

Batconnect: an integrative approach combining molecular, behavioural and ecological data to assess the effects of roads on bats

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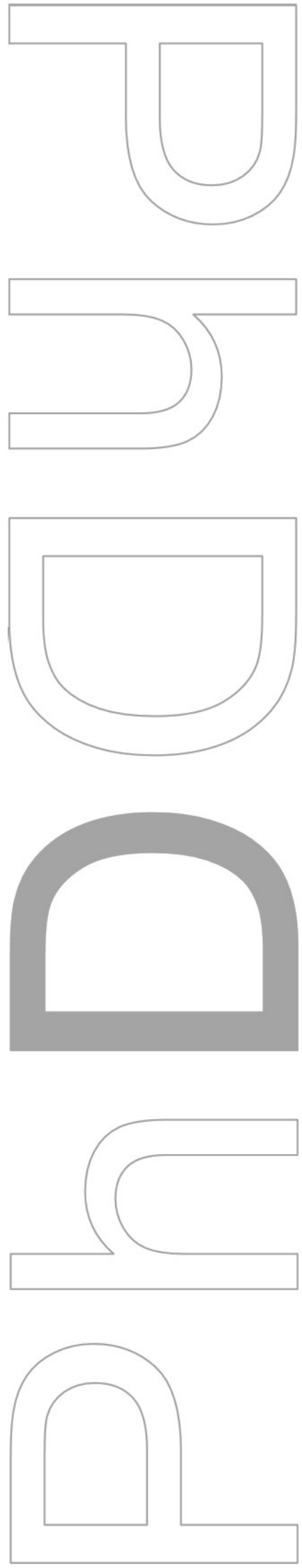
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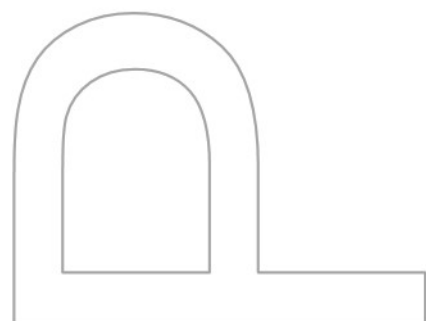
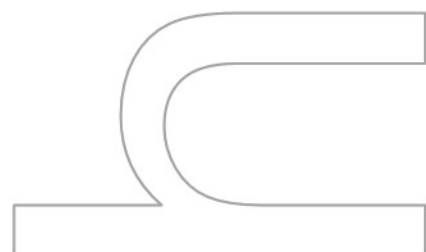
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Para a minha avó Gertrudes.

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After 8 years of starting this journey (excessive amount of time to finish the thesis,...I know), I found a blank page. I think its time to do a retrospective analysis and to do a kind of balance about last years. Firstly, if I start now, maybe the journey is completely different. I think that could have waited to be sure if I want to get a PhD, maybe not. But, I hope that can be the beginning of the next battles.

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Resumo

O desenvolvimento das infraestruturas de transporte é uma das principais pressões antropogénicas na vida selvagem, devido à fragmentação ou degradação do habitat, mortalidade direta provocada pela colisão com veículos e à barreira ao movimento dos indivíduos entre as parcelas de habitat. Estes fatores quando combinados podem levar a uma redução da conectividade da paisagem e consequentemente do fluxo genético entre populações e condicionar a viabilidade populacional a longo prazo. A influência destes fatores negativos pode estender-se até várias centenas de metros para além da estrada. A perceção de como as estradas afetam a vida selvagem é essencial para desenvolver um conjunto de estratégias que minimize os seus efeitos negativos, particularmente para espécies que sofreram recentemente declínios populacionais.

Nesta tese, foram combinadas técnicas moleculares e informação recolhida no campo para avaliar os efeitos ecológicos das estradas na comunidade de morcegos. Este grupo tem um conjunto de características que os torna altamente suscetíveis aos efeitos potenciais das estradas e são um dos grupos de vertebrados mais ameaçado na Europa. Os objetivos específicos da tese são os seguintes: i) entender a importância do contexto da paisagem na dinâmica dos padrões de mortalidade por atropelamento; ii) quantificar a distância de efeito da estrada para morcegos com características ecológicas distintas e avaliar como varia este efeito da estrada em diferentes contextos de paisagem, e iii) identificar os elementos paisagísticos que afetam o fluxo genético e a estrutura de parentesco para uma população de morcego-de-ferradura-pequeno (*Rhinolophus hipposideros*).

A combinação entre os dados de atropelamentos de morcegos e a informação de deteção remota mostraram que os pontos-negros de mortalidade podem mudar ao longo do tempo acompanhando as alterações da adequabilidade do habitat nas zonas envolventes da estrada. Estas pequenas alterações locais da adequabilidade estão sobretudo relacionadas com a diminuição da produção vegetal e o aumento do stress hídrico. Os dados mostram também que a atividade dos morcegos diminui com o aumento da distância à estrada e que a área de influência da estrada depende das características ecológicas dos morcegos. Os dados genéticos mostram que as estradas podem afetar o fluxo genético, de forma diferente para cada um dos sexos. As fêmeas que têm abrigos mais próximos das estradas são menos relacionadas geneticamente

do que as fêmeas que ocorrem em abrigos afastados destas infraestruturas. A cobertura arbórea e a homogeneidade das parcelas de floresta entre os abrigos promovem a conectividade genética.

Em suma esta tese sublinhou a evidência que as estradas podem ser uma forte ameaça à conservação dos morcegos, uma vez que têm um papel fundamental na estruturação genética das suas populações, dificultam o movimento e a atividade nas suas proximidades e aumentam significativamente a mortalidade por causas não-naturais. Desta forma o principal desafio é perceber como é que podemos aumentar a conectividade e a permeabilidade das estradas sem aumentar o risco de colisão com os veículos. O nosso trabalho destaca também que é fundamental manter os programas de monitorização de atropelamentos durante longos períodos e esta monitorização deve ser complementada com estudos da dinâmica populacional para perceber os efeitos das estradas a longo-prazo. É necessário promover a conectividade da paisagem e que as medidas de mitigação sejam implementadas o mais breve possível.

Palavras-chave: Conectividade da paisagem, Deteção remota, Efeito barreira, Ecologia de morcegos, Gestão rodoviária, Habitat na envolvimento, Parentesco genético, Ponto-negro mortalidade, *Rhinolophus hipposideros*, Zona de efeito da estrada.

Abstract

The development of transport infrastructure is one of the main human-related pressure on wildlife, mainly due to habitat fragmentation or degradation, direct mortality by collision with vehicles and hampering of the movement of individuals among habitat patches. When combined, these factors can decrease landscape connectivity, reducing gene flow between populations on either side of the road and long-term population viability. Additionally, these negative effects can extend their influence on distances of several hundred meters away from roads, into the surrounding landscape. Understanding how roads affect wildlife is essential to developing strategies that reduce their negative impacts, especially for species whose populations are declining.

In this thesis, I combined molecular and field-based techniques to assess the ecological impacts of roads on bats, one of the most threatened groups of European vertebrates. Bats have several life-history traits that place them at high risk from road impacts. Specifically, the thesis aims at: i) understanding the importance of landscape-context on the dynamics of bat roadkill patterns; ii) quantifying the road-effect zone for different bat guilds in different road-surrounding characteristics; and iii) identifying how landscape affects the gene flow and genetic relatedness structure in a population of the endangered lesser horseshoe bat (*Rhinolophus hipposideros*) population.

