colonies seems to mainly depend on the spatial arrangement of maternity roosts. These results suggest the importance that connectivity among maternity roosts may have in shaping the genetic structure of the lesser horseshoe bat populations. However, information is lacking on which landscape features that may hamper movements between female roosts. Tournant (2013) also showed that even on non-fragmented populations, females from the same roost were more related among themselves than to females from other roosts, and that could lead to population substructure on future generations.

Peterman et al. (2019) recognize that using the most common genetic tools to delimit the geographic boundaries of breeding populations and the assessment of population connectivity can often lead to misleading results, particularly where the population structure is subtle, or in subpopulations that have recent genetic differentiation. These methods may be insufficient to detect the very fine scale and contemporaneity of spatial structuring within non-fully isolated populations (Lowe and Allendorf, 2010; Saenz-Agudelo et al., 2009; Manel et al., 2005). In addition, highly vagile and/or long-lived species like bats are often expected to exhibit a limited population structure, even over large geographic regions. Thus, the analysis of the genetic structure for those species frequently requires genetic markers with a high genomic resolution, such as single nucleotide polymorphisms (SNPs). The advent of high-throughput genotyping technologies also has allowed a large improvement in the ability to quantify fine scale population genetic variation and provide better insights into contemporary subpopulation structure. Furthermore, this information may be associated with landscape features at a fine scale to derive insights into factors affecting population structure (e.g., identification of local or recent barriers) (Balkenhol et al., 2016; Manel and Holderegger, 2013; Wagner and Fortin, 2013; Balkenhol and Waits, 2009). Norman et al. (2017) proposed the “landscape relatedness” method to detect fine scale population structure within a continuous population which provides insights into contemporary genetic processes (e.g., gene flow). This method consists of a new spatially explicit method that allows sorting individuals regarding quantitative relatedness values and detecting divergences from a non-uniform distribution of relatedness throughout the landscape. One key advantage of this kinship-based assignment method is that it characterizes the contemporary population structure based on the existing generations at the time of sampling (Norman et al., 2017; Palsbøll et al., 2010). Differences in relatedness may be attributable to different sex-specific patterns of social organization, as well as to sex-biased dispersal abilities. Several studies about the lesser horseshoe bat have already recognized that differences in dispersal distances travelled by males and females may imply differential genetic structuring of each sex (e.g., Afonso et al., 2016).

For species living in colonies, with highly sedentary and philopatric behaviour, such as the lesser horseshoe bat, it is fundamental to assess inter and intra colony patterns of genetic relatedness, to understand how their social and spatial organization is affected by landscape features. However, no study has yet quantified the potential impact of connectivity loss among lesser horseshoe bat roosts, nor identified how specific landscape characteristics shape genetic relatedness structure, particularly considering females and males separately.

The aims of our study are: (1) to characterize the fine scale genetic relatedness within and among lesser horseshoe bat roosts, separately for females and males; and (2) to identify contemporary landscape features that may be restricting or facilitating gene flow between roosts, for each sex. Therefore, our expectations are that adult females (highly philopatric) will have spots with higher genetic relatedness, promoting population structure among them, while males will display uniform levels of relatedness throughout the landscape. Additionally, we assume that an increase in the amount of unsuitable habitat surrounding roosts and the proximity of roads, will reduce connectivity and consequently decrease genetic relatedness among roosts, particularly for females. Our study clarifies the fine scale spatial genetic relatedness of a threatened bat species, allowing us to identify the key landscape drivers of the contemporary relatedness structure of each sex.

2. Material and methods

2.1. Study area and roost surveys

Fieldwork was conducted in an area comprising approximately 681,000 ha in the Alentejo region, southern Portugal (38°32′24″ to 38°47′33″N; −08°13′33′ to −07°55′45″W; Fig. 1). The topography of the study area is smooth and undulating (150 to 400 m a.s.l.) and the landscape is dominated (>90 %) by open woodlands of cork oak and holm oak (*Quercus suber* and *Q. rotundifolia*), the Portuguese "*montado*", a High Nature Value Farming System, intermixed with extensive open agricultural areas (Paracchini et al., 2008; see also Pinto-Correia et al., 2011 for details of this system). Other land uses are intensive farmland, olive groves, and vineyards interspersed with small human settlements, roads, wetlands, and several small streams with riparian vegetation. The climate is Mediterranean, with the average temperature ranging from 5.8 °C to 12.8 °C in the winter season (January) and from 16.3 °C to 30.2 °C in the summer (July); the average annual rainfall is 609.4 mm (Évora 1971–2000; Instituto de Metereologia, 2010).

Bat roosts were actively searched by surveying thoroughly all favourable sites (e.g., watermills, old buildings, caves) (Medinas et al., 2013). Sites were classified as roosts when lesser horseshoe bats were found inside, flying out, or in their absence, if we found bat droppings. All identified roosts with species presence were visited five times between February 2016 and February 2017. On each roost inspection, we counted all bats inside, along with recording the presence of lactating females and/or young. We captured all individuals with nets and recorded their sex, age, and reproductive state. We also took tissue samples consisting of 3-mm biopsy punches from each wing at a standardized position in the plagiopatagium, while avoiding veins and arteries. We fixed the tissue samples in 70 % ethanol. Bats were then released at the roost within 30 min of capture. When the punch wounds healed up, a distinct scar was visible during the entire sampling period and thus, we were able to differentiate captured bats from recaptures and avoided taking tissue samples from both wings on subsequent roost visits. Capture and handling protocols followed published guidelines for the treatment of animals in research and were performed under licenses (licenses number 667/2016/CAPT and 484/2017/CAPT) from the competent national authority (Instituto de Conservação da Natureza e das Florestas).

2.2. DNA extraction, data processing and population genetic analysis

We collected 327 tissue samples from 36 occupied roosts and extracted DNA for all sampled individuals (captures and recaptures). Total genomic DNA from each bat sample was isolated using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) following the "Isolation of Genomic DNA