Bat casualties and freely available remote sensing data show that bat roadkill hotspot locations may shift along time accompanying spatiotemporal changes in habitat suitability, assessed by change in vegetation productivity and water stress. Our results also show that bat activity increased with increased distance from roads, but road-effect zone was guild-specific. Based on genetic data, we have shown that effects of roads on gene flow may be sex-specific, being more noticed in females. Females from roosts close to roads were less genetically related. Tree cover and landscape homogeneity promote genetic relatedness.

Overall, this thesis underlined the substantial evidence that roads are a potential threat to bat conservation, playing a particular role on the contemporary population genetic structure of lesser horseshoe bat, hampering the activity of bats in immediate vicinity of the road and increasing the non-natural mortality. A key challenge for bat conservation is resolving how to increase the permeability of roads without increasing the roadkill risk. We suggest that monitoring roadkills across several years should be accompanied by

the evaluation of bat populations dynamics and that management measures to increase landscape connectivity would be worth to be considered as early as possible.

Keywords: Bat ecology, Road barrier effect, Genetic relatedness, Hotspot location changes, Landscape connectivity, *Rhinolophus hipposideros*, Road-effect zone, Road-surrounding habitat, Road management, Remote sensing.

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List of Abbreviations

ADS	ANIMAL DETECTION SYSTEM
AI	ARTIFICIAL INTELIGENCE
AIC	AKAIKE INFORMATION CRITERIA
Ajk	INDIVIDUALS' RELATEDNESS COEFFICIENT
ALAN	ARTIFICIAL LIGHT AT NIGHT
CI	CONFIDENCE INTERVAL
ddRAD-seq	DOUBLE DIGEST RESTRICTION ENZYME-ASSOCIATED DNA SEQUENCING
DIC	DEVIANCE INFORMATION CRITERION
DOI	DIGITAL OBJECT IDENTIFIER
e.g.	EXEMPLI GRATIA, FOR EXAMPLE
EEC	EUROPEAN ECONOMIC COMMUNITY
EIA	ENVIRONMENTAL IMPACT ASSESSMENT
EN	ESTRADA NACIONAL
EP	ESTRADAS DE PORTUGAL
ERDF	EUROPEAN REGIONAL DEVELOPMENT FUND
et al.	ET ALII, AND OTHERS
EU	EUROPEAN UNION
FCT	FUNDAÇÃO PARA A CIÊNCIA E TECNOLOGIA
GLM	GENERALIZED LINEAR MODEL
GLMM	GENERALIZED MIXED LINEAR MODEL
HLC	HOTSPOT LOCATION CHANGES
HPMV	HIGH-PRESSURE MERCURY VAPOUR
INLA	INTEGRATED NESTED LAPLACE APPROXIMATION

IUCN	INTERNATIONAL UNION FOR CONSERVATION OF NATURE
KDE	KERNEL DENSITY ESTIMATION
km	KILOMETER
km ²	SQUARE KILOMETER
LED	LIGHT-EMITTING DIODE
LRE	LONG-RANGE ECHOLOCATORS
m	METERS
M	ESTRADA MUNICIPAL
MEM	MORAN'S EIGENVECTOR MAPS
MRDM	MULTIPLE REGRESSIONS OF DISTANCE MATRICES
MRE	MID-RANGE ECHOLOCATORS
NDVI	NET PRIMARY VEGETATION PRODUCTIVITY
NDWI	NORMALIZED DIFFERENCES WATER INDEX
°C	DEGREE CELSIUS
PCoA	ANALYSIS OF PRINCIPAL COORDINATES
PCR	POLYMERASE CHAIN REACTION
R^2_{Adj}	ADJUSTED COEFFICIENT OF DETERMINATION
RADS	ROADSIDE ANIMAL DETECTION SYSTEM
SD	STANDARD DEVIATION
SE ou Std. Error	STANDARD ERROR
SNPs	SINGLE NUCLEOTIDE POLYMORPHISMS
SRE	SHORT-RANGE ECHOLOCATORS
STAMP	SPATIOTEMPORAL ANALYSIS OF MOVING POLYGONS
TEMPERATURE	LAND SURFACE TEMPERATURE

TOA	TOP-OF-ATMOSPHERE REFLECTANCE
UK	UNITED KINGDOM
USA	UNITED STATES OF AMERICA
VIF	VARIANCE INFLATION FACTOR
WAIC	WATANABE-AKAIKE INFORMATION CRITERION
WETNESS	WETNESS TASSELED CAP TRANSFORMATION INDEX
<i>wi</i>	AKAIKE WEIGHTS
WVC	WILDLIFE-VEHICLE COLLISION
μl	MICROLITRE

Chapter 1

Introduction

1.1 Current status and future perspectives of the global road network

Global biodiversity is declining at an unprecedented rate as a result of human-induced disturbances in the environment (Pimm et al. 1995; Sala et al. 2000). Roads are an example of anthropogenic activity that negatively affect wildlife and ecosystems and represent a major driving force of biodiversity loss (Forman et al. 2003). In the last decades, a multitude of factors are promoting rapid road expansion worldwide, including a quest for increased regional and international trade, an easier movement of persons and goods, and better access to valuable resources (timber, mineral or arable land, energy sources and infrastructures, etc.). In developing nations, new roads, or road improvements, such as asphaltting, are expected to sustain greater prosperity of rural livelihoods. In this way, roads can promote substantial social and economic development. However, they also open a Pandora's box of environmental problems. Furthermore, while the road network is expanding and both traffic volume and speed are increasing, roadless areas are shrinking in number and size (Forman et al. 2003).

Roads are undoubtedly a dominant feature of most landscapes worldwide. There are more than 64 million kilometres of roads that fragment landscapes around the world. If current trends continue, by 2050, paved roads will increase by 25 million kilometres worldwide, 90 % of which are in developing nations which still sustain exceptional levels of biodiversity and provide vital ecosystem services (Dulac 2013). In the conterminous United States of America, 83% of the territory is now within 1 km of the nearest road and road density is 0.67 km/km² (Forman & Alexander 1998; Riitters & Wickham 2003). In most of Europe, almost 25% of all land area is located within 500 m of the nearest road and about 50% is within 1.5 km (Torres et al. 2016). Moreover, worldwide road density is 1.95 km/km². The densest road network is in Central Europe, particularly in Belgium, The Netherlands, and Luxembourg with 3.48 km/km², whereas regions with low road density are in the north and in mountainous regions (e.g., Iceland, Andorra, Austria). Global traffic volume is expected to more than triple by 2050, growing from 38 trillion passenger per year in 2017 to more than 105 trillion passengers per year by 2050 (OECD 2019). Most of these passengers will still be in developing countries, which will also have a five-fold increase in the number of vehicles by 2050 (Fulton & Eads 2004; Ree & Smith 2015). The magnitude of these predictions on growth of paved road extension, traffic

volume, and number of passengers may be a matter of debate, because will be strongly conditioned by the availability and pricing of resources (e.g., fuel), as well as development and/or environmental policies (e.g., climate change mitigation) (Laurance et al. 2014). However, the rate of expansion is so high that it often overwhelms the capacity of environmental planners and managers to properly implement measures to soften the impacts of the expansion of the road network on biodiversity (Forman et al. 2003; Laurance et al. 2009). Laurance et al. (2014) have proposed a global and continental scheme to prioritize road construction considering the environmental costs of infrastructure expansion while maximizing its benefits for human development. The challenge for society is to acknowledge whether the need for the enlargement of the world road network road is strictly necessary and, if so, to find efficient solutions to lower its ecological impacts.

1.2 Spatial extension of the impacts of roads: The 'road effect zone'

Roads are a major source of nonnatural wildlife mortality, surpassing hunting. They also create a barrier to individual movements, limiting genetic exchange and jeopardizing ecological connectivity (Forman & Alexander 1998). Roads penetrating into wilderness or frontier areas are a major proximate driver of habitat loss and fragmentation and environmental degradation, often with irreversible impacts on ecosystems (Brown et al. 2006; Jackson & Fahrig 2011). The ecological impacts of roads are not limited to the site of the road itself, but often extend into the surrounding landscape, over a distance known as a 'road-effect zone' (Forman & Alexander 1998).

Knowing the average width of the road-effect zone permits us to estimate the proportion of land ecologically affected by roads and help road practitioners to properly mitigate its ecological impacts (Forman et al. 2003). Roads potentially impact 15 % - 20 % of the territory of the conterminous United States, despite only covering 1 % of its land area (Forman & Alexander 1998). In the European Union, the road-effect zone is estimated to affect 35 % of the territory and 5 % of Natura 2000 area (Claireau et al. 2019b). The road-effect zone is characterized by declines in species richness, population size, and/or individual fitness (e.g., lower fecundity, poor body condition) (Eigenbrod et

al. 2008). The effects are stronger in the proximity of the roads and gradually vanish with increasing distance to the infrastructure (Eigenbrod et al. 2008). The extent to which roads negatively affect species is difficult to quantify because it depends on intrinsic characteristics of the road (e.g., width, surface type, elevation relative to adjacent landscape), traffic (volume and speed), landscape features (e.g., surrounding land uses, vegetation structure), and species traits, (e.g., volant, nonvolant) (Forman & Alexander 1998; Forman & Deblinger 2000). The area of impact can range from distances lower than 100 m, for sedentary species, to more than 5000 m for highly mobile species (e.g., large mammals) (Benítez-López et al. 2010; Bennett et al. 2013; Rotholz & Mandelik 2013)

Studies show that the abundance and species richness of breeding birds often decline near roads, with larger reductions occurring close to high-traffic roads (Palomino & Carrascal 2007; Griffiths et al. 2010). The main explanation is that traffic noise affects bird reproductive rate by reducing foraging time (Quinn et al. 2011). However, road-effect zones have also been documented for other taxonomic groups including insects (Bhardwaj et al. 2018, 2021), amphibians (Eigenbrod et al. 2008; Grace & Noss 2018), reptiles (Murphy et al. 2022) and mammals (Benítez-López et al. 2010; Ascensão et al. 2017a). The magnitude of road impacts is likely to vary over time (Balkenhol & Waits 2009; Kleinschroth et al. 2016). Most impacts are felt since construction and remain throughout the operation phase unless mitigation actions are implemented.

1.3 Impacts of roads and traffic on wildlife and ecosystems

The effects of roads and traffic are diverse and complex, resulting in a range of negative impacts on wildlife, populations, communities and landscapes (Trombulak & Frissell 2001; Fahrig & Rytwinski 2009). Most of the effects of roads are often deleterious and, if severe enough, can reduce the size of wildlife populations and increase the risk of local extinctions (Van Der Ree et al. 2015). Furthermore, multiple impacts can be cumulative, accelerating the potential long-term decline in the population viability (Jackson & Fahrig 2011; Ceia-Hasse et al. 2017). Possible effects of roads on wildlife include direct mortality from vehicle collisions, habitat loss and degradation, barrier to movement, or disturbance from light, noise, and pollutants. Roads often are also a

corridor for invasive species (Brown et al. 2006). The main ecological impacts of roads are shown in Figure 1.1 are detailed hereafter.

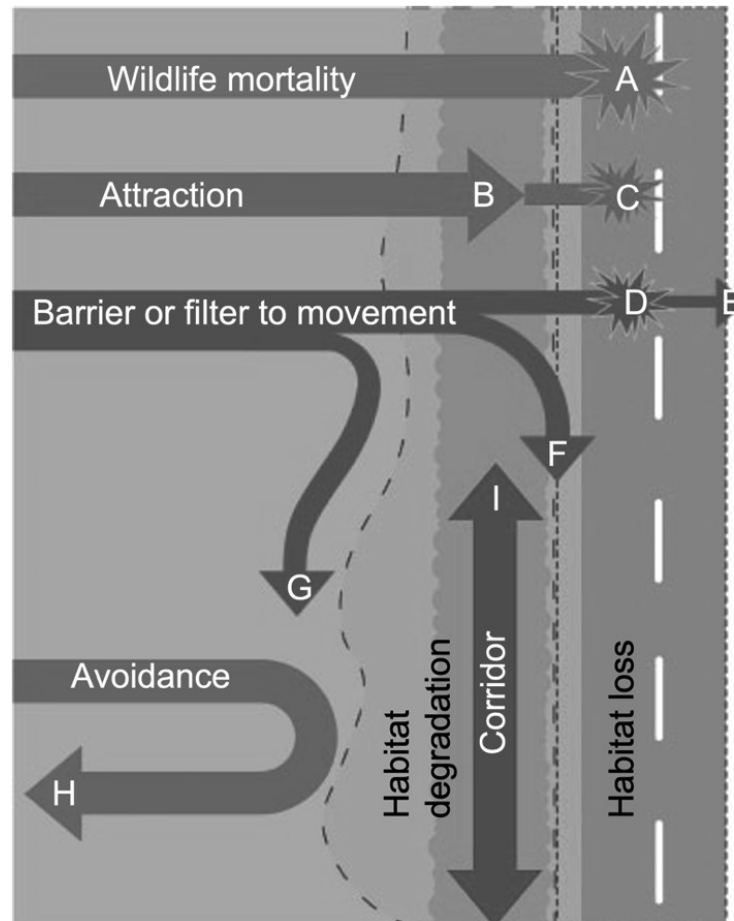


Figure 1.1 - Roads have wide-ranging impacts on wildlife, population and local ecosystems, including: A) wildlife mortality due to wildlife-vehicle collisions (WVC); B) attraction to resources on the road or roadside; C) which may result in death due to WVC; Barrier or filter reduces the movement of individuals across the road that D) attempt to cross are roadkilled or E) some make it across, while F) others are deterred from crossing by the road or G) degraded roadside habitat. H) Other species actively avoid the road or degraded habitat, while some species use the roadside as habitat and/or corridor for movement (adapted from van der Ree et al., 2015).