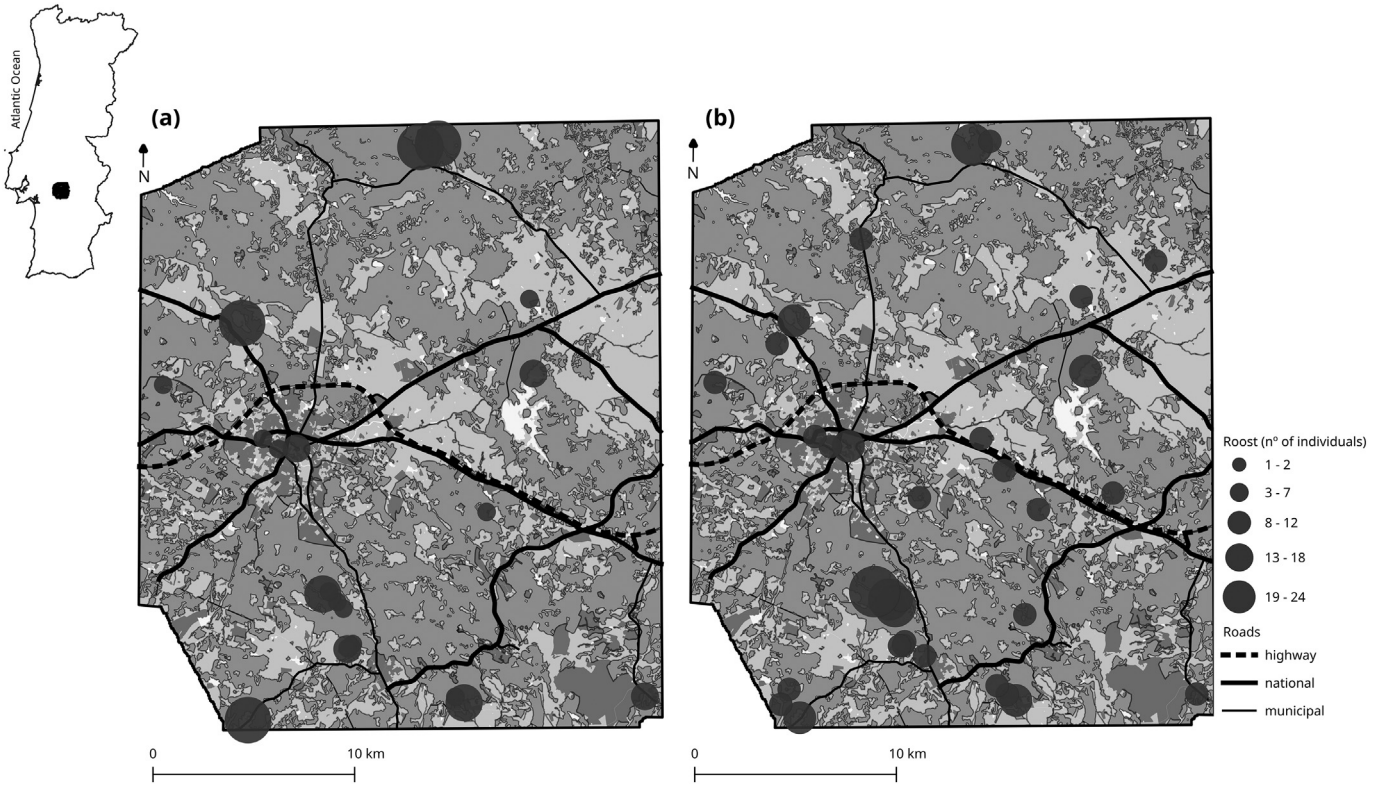

Fig. 1. Map of study area in southern Portugal showing roost locations occupied by (**a**) females (N = 20) and (**b**) males (N = 33). Circles represent roosts and their size represents the number of genotyped individuals per roost. The three main land uses are forest (dark grey), sparse woodland (grey) and open agricultural area (light grey) areas.

from Tissues" protocol. We quantified DNA samples using a *Qubit* 3.0 fluorometer (Life Technologies, Waltham, MA, USA), and normalized them all to 10 ng/μl. Libraries were prepared using a double digest restriction enzyme-associated DNA sequencing (ddRAD-seq) protocol following Peterson et al. (2012). Briefly, DNA in individual samples was cut using the *Mse*I and *Sbf*I restriction enzymes (New England Biolabs), and then individually barcoded using enzyme specific adaptors, where SbfI adaptors had unique 6 bp barcodes for each sample in a 96-well plate. Following a purification step using a 1:1 ratio of AMPure beads (Agencourt) to remove short fragments, we pooled samples in groups of 96, including 42 replicates of randomly selected individuals, resulting in a total of four libraries. For each library, we performed a PCR amplification step with Illumina PCR primers to enrich for fragments including the barcodes and the two enzyme specific adaptors. We pooled the four libraries and performed size selection for 200–400 bp on a 0.2 % agarose gel by manual gel cutting, and the resulting libraries were indexed separately. We then purified the libraries using the MinElute Gel Extraction Kit (Qiagen) and quantified the extracts using quantitative PCR. The libraries were sequenced as single-end reads (using 100 cycles, i.e., amplifying 100 bp per sequence) on four lanes of an Illumina HiSeq 1500 high throughput sequencer at CIBIO-InBIO research centre (Vairão, Portugal).

We demultiplexed the resulting reads using the *process radtags* tool from *stacks* v1.0 (Catchen et al., 2013), and then aligned the reads for each sample against the *R. ferrumequinum* genome (GCA_000465495) using the *mem* function from *bwa* v. 0.7.17 (Li, 2013). Finally, we used *samtools* and *bcftools* to remove PCR duplicates, and perform SNP calling (Li et al., 2009).We used the built-in filters of the software VCFtools for post-processing analyses (Danecek et al., 2011). We selected SNPs to be highly discriminatory with characteristics such as high minor allele frequency and low levels of linkage between SNPs (Norman et al., 2013). Briefly, we excluded loci that had a significant deviation from the Hardy-Weinberg Equilibrium (HWE) and kept in our subset only loci that had a minor allele frequency above 5 %, a minimum quality of the SNP above a phred score of 30, and a maximum of 10 % missing data for both loci and individuals. We further kept SNPs located >10,000 bp of each other to avoid deviations from Linkage Equilibrium. Individuals with multiple sample locations were analysed using only the first capture location. Genetic diversity indices for each sampling location (see Methods: Genetic structure of population and genetic diversity of roost-groups), and pairwise genetic relatedness for all individuals and per each sex (see Methods: Relatedness estimates) were computed.

2.3. Genetic structure of population and genetic diversity of roost-groups

We inferred population structure using two approaches: the Bayesian model-based clustering method implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000) and the analysis of principal coordinates (PCoA) implemented for package dartR in R (Gruber et al., 2018). For STRUCTURE, we determined the most likely number of genetic clusters (K), ranging from 1 to 36 (the number of sampled roosts). We used an admixture model and assumed independent allele frequencies. We performed 10 independent runs, implementing 500,000 iterations following a burn-in period of 100,000 iterations for each value of K. Secondly, to examine genetic structure from a multivariate perspective, we ran a PCoA analysis, not making assumptions about the input data (Jombart et al., 2009). Here, a matrix of genotype distance was created and analysed for genetic differentiation based on the two axes explaining the most variation. Hence, a bi-dimensional plot was made with *gl.pcoa.plot* function in R, to visualize in the first two principal coordinates genetic distances among individuals. We replicated these two approaches to assess the population structure for females and males separately. These analyses were performed to explore whether optimal K was influenced by the structure of females and males within the population.