1.3.1 Road mortality

Although road infrastructure is a relatively recent source of wildlife mortality, it accounts for millions of deaths every year through animal vehicle collisions. In some European countries, the estimated number of birds roadkilled per year is 27 million in

England and 9.4 million in Germany (Erritzoe et al. 2003). In the USA, early estimative were 80 million birds/year (Erickson et al. 2005), but more recent data point to up to 340 million birds/year (Loss et al. 2014). In Australia, 5.48 million frogs and reptiles are roadkilled yearly (Forman et al. 2003), and in Denmark, collisions cause an annual loss of approximately 5-20% of the adult population of spadefoot toad (*Pelobates fuscus*) and brown frogs (*Rana temporaria* and *R. arvalis*) (Hels & Buchwald 2001). These numbers illustrate the magnitude of this human-induced phenomenon.

Roadkill is relatively nonselective and it can affect healthy as well as weak individuals from most terrestrial species, being them rare or common (Polak et al. 2014). A large proportion of the population can be lost, which is particularly worrying for endangered species, like many bats (Gaisler 2009; Fensome & Mathews 2016). Road-associated mortality effects include reducing population sizes and densities (van Langevelde et al. 2009), shifting sex ratios (Kantola et al. 2019), and reducing genetic diversity (Ascensão et al. 2016). Differential road mortality of individuals of different sexes or life stages can affect the dynamics of populations and demography in the long-term.

Most roadkill studies focus on large mammals (e.g., (Kang et al. 2016; Ascensão et al. 2017a) due to drivers' safety reasons. However, currently, road and conservation agencies are increasingly cooperating to implement measures (e.g., building wildlife passages associated with barriers or fences that guide animals toward them) that minimize roadkill for different groups of wildlife, such amphibians, birds, and bats. However, the success of these measures depends on knowledge of the temporal and spatial patterns of roadkill (Forman & Alexander 1998) and can be compromised by the lack of accurate information about location and/or timing of roadkill (Baxter-Gilbert et al. 2015). Road mortality frequently also shows a spatially clumped distribution, with mortality hotspots occurring often along road sections adjacent to high suitability habitats (Gomes et al. 2009; Gagné et al. 2015). Spatial pattern of hotspots it's not stationary overtime, resulting from the interaction of several biotic and abiotic factors (e.g. land use, water availability, road surface temperature, canopy density).

1.3.2 Habitat loss and degradation

Habitat loss is a direct consequence of replacement of the original habitat by road pavement and verges. Moreover, the easier access of people to the roadside encourages further development on those areas (e.g., new agricultural plantations and livestock production), which are an indirect source of habitat loss often resulting in further clearing vegetation, allowing establishment of invasive species or livestock that may cause overgrazing (Gelbard & Belnap 2003; Gelbard & Harrison 2003). The area of indirect habitat loss and its ecological impact can largely exceed the area of direct loss (Claireau et al. 2019b). The open spaces may attract light-demanding species and may be avoided by others (edge effect), which may chronically interrupt or alter species movements, dispersal and migration routes or gene flow (Holderegger & Di Giulio 2010).

1.3.3 Habitat Fragmentation and Road Barrier Effect

Roads cause habitat fragmentation by dividing continuous habitat into separate patches and changing biotic and abiotic conditions in their vicinity (e.g., wind speed, temperature, moisture, pollutant concentration) of the original habitat and at patch edges (edge effect). The change of conditions in hard edges along linear clearings, particularly along forests, affects mostly interior-specialist species that are often replaced by edge-adapted and generalist species (Van Der Ree et al. 2015).

Restricted wildlife movements due to the presence of roads are often linked to an aversion or total impediment of animals in crossing the road. For some species, roads can impede accessibility to resources such as foraging habitats, breeding sites, or mates. Roads create a break in natural habitats, introducing unnatural shapes, wide open spaces, and changed habitat along road verges. They also decrease landscape functional connectivity (facility of species-specific movement across the landscape) (Holderegger & Di Giulio 2010) and promote home range rearrangements (Bissonette & Rosa 2009). Habitat fragmentation can contribute to smaller and more isolated populations. The barrier effect has been documented in a large diversity of taxa including the grizzly bear (Proctor et al. 2019), the caribou (Wilson et al. 2016), squirrel gliders and

koalas (Dudaniec et al. 2013), voles and mice (Grilo et al. 2018), frogs (Grace & Noss 2018), beetles (Rotholz & Mandelik 2013) and bumblebees (Muñoz et al. 2015). Species with large territories (e.g., carnivores), travelling long daily distances to feed (e.g., bats), or migrating species (e.g., caribous) seem to be particularly affected (Dyer et al. 2002; Kantola et al. 2019).

1.3.4 Road avoidance

Avoidance of roads, that is, refraining from the use of the road and the road effect zone, has been demonstrated for many wild species. Avoidance behaviour can be due to three main causes: (1) road surface avoidance; (2) traffic avoidance; and (3) vehicle avoidance. Species with road surface avoidance may approach the road but will be reluctant to venture onto the road surface, resulting in a barrier to movement. Road surface avoidance may depend on the surface material. For example, cactus mice *Peromyscus eremites* avoid paved surfaces, but will cross dirt roads of the same width (Brehme et al. 2013). This component of road avoidance is a short-range phenomenon, as it occurs only at the physical border of the road. Traffic avoidance includes wildlife refraining from any long-ranging traffic disturbance, such as noise, light and vibrations (Reijnen et al. 1996; Forman & Alexander 1998). It may extend to the entire road-effects zones, causing individuals to avoid areas from a few meters to several kilometres from roads (Benítez-López et al. 2017). Species with high noise avoidance, such as birds, will stay away from noisy roads and will avoid crossing (Reijnen et al. 1996). Vehicle avoidance is a short-range and circumstantial behavioural response in which species avoid approaching or crossing roads when vehicles are passing. It may depend on the speed of the approaching vehicle (Erritzoe et al. 2003).

Road avoidance is species-specific with species with a larger relative brain size having a higher ability to evaluate the risk associated with roads and traffic (Husby 2017). High road avoidance means that individuals undertake fewer attempts to cross the road. This will reduce traffic mortality, but will also reduce gene flow and increase inbreeding chances, hindering long-term population viability (Epps et al. 2005).

1.3.5 Attraction: road verges as refuge or corridor

Roads and roadsides can also attract some species by providing resources or improved opportunities. In some highly modified landscapes (e.g., intensive agricultural or plantation areas), road-related habitats (e.g., road verges) may act as last remnants of natural or semi-natural habitats being valuable for biodiversity conservation. (Vandeveldt et al. 2014). In those circumstances, survival of individuals in a particular landscape may depend on the existence of verges. For plants, including invasive species, and small fauna (e.g., insects, snails, small mammals, lizards, snakes), vegetated road verges may provide relevant resources including feeding opportunities, refuge and/or movement corridors (Carthew et al. 2013; Rotholz & Mandelik 2013; Bernes et al. 2016; Galantinho et al. 2017). However, the value of verges for conservation depends on their vegetation management. For instance, regular clearcuttings to prevent fire ignition, which are now mandatory in Portugal, strongly reducing the potential of verges for biodiversity conservation.