To assess the genetic diversity, we spatially clustered bat samples from roosts within a radii of 1500 m (hereafter called roost-group) and excluded roost-groups with <2 individuals. The adjacent roosts were clustered to reduce bias associated with small samples. The radii of 1500 m was chosen because this is the average nightly foraging distance of lesser horseshoe bats in a similar landscape (Bontadina et al., 2002). Genetic variation was first assessed by calculating the total number of alleles (*A*) per roost-group, representing the total number of variants for all genotyped loci, the observed (H_O) and expected (H_E) heterozygosities and inbreeding index (F_{IS}) for each roost-group. All these analyses were performed using the *hierfstat* R package (Goudet, 2005). To determine inbreeding within the roost-group, we further used the identity disequilibrium statistic (g2), which measures heterozygosity covariance across loci (David et al., 2007). We also tested a null-hypothesis of no variance in inbreeding in the sample (H_O: g2 = 0), calculating the 95 % confidence interval (CI), via 1000 bootstraps over individuals and the approximate *P*-value via 1000 permutations. All inbreeding analyses were carried out using the *inbreedR* R package (Stoffel et al., 2016).

We also tested for genotypic differentiation between roost-groups for each subset of data analysed (males and females). Levels of differentiation were quantified by pairwise comparison of each subset using F_{ST} estimator values following Weir and Cockerham (1984) and using the calculations in *adegenet* package in R (Jombart and Ahmed, 2011). To evaluate whether levels of differentiation of pairwise population were significant, we compared the distribution of observed F_{ST} *values* with the distribution of F_{ST} *values* obtained from 1000 permutations among roost-groups using a Mann-Whitney *U* test (Sagot et al., 2016). We also tested whether genetic variation was distributed among roost-groups using analysis of molecular variance (AMOVA, Excoffier et al., 1992). Separate analyses were also performed for females and males.

Effective population size (N_e) was estimated for the entire sampled population using the Linkage Disequilibrium approach (Waples and Do, 2010) and considering a random mating model. The Jackknife method was used to estimate the confidence interval. The lowest allele frequency was defined as 0.05 to further reduce bias. The effective population size analyses were carried out in NeEstimator v. 2.01 (Do et al., 2014).

2.4. Relatedness estimates

Individuals' relatedness was derived using the Ajk coefficient (hereafter Ajk-values) (Yang et al., 2010), which estimates how much of the genome two individuals share. This allowed us to infer the most probable relationship between individuals. Ajk-values are expected to be zero for unrelated individuals, and one for an individual with itself. Moreover, first-degree relatives share approximately 50 % of their genome and show Ajk-values close to 0.5. Such relationships include the individual's parents, as parent-offspring or full-siblings. Second-degree relatives (share 25 % of their genome) will have Ajk-values around 0.25, which includes individuals, such as half-siblings or grandparents and grand-offsprings. As lesser horseshoe bats typically display natal breeding fidelity, we expected a higher probability of relatedness between individuals from the same roost than individuals sampled from different roosts.

2.5. Interpolations and Integrated Nested Laplace Approximations (INLA) procedures

The interpolations of genetic relatedness across the study area to detect non-uniformity in the spread of relatedness between roosts, and to detect fine scale structuring, were conducted using INLA (Rue et al., 2009) with the package INLA (version 19.04.16) implemented in R (R Development Core, 2016). INLA handles Bayesian models, which approximate posterior probability distributions via numerical integration as does Markov Chain Monte Carlo (MCMC) simulations, but gaining in computational time (Blangiardo and Cameletti, 2015; Blangiardo et al., 2013). Interpolations were calculated based on one focal roost at a time and pairwise relatedness among roosts was represented by their 95th quantile Ajk-value. We repeated the analysis for each roost (N = 36) so that every roost location was included in an interpolation N-1 times (Norman et al., 2017). Firstly, we ran INLA models with different families to ensure that the Gaussian

distribution was the most appropriate for the 95th quantile values of roost-pairwise Ajk. All interpolations were overlaid with the overall result being the sum of the roosts-based relatedness values across the landscape. Maps for mean relatedness and standard deviations were created using the *levelplot* function from the *lattice* 0.20–38 R package (Sarkar, 2008) at 100 m × 100 m grid. To identify the areas of statistical significance, we divided the mean relatedness values at each grid point by root-mean-square of the standard deviation. We used a two-tailed test with alpha level 0.05 to identify the areas with significant values (values >0.975 or <0.025). Relatedness interpolations for females and males and their areas of significance were in turn analysed following the same procedure as for the dataset with all individuals.

Following Lindgren and Rue (2015) approach to model the spatial correlation, prior to running the interpolations, we integrated on each model a spatial Matérn covariance using the SPDE procedure (Spatial). Therefore, a bi-dimensional mesh was created assuming that roost locations were the initial vertices for the triangulation and additional vertices were added heuristically to minimize the number of triangles needed to cover the region subject (Muñoz et al., 2013). Furthermore, these extra vertices were used as prediction roost locations. The priors and hyperparameters currently implemented in R-INLA were used as default. Additional models were run, including Euclidean distance between roosts (*Dist*) as a covariate and the roost identification as a random factor (*Roost*). Best candidate models were selected based on lowest Deviance information criterion (DIC) (Spiegelhalter et al., 2002) and Watanabe-Akaike information criterion (WAIC) (Watanabe, 2010).

2.6. Landscape relatedness analysis

2.6.1. Landscape genetics-resistance modelling

We assessed relatedness patterns of lesser horseshoe bat roosts across several environmental continuous gradients (e.g., tree density at different scales and distance to roads with different traffic volumes) to identify potential fine scale drivers of bat genetic relatedness patterns (Fig. 2). These analyses were applied for the 95th quantile pairwise Ajk-value between roost pairs considering all individuals and subsequently partitioned for females and males. We followed Roffler et al. (2016) procedures for the selection of resistance surfaces. We used a roost-based approach combined with spatially explicit landscape resistance methods to determine the best predictors (hereafter landscape variables). We calculated a set of 59 landscape resistance surfaces representing different composition and configuration of main land cover types in the study area, e.g., tree cover density at different spatially explicit scales, and distances to streams, human settlements and roads (Ducci et al., 2015; Tournant et al., 2013; Bontadina et al., 2002) (Supplementary Material - Table S1). To calculate resistance surfaces for tree density and land cover maps, we implemented a multi-scale approach by using a moving window analysis. Five spatial scales were selected for tree density (circular windows of 50, 100, 150, 250 and 500 m radii) and two scales for land use (circular windows of 1500 and 3000 m radii). Such scale selection has previously been shown to be ecologically meaningful for lesser horseshoe bat movements and reflecting the core area of the foraging zone (Jan et al., 2017; Tournant et al., 2013).

Explanatory variables were derived from tree density and land cover maps at a 50 m resolution from the Tree Cover Density 2015 Pan-European Copernicus product (Copernicus Land Monitoring Service) (EU-TDC, EEA, 2017) and COS2010 (DGT, 2018), respectively. First, owing to the high number of land cover types occurring in COS2010 [see details for land cover reclassification in Herrera et al., 2016], we pooled the land cover types into only three broad categories: (1) forest (i.e., dense forests of cork and holm-oak with >30 % tree cover); (2) sparse woodland (i.e., natural woodland with tree density < 30 %) and (3) open agricultural areas (i.e., crops such as cereals, vegetables, vineyards and cut forests). To characterize the amount and the spatial distribution of each land cover type and considering the two scales mentioned above, we computed seven landscape configuration indices within the FRAGSTAT 4.1 software (McGarigal et al., 2012) (Supplementary Material - Table S1). In addition, we measured the distance from roosts to highway, national and regional roads, considering all roads together, and only roads with high traffic volume (national and highway). We also measured the distance from roosts to urban areas, human settlements and streams with and without riparian gallery. All resulting maps were transformed into rasters with 50-m pixel size.

2.6.2. Testing and optimizing models of isolation: distance or environmental resistance

To examine the landscape effects on the genetic relatedness structure of the studied lesser horseshoe bat population, we tested patterns of isolation by distance (IBD) (i.e., geographic distance) and isolation by resistance (IBR) (i.e., landscape resistance distance) (Etherington, 2016). We used the 95th quantile Ajk-values among each roost-pair (as response variable) and repeated this framework for each subset analysed (all individuals, females, and males separately). For the IBD analysis, we calculated geographic distances as straight lines between roost-pairs. For IBR, we developed resistance surfaces derived from the inverse of the pairwise least-cost resistance distance, using the *gdistance* R package (van Etten, 2017), of each individual landscape resistance surface. Additionally, the roost pairwise Euclidean distance - considering all pixels in the resistance surface as 1 - was used as the null model. We performed simple Mantel tests to assess whether effective distance was significantly correlated with relatedness and two Partial Mantel tests to evaluate the sign and significance of the relationships: (1) between genetic relatedness distance and resistance distance matrices, while controlling for the effect of Euclidean distance (null hypothesis); and (2) between relatedness distance and Euclidean distance, while accounting for the effects of the landscape resistance surface (Guillot and Rousset, 2013). Significance was assessed using 10,000 randomizations. We used a Mantel test implemented in the *ade4* R package (Dray and Dufour, 2007) and Partial Mantel tests were conducted using the *vegan* R package (Oksanen, 2018).

2.6.3. Multivariate resistance model to map the spatial genetic structure

We built our multivariate resistance surface using only variables with significant results in the Partial Mantel tests while controlling for Euclidean distance, and non-significant results while controlling for resistance surfaces (Koen et al., 2012; Cushman et al., 2010; Wasserman et al., 2010). To account the possible effect of distance among roosts on spatial relatedness structure, we also included the Euclidean distances between roost-pairs. Further, prior to fitting multiple regression models, we excluded the least ecologically meaningful resistance surface from pairs with Pearson's correlation (r) > 0.7 (Hosmer and Lemeshow, 2000). We standardized the resistance surfaces using a z-transformation to allow the parameter estimates to be comparable (Roffler et al., 2016). Finally, to determine the relative importance of optimized landscape resistance distances on relatedness distance, we used multiple regressions of distance matrices (MRDM) applying 10,000 permutations for significance tests (Legendre et al., 1994). The strength of the correlation score was assessed with r (Pearson's correlation coefficient) and model fit assessed with R^2 (Determination coefficient). Models were assessed for multicollinearity and variables with variance inflation factor (VIF) score > 4 were dropped from the candidate model set. We used the Akaike's information criterion corrected for small samples (AICc) and the corresponding Akaike weights (*wi*) to rank candidate models (Burnham and Anderson, 2002). For models with ΔAICc <2, we performed a model averaging approach, averaging parameters, unconditional standard errors (SE) and 95 % confidence intervals (CI) (Burnham and Anderson, 2002). Variable coefficients whose confidence limits included zero were considered not significant (Burnham and Anderson, 2002). To test our first hypothesis that landscape features influence males and females differently, we fitted an identical set of models for each sex data subset. Multi-model inference was implemented using the MuMIn R package (Barton and Barton, 2020). Additionally, MRDM were also performed to control the variation on inter-roosts relatedness associated with a highway (A6) as a putative barrier. Roost pairs were coded into a barrier matrix with a binomial variable representing roosts on the

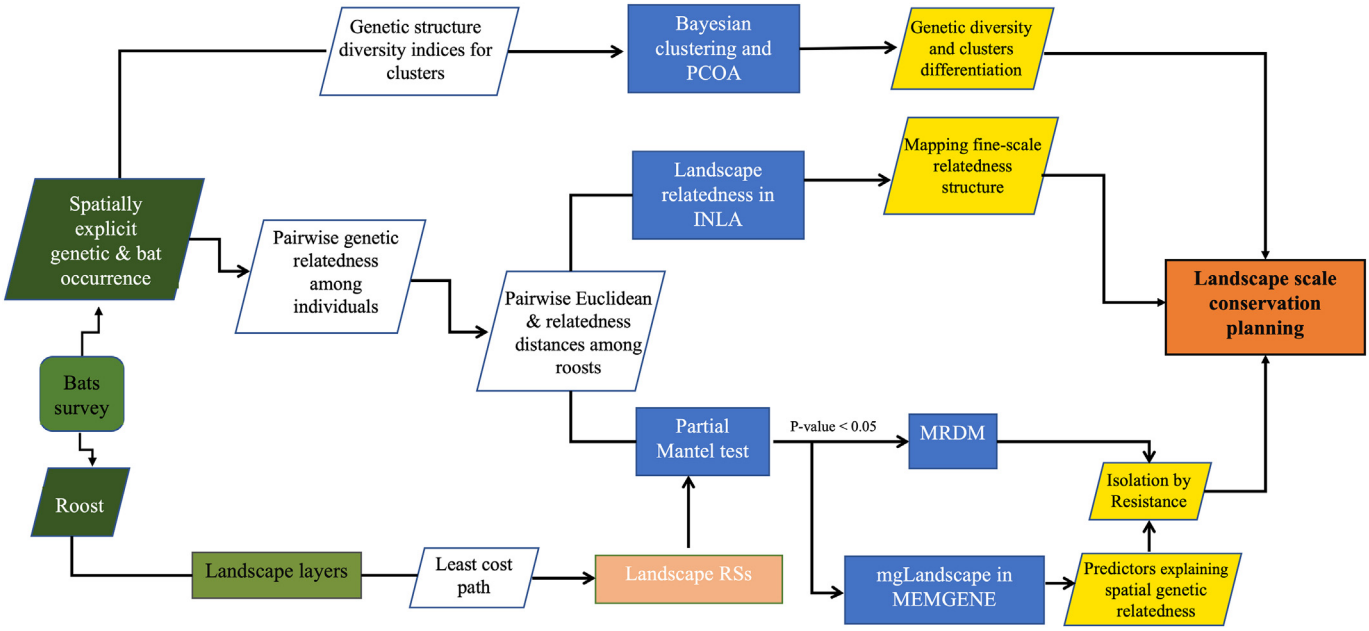

Fig. 2. Simplified flowchart of the methodology. The flowchart depicts inputs (green), intermediate processes and preparatory analysis (white), statistical analysis (blue), results (yellow) and intermediate objectives and conservation issues (orange). Explanation of acronyms: Resistance surfaces (RS), Principal coordinates (PCOA), Integrated Nested Laplace Approximation (INLA), Multiple regression of distance matrices (MRDM), Moran's Eigenvector Maps (MEMGENE). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

same side (0) or opposite sides (1) of the highway that cross the study area. MRDMs were performed using the *ecodist* R package (Goslee and Urban, 2007).