1.4 Road effects on genetic structure (genetic diversity, genetic differentiation, and gene flow)

When individuals interact with roads, mortality, barrier effect, and avoidance behaviour are three well-known causes of gene flow reduction (Forman et al. 2003). All of them limit dispersal and the exchange of individuals and alleles between populations from either side of the road, potentiate genetic drift and inbreeding (Remon et al. 2019). They can lead to the loss of genetic diversity within populations while increasing divergence between populations. Roadkill also produces a continual reduction in abundance, which can have a large long-term negative impact on genetic diversity. When its rate is high, it contributes to accelerating genetic divergence in populations separated by roads (Keyghobadi 2007). Additionally, in small populations, inbreeding tends to be higher, and the chances of expression of recessive detrimental alleles and the loss of rare alleles is high, reducing the fitness of affected individuals (Manel & Holderegger 2013). However, the negative effects of inbreeding and genetic drift could be hampered if individuals and genes are regularly exchanged among populations from different locations (i.e., the two sides of the road) (Holderegger & Di Giulio 2010). In the

last decades, there has been an increased interest in understanding the effects of roads on the genetic composition and structure of populations (Carvalho et al. 2018; Tay et al. 2018; Westekemper et al. 2021). Available research range from plants and ground beetles or to amphibians and mammals (see review in Holderegger and Giulio, 2010). Overall, these studies showed that roads, mainly highways and high-traffic roads, promote genetic structure in a wide range of species with different life-history traits. From habitat specialists, such as koala *Phascolartos cinereus* (Dudaniec et al. 2013), grizzly bear *Ursus arctus* (Proctor et al. 2019), and roe deer *Capreolus capreolus* (Hepenstrick et al. 2012) to habitat generalist like coyote *Canis latrans* (Riley et al. 2006), badger *Meles meles* (Frantz et al. 2012); or from low mobility species like red-backed salamander *Plethodon cinereus* (Marsh et al. 2008), timber rattlesnake *Crotalus horridus* (Clark et al. 2010) and, bank vole *Myodes glareolus* (Gerlach & Musolf 2000) to highly mobility species as lesser horseshoe bat *Rhinolophus hipposideros* (Medinas et al. 2023) or European wildcat *Felis silvestris* (Westekemper et al. 2021). However, some species and populations are more vulnerable to roads than others, and the complex combination of road and landscape-related features (i.e., road density, land uses) with species traits (i.e., locomotion features, habitat specialization) plays an important role on the level of gene flow and on the putative influence of roads on fitness-relevant genetic variation. Thus, the effects of roads on population genetic patterns and of populations living in their surroundings still need clarification. Moreover, the current genetic structure may reflect historical rather than present landscape composition and configuration (Holderegger & Di Giulio 2010). Several studies highlight that negative effects of small population size such as genetic isolation, particularly on long-lived species, require time to become detectable, i.e., several generations are needed for spatial genetic structure to be built up (Murphy et al. 2008). Many roads have only recently been built (less than 50 years). Thus, only a small number of generations have passed since construction. This suggests that many current wildlife populations may still be weakly genetically differentiated (Murphy et al. 2008; Holderegger & Di Giulio 2010). In addition, most studies report that roads and even highways and motorways are rarely complete barriers to animal movements (Pritchard et al. 2000; Riley et al. 2006). Thus, roads may be acting like a semi-permeable filter, slightly reducing movement and gene flow across time. However, Riley et al. (2006) reported that coyotes, despite often crossing the road, did not necessarily mate with individuals on the opposite side, shaping the structure of population.

Until the end of the 20th century, it would be difficult to detect any genetic effects of roads by using standard population genetic approaches, particularly for highly vagile and/or long-lived species (Holderegger & Di Giulio 2010). Most of these methods readily detect subpopulations that are highly differentiated (i.e., with little gene flow across them), but the subtle road barrier effect can only be detectable by new and fine-scale methods that allow infer the contemporary population structure and recent migration of animals across roads or highways (Manel & Holderegger 2013; Rolland et al. 2015). These rely on high-throughput genotyping technologies that allow sequencing of large sets of genome data (e.g., single nucleotide polymorphisms - SNPs), and parentage level analysis that detected relationships among all population individuals providing reliable estimates, at a fine-scale, of the contemporary genetic structure (Palsbøll et al. 2010; Jensen et al. 2019).

1.5 Roads and bats

Nowadays, human activities negatively affect the well-being and survival of bat populations, and many species are endangered in many regions of the world (Jones et al. 2009; Voigt & Kingston 2016), including areas with dense anthropogenic infrastructures such as the conterminous United States and Western Europe (Fensome & Mathews 2016; Laforge et al. 2022). Bats have several ecological traits that make them highly susceptible to the impact of roads and traffic. Bats use large areas of the landscape to forage, and some species have seasonal migrations - between winter and summer roosts - of usually 50 or 100 km, or annual migrations over thousands of kilometres (Hutterer Rainer et al. 2005). In general, more mobile species are more susceptible to roadkill. Bats have low reproductive rates and long generation times. Species that have low reproductive rates but long generation times (bats frequently have generation time about 5 years (Mundinger et al. 2022) (Altringham 2011) tend to be more vulnerable to habitat loss and will recovery slowly from disturbance events (Forman & Alexander 1998; Schorcht et al. 2009).

1.5.1 Bat life history

Bats are an extremely diverse group inhabiting a wide variety of habitats, having, among mammals, a unique combination of adaptations, including active flight, echolocation, and hibernation. There are more than 1,450 bat species distributed throughout the world, comprising more than one fifth of all living mammal species (Simmons & Cirranelo 2023). They are the second largest order of mammals (Dietz, C., Nill, D. and von Helversen 2009). Although they are small mammals (the Bumblebee bat weighs about three g and fruit bat can reach up to 1,500 g, they have several life history traits in common with many large mammals. They have a long lifespan and take several years to reach sexual maturity, and often produce only one pup per year (Fenton 1997). Therefore, they may be unable to withstand even moderate increases in mortality, without avoiding population declines (Schorcht et al. 2009). In addition, many bat species are gregarious, living in colonies that concentrate thousands of individuals in a unique location. If this is disturbed or destroyed, many individuals will be affected simultaneously.

1.5.2 Bat Conservation Status

The combination of all the traits mentioned above makes bats very vulnerable to human impacts. Thus, it is not surprising that in the European Union all bat species are protected by law (Annexes II and/or IV of the Habitat Directive – Council Directive 92/43/EEC). Similar laws apply to bats in other European countries, North America, and Australia. This legislation strictly protects all bats from being killed, injured, taken or disturbed, and their roosts are protected from damage or destruction. Despite this, in the last decades there has been a dramatic worldwide decline in bat populations. Globally, 40% of all bat species are currently listed as threatened with extinction (Vulnerable, Endangered, Critically Endangered), Near Threatened, or Data Deficient by the International Union for the Conservation of Nature (IUCN 2023).

There are 28 bat species reported in continental Portugal, and all are protected by EU legislation. Among these six are Threatened, three are Near Threatened, and four

species are categorized as Data Deficient (Mathias et al. 2023). The major threats to bats identified by IUCN assessments are land use changes (e.g., agricultural intensification, logging, livestock raising), urbanization, persecution, quarrying, and general human intrusions on habitats (Voigt & Kingston 2016). Bats are also particularly vulnerable to habitat loss, disturbance and non-natural mortality, including mortality induced by collisions with vehicles (Fensome & Mathews 2016).

1.5.3 Effects of roads on bats

Despite the amount of evidence that roads can significantly affect bats, only in the last 20 years significant attention has been given to this topic (Fensome & Mathews 2016; Ramalho & Aguiar 2021). The few existing studies have been carried out in North America (Zurcher et al. 2010; Bennett & Zurcher 2013; Russo-Petrick & Root 2023) and Europe (Gaisler 2009; Medinas et al. 2013; Claireau et al. 2019b) and more recently in South America and Asia (Silva et al. 2020; Ramalho et al. 2021).