Mantel tests are not fully independent of distance data and do not allow estimation of the amount of genetic variation explained by the spatial pattern of each landscape resistance surface (Legendre and Fortin, 2010). Hence, to further detail the results obtained from the MRDM analysis, the resistance surfaces were re-analysed using the distance based on Moran's Eigenvector Maps (MEM) calculated by a modified version of *mgLandscape* function from the MEMGENE R package (Galpern et al., 2014). This redundancy analysis was applied using the MEM eigenvectors as explanatory variables and the relatedness distance matrix as response variable. This method identifies the amount of variation in genetic distance that is explained by the spatial patterns of each resistance surface and was estimated as the adjusted coefficient of determination R^2 (R^2_{Adj}). This method can detect complex and relatively weak spatial patterns and has been recommended as a powerful alternative to other common analyses applied in landscape genetic studies (Legendre and Fortin, 2010).

3. Results

3.1. Sampling roosts and SNP genotyping

We inspected 97 potential roosts of which 36 were occupied by lesser horseshoe bats. Roosts were found in buildings (55 %), old ovens (22 %), caves (17 %) or abandoned watermills (6 %), in areas surrounded by woodland patches and/or near streams with riparian galleries. A total of 327 bat tissue samples were genotyped and, after removing duplicate individuals (N = 59), were associated to 228 distinct individuals. Among the individuals identified, 132 were females, and 96 were males. The number of resamples per individual varied from one to three. On average, 9.8 individuals were sampled per roost, with colonies comprising one to thirty-one bats (Fig. 1).

The average number of raw RAD-tags per individual was 1,587,224 (min. 6908; max. 8,414,170; SD 1,354,845). After filtering, we obtained a final dataset of 2837 SNPs for 287 samples. Considering the 34 replicates included (and the 59 duplicated samples), we observed a replicability of genotype calls >90 %. All kept loci were in Hardy-Weinberg equilibrium.

3.2. Structure of population and diversity of roost-groups

The 10 independent runs of the STRUCTURE indicate a single genetic cluster and lack of population boundaries among all individuals or among each sex when analysed separately (Supplementary Material - Fig. S1). The PCoA results also show no apparent substructuring between the sampled roosts (Supplementary Material - Fig. S2). After pooling the roosts located within a 1500 m radius, we obtained a total of 21 roost-groups for all individuals (13 roost-groups for females and 20 roost-groups for males). The AMOVA analysis indicated that most of the genetic variation was due to differences among individuals within roost-groups (97 %), and differences among roost-groups explained only 3 % (*P-value* = 0.010). For males, the variation within roost-groups was higher than for females (males = 99 %; females = 95 %, both with *P-value* = 0.010). Locus diversity statistics for each roost-group are presented in Supplementary Material - Table S2. The expected heterozygosity (H_e) was similar across the roost-groups and ranged from 0.31 (roost-group 13) to 0.25 (roost-group 8). For males H_e values ranged from 0.31 (roost-group 15) to 0.23 (roost-group 9) and for females ranged from 0.31 (roost-group 6) to 0.26 (roost-group 7). F_{IS} values were negative in 6 roost-groups for overall bats, in 5 roost-groups for males and in 4 roost-groups for females (Supplementary Material - Table S2). Weak but significant identity disequilibrium was detected in the genotype dataset (g2 = 0.01, CI = 0.01–0.02, *P-value* = 0.012 based on 1000 permutations) considering all individuals. Females also had a significant disequilibrium (g2 = 0.01, CI = 0.01–0.02, *P-value* = 0.011 based on 1000 permutations), while for the males were non-significant (g2 = 0.21, CI = 0.01–0.02, *P-value* = 0.165 based on 1000 permutations).

Pairwise F_{ST} between roost-groups were low to moderate (F_{ST} = 0.014 ± 0.022) and revealed a weak and non-significant level of genetic differentiation among the sampled roost-groups (all Mann-Whitney tests *P-value* > 0.050).

Finally, we estimate that the effective population size ranges from 342 to 517 individuals (95 % confidence interval) with an average of 413 individuals.

3.3. Relatedness estimates and interpolations

We calculated Ajk-values for every possible pair among the 228 individuals resulting in 25,878 Ajk-values. These values ranged from −0.08 to

0.65 with a mean of 0.00 (SD = 0.03). We detected 23 first-order relationships (full-siblings or parent-offsprings) of which over 75 % were from individuals captured in the same roost (N = 18 bat-pairs). We also identified 59 second-order relationships (half-siblings or grandparents - grand-offsprings), from which 43 bat-pairs were sampled in the same roost. Females represent about 70 % of both full- and half-siblings. The 95th quantile Ajk-values between different roosts ranged from −0.05 to 0.64. For males this value was higher than for females (females: 0.53; males: 0.64).

The generated heatmap interpolations based on the best INLA models (Supplementary Material - Table S3) are shown in Fig. 3a, c and e in which the orange and light purple areas highlight levels of unrelatedness that would be expected in panmictic populations (Ajk-values ~0). On the other hand, areas with a high degree of relatedness (Ajk-values >0) or unrelatedness (Ajk <0) are represented in yellow and dark purple, respectively. Fig. 3b shows areas that are statistically significant considering all individuals. Unrelated areas are depicted in red and are mainly concentrated in the centre of the study area, while the eastern and western part of the study area show large areas with a significant degree of relatedness, depicted in blue. The sex partitioned analysis yields different patterns (males - Fig. 3e and f; and females - Fig. 3c and d). Males broadly follow a pattern of relatedness similar to the pattern of all individuals, whereas females show a significantly higher degree of relatedness in opposite sites, north and south, of the study area, with a consistently lower relatedness in the central area, which includes the highway, national roads with high traffic, and their surrounding areas.

3.4. Relative effects of distance and landscape features on relatedness

Mantel test results for IBD showed a non-significant correlation between geographic distance and relatedness (95th quantile pairwise Ajk-values) among roosts for all individuals ($R = -0.053$, *P-value* = 0.819), or considering females and males separately (females: $R = -0.100$, *P-value* = 0.877; males: $R = -0.010$, *P-value* = 0.554). To contrast with the IBD null expectation tested above, Partial Mantel univariate tests were applied to test for IBR using each of the landscape factors of interest and controlling for the effects of Euclidean distance. The correlation between relatedness and IBR (Mantel r-value) was considerably higher for landscape variables characterized within 1500 m radii (Supplementary Material - Table S4). For all individuals the correlation was significant for Euclidean nearest neighbour distance between forest patches (ENeig_FOR) and Aggregation index of forest patches (AInd_FOR) and was negatively correlated with Patch richness (PRic), while for the females' subset it was significant for aggregation index of forest patches (AInd_FOR) and Contiguity index of woodland patches (CInd_WOOD). For males, the genetic relatedness was significant and negatively correlated with Patch richness (PRic) and Euclidean nearest neighbour distance between forest patches (ENeig_FOR). Resistance surfaces describing tree density at 250 m radii (Tden250) were positively and generally better correlated with genetic relatedness for all individuals, as well as for males and females separately. In addition, relatedness among females is lower among roosts in the proximity of national roads (DRnat). All results of the Partial Mantel tests to assess the effect of Euclidean distance on genetic distance, controlling for each variable, were not statistically significant (*P-value* > 0.050) (Supplementary Material - Table S4). The best multivariate model (MRDM) for all individuals showed that relatedness among roosts was positively related in areas with higher tree density and higher aggregation of forest patches and decreased in heterogeneous landscapes. Relatedness for all individuals was also positively related in areas with high neighbourhood of forest patches (contiguous forest) (Table 1; Supplementary Material - Table S5). However, the 95 % confidence interval of this variable overlapped zero and thus the coefficient was deemed not significant (Supplementary Material - Fig. S3). The best models for females, showed strong associations of relatedness to areas with higher tree density and between roosts located further away from roads. Females' relatedness was negatively correlated with contiguous woodland areas and positively related in areas with higher aggregation of forest patches, however none of the coefficients were significant. The best MRDM models for males were uninformative because the confidence intervals for estimated variables coefficients overlapped zero (Table 1; Supplementary Material - Fig. S3). In addition, considering all individuals, the MRDM analysis suggests a slight relatedness structuring of the population, with individuals being less related on opposite sides of the highway, compared to those located on the same side of the highway ($R^2 = 0.016$, *P-value* = 0.073), despite the ratio of the spatial variation on relatedness being low. For females and males analysed separately, there was a non-significant isolation-by-barrier effect between roosts on opposite sides of the highway (females: $R^2 = 0.002$, *P-value* = 0.512; males: $R^2 \leq -0.001$, *P-value* = 0.443).

Table 1
Models explaining relationship between landscape surface resistance and lesser horseshoe bat (*Rhinolophus hipposideros*) relatedness distance (Ajk). Mantel coefficients (r) and *P*-values area shown for relatedness distance in multiple regression of distance matrices (MRDM) models. MRDM model fit (R^2) and *P-values* are also shown. *P-values* < 0.05 are highlighted in bold.

Model	r	*P*	R^2	*P*
All individuals				
intercept	0.013	<0.001	0.049	<0.001
Tden250	**0.002**	**0.016**		
AInd_FOR (1500 m)	**0.003**	**0.012**		
PRic (1500 m)	**<−0.001**	**0.019**		
ENeig_FOR (1500 m)	−0.002	0.121		
Euclidean distance	−0.001	0.379		
Females				
intercept	0.007	0.788	0.053	<0.001
Tden250	**0.001**	**0.029**		
AInd_FOR (1500 m)	0.001	0.726		
DRnat	**0.002**	**0.010**		
CInd_WOOD	−0.002	0.052		
Euclidean distance	0.001	0.347		
Males				
intercept	0.009	0.251	0.012	0.140
Tden250	0.001	0.554		
ENeig_FOR (1500 m)	−0.001	0.168		
PRic (1500 m)	−0.001	0.364		
Euclidean distance	<0.001	0.542		

Variables described in Table S1: Tden250 = Tree density at 250 m scale; ENeig_For (1500 m) = Mean distance of nearest forest patches neighbour at distance of 1500 m scale; AInd_FOR (1500 m) = aggregation index of forest patches at 1500 m scale; DRnat = distance to national roads; PRic (1500 m) = Patch richness at 1500 m scale and CInd_WOOD = Contiguity Index woodland patches at 1500 m scale).

3.5. Spatial explicit mapping of genetic structure

Moran's eigenvectors (MEM) derived from Euclidean distances ([abc]) (Supplementary Material - Table S6) explained a slightly lower proportion of spatial relatedness variation for all individuals ($R^2_{adj[abc]} = 0.147$) than the MEM eigenvector derived from the Patch richness resistance surface (with $R^2_{adj[abc]} = 0.185$). Resistance surfaces that best explain the spatial relatedness between females were highly associated with the MEM eigenvector based on distance to national roads (DRnat) ($R^2_{adj[abc]} = 0.197$) and Tree density-based resistance surface (Tden250) ($R^2_{adj[abc]} = 0.147$) (Supplementary Material - Table S6). Spatial relatedness between males was better explained by the MEM eigenvector based on Tree density-based resistance ($R^2_{adj[abc]} = 0.281$). All models indicate a good fit, as fractions of spatial genetic distance that are explained by resistance surfaces [a], are higher than the fractions explained by coordinates [c] (Supplementary Material - Table S6).

4. Discussion

This study is the first to evaluate, at a fine scale, the effect of landscape resistance to movement on the genetic relatedness among bat roosts. We used the endangered lesser horseshoe bat (*Rhinolophus hipposideros*), a