1.5.3.1 Habitat loss and degradation (light, noise, and chemical pollution)

Roads are a major threat for bats, as they fragment and degrade natural areas and reduce access to important habitats (Kerth & Melber 2009; Bennett & Zurcher 2013; Berthinussen & Altringham 2017; Laforge et al. 2022). Furthermore, migration routes and home ranges or territories are bisected by roads, which may reduce landscape connectivity for some bat species, due to the road barrier effect (Zurcher et al. 2010). Road construction and auxiliary structures directly destroy and degrade foraging habitat (Laforge et al. 2022). A typical double carriageway (2+2 lines) road replaces on average two ha of habitat per kilometre. The addition, hard shoulders, junctions, service areas, and other structures can remove even more habitat for bats. Many roosting sites may also be lost due to tree cutting, particularly at hedges and along water courses, and old buildings demolition (Abbott et al. 2012a). Some bat species also avoid landscapes that are artificially lit at night (Stone et al. 2012; Zeale et al. 2018), and many are less active in areas with traffic noise (Siemers & Schaub 2011; Bennett & Zurcher 2013) (please see details below), both contributing to reduce bat abundance and diversity.

Light –

The influence of artificial lighting is likely to be one of the most significant factors determining bat movements across the landscape (Blake et al. 1994; Hale et al. 2015; Stone et al. 2015; Elmeros et al. 2016). Roadside lighting has a species-specific effect (Stone et al. 2009). Slow-flying species, such as *Rhinolophus* spp., avoid lit landscapes (Threlfall et al. 2012). On the contrary, fast-flying (e.g. *Eptesicus* spp.) and generalist (e.g. *Pipistrellus* spp.) species show an attraction to light and can exploit lit roads because of the rich food resource they may provide (Threlfall et al. 2012). Stone et al. (2015) argue that increased bat foraging activity near roads, especially in open areas, should mainly be due to artificial lighting, including car headlights, which attract insects (Bhardwaj et al. 2021). Regarding street (road) lighting, Blake et al. (1994), in England, reported that roads equipped with orange (low-pressure sodium) lamps were associated with a two-fold increase in bat activity compared with unlit roads. In roads with blue-white lamps (high-pressure mercury vapour, HPMV), a five-fold increase in activity was reported. In Germany, species responded differently when HPMV lamps were replaced by LEDs (Lewanzik & Voigt 2017). *P. pipistrellus* strongly reduced activity with LEDs, while light-averse *Myotis* spp. increased its activity at the same sites (Lewanzik & Voigt 2017).

Elmeros et al. (2016) suggest the use of artificial lights to induce avoidance or attraction behaviour in bats toward roads and safe passages, respectively. However, these alterations may affect bat species differently (please see Section 1.5.8 Implications for conservation and mitigation measures). In some circumstances, they may increase insect availability near the roads, could increase the roadkill risk. For example, artificial lighting in bridges reduces the effectiveness of bat safe crossings and increases the risk of mortality (Bhardwaj et al. 2020). Moreover, adding lighting to wildlife crossing structures would make them more accessible to human-use, increasing disturbance and hampering their use by other nocturnal wildlife. Thus, despite the potential of manipulating bat behaviour and reducing roadkill and barrier effects, using different lightening schemes, more research is needed before it can be widely used in road mitigation programs.

Noise –

Noise induced by traffic disturbs bat foraging efficiency and movements of bats (Siemers & Schaub 2011; Bennett & Zurcher 2013). Species that use passive listening, i.e., those that rely on listening for prey rustling sounds to find food, such as *M. myotis*, significantly reduce their foraging efficiency in environments with high levels of noise (Schaub et al. 2008). Thus, it is likely that habitats adjacent to noisy roads would be unattractive as feeding areas for some bat species (Finch et al. 2020). However, traffic noise is likely to have a significant effect only over relatively short distances, as high-frequency sounds are attenuated rapidly. Siemers and Schaub (2011) reported that degradation of habitat due to noise for foraging of passive listening species extends up to 60 m from roads. Furthermore, noise may also affect the acoustic characteristics of bat sound. Bats exposed to noise increased the number of daily echolocation sequences and decreased the number of social calls for intra-specific communication (Song et al. 2019). The sound and movement of vehicles may be interpreted as a threat by some bat species, causing them to adopt defence behaviours against predators, deviating from the road (Baxter et al. 2006).

Chemical Pollution –

Chemical pollution is another significant factor that can affect bat fitness and behaviour in the vicinity of roads. Some studies have evaluated the direct exposure of insects, and indirectly bats, to chemical pollution. However, a road effect zone and its impacts on bats have not yet been quantified. Road developments may also pollute and degrade wetland foraging habitats by runoff from the pavement. Abbott et al. (2009) report that a decline in river water quality led to a reduction in bat activity and in foraging activity of *P. pipistrellus*.

1.5.3.2 The road-effect zone

The road-effect zone on bats is widely documented and quantified for several species with different habitat requirements, road types, and different landscape contexts (Zurcher et al. 2010; Berthinussen & Altringham 2012; Kitzes & Merenlender 2014; Medinas et al. 2019; Ramalho & Aguiar 2021). Studies worldwide show that activity and species

richness decline with proximity to major roads. Gleaning and slow-flying species tend to be less active near major roads (Zurcher et al. 2010; Kitzes & Merenlender 2014). On the contrary, fast-flying and aerial hawking bats may have higher activity on road edges than in the surrounding agricultural habitat (Laforge et al. 2019). Berthinussen and Altringham (2014) report effects up to 1,600 m from a six-lane motorway in north-west England (30 – 40,000 vehicles/day) even for the most common species (e.g., *P. pipistrellus*). In North America, Kitzes & Melber (2014) show that bat activity 300 m from the road was twice as high as bat activity adjacent (zero meters) to the road. However, the relationship between bat activity and the distance to the roads is nonlinear and highly dependent on the surrounding habitat and functional traits of the species. Medinas et al. (2019) found that when roads cross high-quality habitats (e.g., Mediterranean woodland – Montado), bats tend to avoid the immediate proximity of roads. On the contrary, when they cross open agricultural areas, where resources are scarce, bat activity near the road is similar to the activity registered 1,000 m away from it. These results suggest that road verges may be a valuable habitat, likely to be used for feeding, when roads cross open agricultural fields (Laforge et al. 2022). Similarly, Vandeveld et al. (2014) show that bat activity is higher over railway verges than in the surroundings intensive agricultural habitat, particularly for *Nyctalus* spp. and *Eptesicus* spp.

Long-range echolocators (e.g., *Nyctalus* spp. and *Eptesicus* spp.), which generally forage in wide open spaces, as well as above the level of the tree canopy level are more tolerant to the presence of roads (Claireau et al. 2019b). On the other hand, short-range echolocators (e.g. *Myotis* spp. and *Plecotus* spp.), which are adapted to fly in woodlands and hedgerows (Frey-Ehrenbold et al. 2013), consistently select woodlands away from roads to feed, avoiding areas along road edges. Overall, the road-effect zone for insectivorous bats is likely to depend on the availability of prey on road verges compared to the surrounding landscape, tending to be smaller when the surrounding landscape is less vegetated or corresponds to large homogeneous agricultural areas, where insect availability is often poor (Bhardwaj et al. 2018).

1.5.3.3 The road-barrier effect

Roads may act as a barrier by interrupting bat flight lines and restricting access to important roosting and foraging sites. A study in Germany found that motorways can restrict habitat accessibility for *M. bechsteinii* females, resulting in smaller foraging areas (Kerth & Melber 2009). Migration routes may be constrained by the presence of the road (Zurcher et al. 2010), reducing ecological connectivity among bat populations.

Roads that interrupt bat flight lines along linear habitats (e.g., treelines and hedgerows) are particularly problematic for bat movements. Bats have been found to reverse their course when approaching roads, and even small gaps of less than 5 m in treelines along flight routes may cause some bats to change direction and veer away from their commuting routes (Russell et al. 2008; Zurcher et al. 2010; Bennett & Zurcher 2013). However, Abbott et al. (2012b) observed that many bats passed above the road where mature hedgerows had been severed, even when the gap induced by the road was more than 50 m. This study also has shown that the rate of bat crossings decreased with increasing distance between hedgerows on opposite sides of the road. The presence of vehicles during commuting and foraging promotes the barrier effect, although this effect is probably species specific. Zurcher et al. (2010) and Bennett & Zurcher (2013) showed that more than a half (60% and 68%, respectively) of Indiana bats, *M. sodalis*, abort crossing attempts in response to approaching vehicles. However, it is unclear whether this effect is due to the influence of noise, vehicle headlights, or both. The flight height also influences road crossing attempts. Bennett & Zurcher (2013) report that the higher height bat flies when approaching the road, the more likely it is that they cross it. High-flying species (e.g. *N. leisleri*) or generalists and open-edge-adapted species (e.g. *Pipistrellus* spp.) cross the road much more frequently than low-flying clutter-adapted species (e.g. *Myotis* spp. and *Plecotus* spp.) (Abbott et al. 2012a). Low-flying bats preferred following severed treelines (excluding the range-restricted *R. hipposideros*) or less exposed river bridges and underpasses to cross the road (Abbott et al. 2012b), when compared with gaps in the treeline. This behaviour could lead to longer journeys that consume time and energy or even restrict access to some habitats.

According to Berthinussen and Altringham (2012), bats' avoidance of the neighbourhoods of high-traffic roads could also be a response to the physical properties of motorways, such as road width (Fensome & Mathews 2016). However, direct evidence

for the influence of road width on crossing behaviour is still lacking. Roads that act as barriers to wildlife can have negative genetic effects by increasing the functional isolation of populations (Holderegger & Di Giulio 2010) (please see details in Section 1.5.3.5 Genetic isolation).

1.5.3.4 Roadkill

Roadkill is a major source of non-natural mortality contributing to the reduction of bat populations size. Although the number of bat casualties could be small compared to other vertebrates, such as birds and amphibians (Erritzoe et al. 2003; Arevalo et al. 2017), the life-history and ecological traits of bats make them particularly vulnerable to added mortality due to collision with vehicles (Schorcht et al. 2009; Fensome & Mathews 2016).

Many studies have focused on quantifying bats road casualties worldwide (Rackow & Schlegel 1994; Capo et al. 2006; Lesiński 2008; Russell et al. 2008; Gaisler 2009; Lesiński et al. 2011; Medinas et al. 2013; Iković et al. 2014; Ceron et al. 2017; Secco et al. 2017; Ramalho et al. 2021). Lesiński (2008) in Poland reports that 6.8 bats/km/year are being killed along road stretches near Warsaw, with 14 species being affected. In the Czech Republic, Gaisler et al. (2009) performed a bat mortality survey at weekly intervals for six consecutive months along an 8 km highway and collected 119 bat casualties belonging to 12 species. In USA, Russell et al. (2009) searched for weekly bat casualties along a 4.4 km highway and founded 29 *Myotis* bats roadkilled. In the southern region of Brazil, Ceron et al. (2017) recorded 11 individuals belonging to at least six species and in Montenegro, Ikovic et al. (2014) reported that 0.03 bats/km/year are killed. In our study area, about 4 bats are killed per kilometre each year (Medinas et al. 2013). However, it is difficult to reliably quantify the number of bat casualties on roads due to the poor detection of small body carcasses (Bafaluy 2000) and high removal rates by scavengers (Slater 2002; Santos et al. 2011; Teixeira et al. 2013). Additionally, many bat carcasses will be thrown off the road when individuals are hit by vehicles. Therefore, roadkill counts often represent severe underestimates due to ineffective research. Slater (2002) estimates that bat roadkill rates should be 12-16 times higher than accounted

based on the number of carcasses found. Teixeira et al. (2013) report that roadkill estimates in Brazil increased 2-40 times when scavenging and low detectability were accounted.

Depending on their foraging strategies, different bat species may be more vulnerable to vehicle collision (Kerth & Melber 2009). Although agile and maneuverable in flight, most bat species fly at low speeds (< 20 km/h) and many fly close to the ground (0 - 4 m), particularly when crossing open spaces on the landscape (Fensome & Mathews 2016). Cluttered-adapted species (e.g. *R. hipposideros*) should be most affected due to their characteristic low and slow flight (Fensome and Mathews (2016). The risk of collision for high-flying species (e.g. *N. leisleri*) is considered negligible because they generally fly above vehicles (Zurcher et al. 2010; Bennett & Zurcher 2013). On average, they represent about 17 % of all bat casualties (Fensome & Mathews 2016).

Regarding the spatial pattern of bat roadkill, previous studies have shown that high rates of bat mortality have been found where flyways cross roads and where there is a high suitability habitat for bats on both sides of the road, such as water bodies and woodlands (Medinas et al. 2013; Iković et al. 2014). Furthermore, linear landscape elements, such as riparian galleries, and their intersection with roads play an important role in concentrating the risk of roadkill (Lesiński 2008). These roadkill hotspots are often described in a static way without considering interannual variations in their location (e.g., land cover, traffic volume, population fluctuations), despite for other mammal species, marked temporal and spatial changes on roadkill hotspots locations have been often reported (Barthelmess 2014; Garriga et al. 2017; Lima Santos et al. 2017).

Roadkill risk may be different for each sex, depending on season. Most often, a higher number of male fatalities are recorded. Males disperse at greater distances and therefore have a higher probability of crossing roads (Medinas et al. 2013). The higher number of males fatalities could also be due to female-biased philopatry and male-biased dispersal. However, in late spring and early summer, the time of pregnancies and lactation, when females' higher body mass interferes with their flight manoeuvrability and they need to start foraging earlier in the day, exposing them to the peak traffic period (Fensome &

Mathews 2016), female roadkill risk may be higher than the one of males. Several studies indicate that young individuals usually flying at a lower height and slower than adults are killed more frequently (Haensel & Rackow 1996; Stratman 2006; Lesiński 2008).