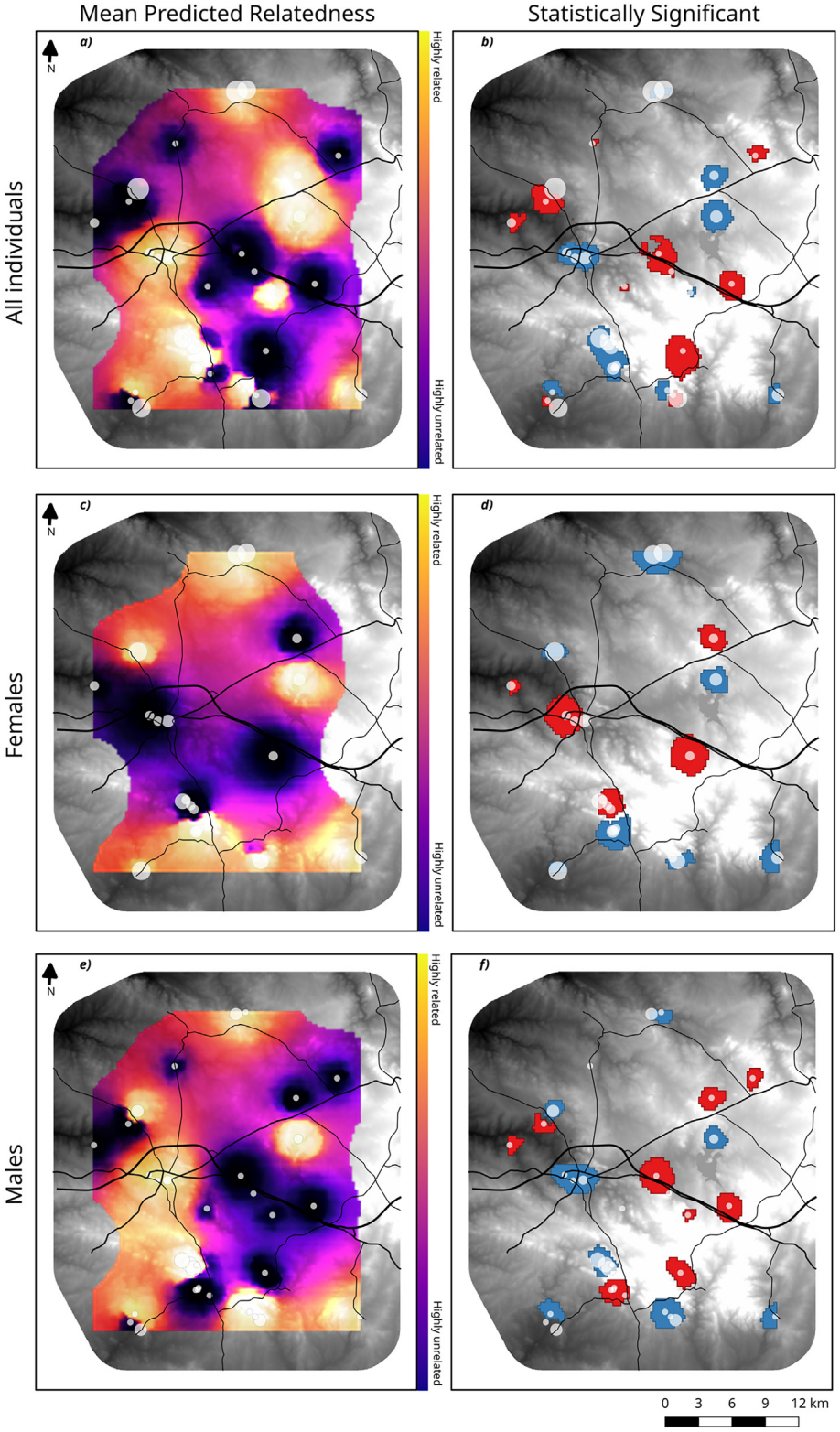

Fig. 3. Relatedness interpolation maps. The *first column* shows interpolations of the entire study area ("global") for maximum pairwise relatedness of all individuals (a), females (c), and males (e) per roost, where yellow areas represent a high degree of relatedness between individuals (Ajk values >0) and purple areas represent a low degree of relatedness between individuals (Ajk values <0). The *second column* shows areas of statistical significance derived when the cumulated mean over the root-mean-square falls within the alpha level of 0.05. Areas that are significant indicate that individuals in these areas are significantly more (*blue*) or less (*red*) related to the population than expected by chance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clutter-foraging bat, highly susceptible to roadkill, as a case study. Spatially explicit methods were used to assess relationships between landscape features and genetic differentiation considering all individuals, as well as females and males separately. Our results show that there is no clear genetic differentiation on the overall studied population and that no subpopulations can be inferred (e.g., PCoA and STRUCTURE results). Nevertheless,

we found a slight differentiation in fine scale genetic relatedness: females from roosts closer to roads and separated by open areas (lower tree cover) were less genetically related. Tree density promotes gene flow, with individuals from roosts located on homogenous habitats linked by forest patches being more genetically related. Additionally, the analysis of fine scale genetic relatedness revealed a decrease on parentage levels among roosts located on opposite sides of the highway, comparing to those located on the same side of the highway.

4.1. Road impacts on relatedness structure

Previous studies based on mtDNA and microsatellite data have shown that genetic differentiation of lesser horseshoe bat populations was solely found in populations separated by major geographic barriers, such as the Pyrenees or the Alps (Dool et al., 2013), and in other *Rhinolophus* species, in populations separated by large distances, e.g., 150 km for greater horseshoe bat (Rossiter et al., 2000) and 200 km for Mehely's horseshoe bat (*R. mehelyi*) (Dragu and Borissov, 2011). Our broad population genetic analysis agrees with these findings. However, using fine scale genetic analysis, with highly discriminatory genetic markers (SNPs) and parentage analysis, our study points out that proximity to major roads, as well as habitat suitability (non-fragmented landscape with high tree cover), may play a role in the genetic structuring of the bat population, acting differently for females and males. It is possible that the suggested landscape effects on slowdown of gene flow may still be too weak to identify a clear genetic differentiation. Thus, the time lag (~20 years) since the construction of the putative barrier (A6 highway was built in 1999), may be too short for genetic patterns to become apparent, considering the long generation time (2 years on average with range of 1–3 years) of lesser horseshoe bats (Gaisler, 1966). Several studies highlight that negative genetic effects of roads need time to become detectable, i.e., dozens of generations, for spatial genetic structure to build up, especially when considering long-lived species (Murphy et al., 2008; Barton and Wilson, 1995). This suggests that many actual wildlife populations separated by roads may already have a weak genetic differentiation (Holderegger and Di Giulio, 2010; Berry et al., 2004) although not yet translated in a formal genetic structuring in subpopulations. Additionally, roads are not a complete barrier to movement in the study area, as suggested by several bat movements between roosts on opposite sides of national roads and highway. Thus, the roads may be acting as a semi-permeable filter, slightly reducing movement and gene flow across space and time. Indeed, the results of the relatedness analysis suggest that a long-term structuring of lesser horseshoe bat populations on opposite sides of the road may be ongoing, but may only be fully noticed further ahead. Other studies on the genetic effects of roads have shown that population structure can arise relatively quickly whenever there is a strong barrier to gene flow, which can happen when several strong impacts (e.g., road mortality, Clark et al., 2010; road avoidance, Ascensão et al., 2017; road barrier, Epps et al., 2005) concur simultaneously and particularly concerning species with low population sizes (Holderegger and Di Giulio, 2010; Frankham, 2005). We estimate a low population size for the lesser horseshoe bat in the study area (effective population size <500 individuals) and thus, road mortality should be also a major conservation concern. While the influence of road mortality in hampering gene flow between populations has been demonstrated for highly vagile and non-volant mammals (e.g., bobcats; Riley et al., 2006), to the best of our knowledge, no studies have assessed the potential impact of road casualties on genetic diversity of bat populations. Previous bat roadkill studies have shown that clutter-foraging species such as the lesser horseshoe bat, that fly close to the ground (heights varying from 0.15 m to 4 m), are particularly vulnerable to vehicle collisions (Fensome and Mathews, 2016; Medinas et al., 2013). Our roadkill surveys in the studied area, found that the lesser horseshoe bat is the threatened species with the highest mortality rate (0.1 bat/km/year, Medinas et al., 2021). Thus, road mortality often results in reduced local abundances and decreased rates of individual exchange among populations on different sides of roads and may lead to adverse genetic effects (decreased genetic diversity and increased inbreeding) (Fahrig and Rytwinski, 2009; Frankham, 2005).

4.2. Influence of sex-biased dispersal on relatedness

Also consistent with our results is the fact that a fine scale local genetic structuring can occur and be triggered by social mechanisms not linked to the movement ability of lesser horseshoe bat, i.e., can be affected by sex-specific traits and social barriers. Overall, uniform levels of genetic relatedness among males throughout the study area indicate that males may disperse further and more frequently. On the contrary, relatedness in females tends to be concentrated in some kin clustering, with female social groups being located mainly in maternity roosts. Moreover, our genetic relatedness results support a strong philopatric behaviour of females (Schofield, 1996) and that gene flow between distant female roosts is limited (Dool et al., 2016). Although the information on the reproductive behaviour of lesser horseshoe bat is still scarce, we can reasonably assume a polygynous mating system, with bats living in separate sex-groups, such as the system found in the greater horseshoe bat *Rhinolophus ferrumequinum* (Rossiter et al., 2002, 2000), or in the Schreiber's bat *Miniopterus schreibersii* (Pereira et al., 2009). In the lesser horseshoe bat, breeding females from a roost are mostly offsprings of females from the same roost, and family units are probably matrilineal. Although the formation of cohesive familiarity groups seems to improve bat fecundity and breeding success (Clutton-Brock and Lukas, 2012; Kerth, 2008), it also increases the risk of inbreeding, especially when populations are small and isolated. Our inbreeding tests showed that females are more genetically related among them comparatively to males. This result also suggests a reduced individual exchange between different roosts or that male offsprings may disperse to adjacent areas, and consequently mate with related breeding females (decreasing pairwise F_{ST} and increasing pairwise Ajk). Based on our genetic data covering a 1-year period, we recorded a low number of inter-roost individual exchanges (3 inter-roost movements for each sex), all movements with short distances travelled (all individuals: mean = 7.1 km). Hesketh (1951) and Hooper and Hooper (1956) also recorded that in small populations of lesser horseshoe bat, individuals rarely move >5 km. Additionally, considering the low proportion of male dispersal and short distances between father-offspring roosts (mean = 7.0 km, Medinas, unpublished data), it is plausible that father-daughter mates may also occur. Females covered a maximum distance larger than males (14.2 km and 9.4 km, respectively), although the low number of inter-roost individual exchanges registered prevent us from gathering robust conclusions about this issue. Other study also reported that females are able to fly >12 km on dispersal movements and return to their maternity roost after two days (Weinberger et al., 2009).

Furthermore, our data suggests that reduced landscape connectivity may be constraining mating between individuals from different and geographically distant roosts. Mating seems to mainly occur between single males from geographically close roosts and females from the same matrilines over several generations (Medinas, unpublished data). This mating structure is not concordant with the pattern of non-random mating to avoid inbreeding, recorded in France for lesser horseshoe bat (Tournant et al., 2013) or in England for the greater horseshoe bat (Rossiter et al., 2000), whereupon matings occurred between breeding females and males from different matrilines or immigrant males (Storz, 1999).

4.3. Impacts of landscape features on genetic relatedness

Overall, the configuration and extension of forest area among roosts (e.g., large forest patches with high tree density and proximity of other forest patches) are the main landscape features that promote genetic relatedness. On the contrary, higher landscape heterogeneity, representing intermixed small patches of woodland and large open agricultural fields, seem to restrict bat movements, particularly for females (Bontadina et al., 2002; Motte and Libois, 2002). Proximity to roads also appears to limit female movements contributing to a lower relatedness among roosts located

near these infrastructures. For males the landscape features create relatively little resistance to gene flow. The presence of unsuitable habitat can be partially overcome if there are scattered roosts (old buildings in our case) across the landscape that could act as stepping-stones for long distance movements or as satellite roosts, which bats use temporarily during night-time (Tournant et al., 2013; Holzhaider et al., 2002). Tournant et al. (2013) in Franche-Comté region, suggested that the low genetic differentiation on lesser horseshoe bat populations was due to the high landscape suitability context, characterized by isolated old farm buildings surrounded by large forest patches, facilitating male long-distance dispersal movements and access to other potential maternity roosting sites. Our study area includes numerous old farm buildings, though most of them are usually unsuitable for bat roosting owing to human abandonment, coupled with a prolonged degradation (frequently with no roof) (Voigt and Kingston, 2016; Sachanowicz and Wower, 2013). Thus, a scarcity of roosting places exists, which besides contributing to the low effective population size (342 to 517 individuals), may also decrease landscape connectivity and hinder gene flow between roosts.

4.4. Conservation implications

High rates of gene flow between roosts through the promotion of individual interchange are essential to minimize inbreeding depression and to improve long-term survival of bat populations (Rossiter et al., 2012). Indeed, even vagile and flying species, can have their movements narrowed, and hence have gene flow between subpopulations partially constrained by barriers (e.g., roads). These effects are of particular concern for clutter-foraging species, such as the lesser horseshoe bats. Promoting landscape functional connectivity and gene flow are key actions to mitigate landscape resistance to movement and road-barrier effects. This can be achieved through protection or restoration of potential movement corridors and by encouraging bats to use road underpasses or cross at secure flight heights. Although our results show only a weak indication of relatedness structuring due to the presence of roads and less suitable habitats around roosts, they should be interpreted as a warning signal. Roads can act as a semi-permeable filter over long periods of time, consolidating genetic isolation of bat populations on either side of the road, and being a progressive silent driver to subpopulation structuring or local extinctions. Thus, to fully understand the magnitude and importance of these possible threats, additional research is required, particularly in identifying key factors that may influence bat behaviour along roads and in developing/improving road mitigation actions for bats. Some conservation measures to facilitate bat movement across roads such as the construction of specific overpasses or underpasses which manipulate bat behaviour (e.g., Zeale et al., 2018), have already been essayed. Recent studies have suggested that the efficiency of these measures increases when they spatially overlap commuting routes of clutter-adapted species (Claireau et al., 2019; Laforge et al., 2019; Voigt and Kingston, 2016; Abbott et al., 2012). However, it is still unknown whether these devices are truly effective in reducing road-barrier and landscape resistance effects on bat movements, and thus how they are contributing to sustain long-term population viability and genetic connectivity.

5. Conclusion

Our results concur with other studies suggesting that roads, unsuitable habitat, and sex-specific traits (e.g., high roost fidelity and strong female philopatry) may limit connectivity among lesser horseshoe bat and may be major shapers of contemporary population genetic structure for females. However, the time lag since road construction and/or species long generation time may hamper the detection of an early and clear genetic differentiation. These findings are likely extendable to other philopatric and clutter-foraging bats, many of which are threatened, for which the role of subtle barriers and sex-biased characteristics warrant further investigation to determine their relative effects on population structuring and whether their continuous impact on movements may lead to significant population structuring in future generations.

Combining animal movement data with patterns of genetic relatedness can ultimately ensure a deeper understanding of the spatial, social and population dynamics of a wild species. Moreover, incorporating multiple alternative pathways (e.g., circuit theory) on analyses, which more realistically approximates dispersal behaviour, can increase the accuracy of the conclusions. The effect of roads on fine scale genetic structuring among roosts suggests that management measures, aiming to increase across-road and landscape connectivity for bats, would be worth to be considered as early as possible. Our findings also demonstrate that, based on powerful genetic markers, we can infer contemporary processes that shape population structure and provide insights at a fine scale about the main drivers of genetic relatedness. Thus, our approach can be a powerful tool in future research efforts in ecology, management, and conservation-oriented programs.

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Credit authorship contribution statement

D.M., J.T.M., S.S. and A.M. conceptualised the research; S.S. and A.M. supported with resources; D.M., H.R., J.P. and A.M. developed the framework; D.M., V.R collected the data; D.M., F.V. and S.B analysed the data; D.M. led the manuscript writing. All authors significantly supported to the writing and reviewing and agreed for the definitive manuscript version to be eligible for publication.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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