Despite the numerous studies on bat roadkill numbers, it is difficult to draw a conclusion about the effects of roadkill rate on local populations and studies that address this issue are rare. Russell and colleagues (2008), estimate that the annual bat mortality rate on roads near roosts is about 5 % of the population. However, the authors recognize that the authentic rate may be higher due to the low detectability of carcasses and because bats when leaving the roosts generally follow linear elements in the landscape and may be killed away from the roosting sites (Ramovš et al. 2010). We must be cautious about the risks for population even with moderately increase bat road mortality because recovery can be slow, jeopardizing the long-term viability of bat populations.

1.5.3.5 Genetic isolation

The genetic effects of roads on bat populations were mostly unknown and, as a consequence, the effectiveness of mitigation measures on the genetic structure of bats has often been poorly monitored. However, there is considerable evidence to suggest that roads act as barrier to bats during foraging and roost switching (Kerth & Petit 2005). Therefore, roads can act as a filter over long periods of time, consolidating genetic isolation leading to genetic structuring. However, several studies highlight that effects of roads need time to become detectable; thus, the time lag since the construction of roads may be too short for local population effects (e.g., genetic differentiation on either side of the roads) become apparent. This suggests that many bat populations separated by roads may show weak genetic differentiation, not yet translated into formal genetic structure in subpopulations. For example, Kerth and Petit (2005) have shown that although motorways can restrict habitat accessibility, local populations of *M. bechsteinii* have only weak genetic differentiation. However, using fine-scale genetic analysis, with highly discriminatory genetic markers (SNPs), Medinas et al. (2023) found that proximity of roads may play a role in the genetic structure of *R. hipposideros*. Females from roosts closer to roads were less genetically related. The proximity to the roads also seems to

limit the movements of females, contributing to a lower level of relatedness among females in those roosts. Importantly, Kerth and Melber (2009) reported that females' colonies of *M. bechsteinii* bounded by a motorway had lower reproductive success than other females, showing evidence of the adverse effects of roads on reproductive output, and as a consequence on long-term survival of bat population. The genetic effects of roads on populations can arise quickly when there is a strong barrier to gene flow, which can occur when several strong impacts, such as roadkill and road avoidance, occur simultaneously.

1.5.4 Species-specific effects

Road effects often species-specific. Large and fast-flying species that typically fly high above the ground and are adapted to foraging in open areas appear to be less affected by roads (Kerth & Melber 2009; Abbott et al. 2012b; Fensome & Mathews 2016). Smaller, slower-flying and clutter-adapted species are more manoeuvrable and typically fly hovering and close to the ground (typically up to 4 m from the ground). These species tend to forage or commute within closed habitats (for example, forests) and are less likely to cross open areas. These habitats provide more protection from predators and easier access to insect prey but make them less efficient flyers. Low-flying species tend to be the most prone to the deleterious effects of roads. Some studies report that they are most affected by vehicle collisions because they fly at the same height as most cars (Stratman 2006; Fensome & Mathews 2016; Novaes et al. 2017). For example, in France, low flying species such as *Myotis* spp. are the most affected by road fatalities (Capo et al. 2006). Other studies also reported that low-flying and clutter-adapted bat species reduce foraging activity when approaching major roads (Berthinussen & Altringham 2012; Bennett & Zurcher 2013). Some clutter-adapted, like *R. hipposideros*, often live in high-density forest environments, being less tolerant to noise and artificial lighting, which makes them more likely to have a road avoidance behaviour (Ducci et al. 2015). Kerth and Melber (2009) also revealed that areas close to roads with high traffic were poor foraging areas and that roads act as flight boundaries for *M. bechsteinii*. On the other hand, an habitat specialist, *Barbastella barbastellus*, integrates roads into their foraging areas and regularly cross these infrastructures (Kerth & Melber 2009).

1.5.5 Effects of road type and traffic speed

Highways and motorways appear to be more harmful than minor roads, perhaps as a result of their high traffic volume and greater width (Fensome & Mathews 2016; Myczko et al. 2017). The extent to which traffic might repel bats from the roadside is not clear, but as bats avoid locations with loud background noise, intense traffic noise may reduce foraging efficiency near high traffic roads (Siemers & Schaub 2011; Finch et al. 2020). Furthermore, high traffic volume increases the risk of collisions of bats with vehicles (Fensome & Mathews 2016; Ramalho et al. 2021). Ikoovic et al. (2014) report that the majority of bat casualties (about 95 %) were found on high-traffic roads (10,300 vehicles/day) compared to roads with low traffic volume (1,100 vehicles/day). Previous research has also shown that the probability of bats successfully crossing the roads depend on traffic volume (Zurcher et al. 2010). These authors showed that Indiana bats *M. sodalis* readily crossed low-traffic roads, but only rarely crossed large highways with high traffic volumes. Furthermore, road crossings increase two times in locations where there is no traffic during the crossing moment. In the UK, Knight (2006) confirmed that high-traffic roads were likely to form a significant barrier to the movement of *R. hipposideros*. However, this study also reports that movements around roosts were not restricted by low traffic roads with single carriageway (Knight 2006; Knight & Jones 2009). On the other hand, other studies report that even minor roads are avoided by many bat species (Bellamy et al. 2013; Bennett & Zurcher 2013). Bellamy et al. (2013) have used habitat suitability modelling to show that woodland specialists, such as *Myotis spp.* and *Plecotus spp.*, avoid all types of roads, while generalist species, such as *Pipistrellus spp.* and *Nyctalus spp.* often use areas around small and low traffic roads. In Indiana (USA), bat activity is higher on rural unpaved roads compared with paved roads (Pourshoushtari et al. 2018). Furthermore, local roads in forested landscapes may be habitat edges and may serve as corridors, being used by many species of bats to move or forage (Fensome & Mathews 2016; Myczko et al. 2017). Although low-traffic roads comprise most of the world's roads, their impact on wildlife is likely underestimated and there is still a lack of information on this issue. Although there is no direct evidence that vehicle speed has influence on bat willingness to cross the road, research on birds shows that a large proportion of individuals tend to fly away from the road when vehicle speed is higher (Husby & Husby 2014).

1.5.6 Cumulative effects of roads on bat populations

Road expansion is a complex phenomenon with multiple deleterious effects on bat populations that are often cumulative among themselves and with other deleterious effects (e.g. roost destruction by logging, foraging area loss by livestock farming, hunting, or persecution). Road mortality often results in reduced species abundances that decrease genetic exchange rates among subpopulations on different sides of the road (Frankham 2005; Fahrig & Rytwinski 2009). Roadkill can jeopardize the long-term viability of bat populations, particularly for rare species (Claireau et al. 2019b). Moreover, when a road is built in the proximity of bat roosts, it may reduce the suitability of surrounding foraging area through both habitat destruction and degradation, hampering the use of commuting routes. Alternatively, colonies can abandon the roosts and relocate away from the roads. All of these issues are likely to increase mortality and reduce reproductive output, contributing to a higher risk of population decline.

The cumulative impacts of habitat loss and degradation, roadkill, and barrier effects on bat populations living in road surroundings have not yet been adequately assessed, although they are very likely to increase the risk of extinction of local populations (Figure 1.2). Furthermore, those impacts may take time to become perceptible, which may jeopardize the implementation of conservation programs.

1.5.7 Can roads benefit bats?

Roads may also attract wildlife to their surroundings, as they can provide favourable habitat along roadside verges (Penone et al. 2012), providing foraging opportunities, movements corridors (Spoelstra et al. 2017) or a new roosting opportunities (e.g. bridges).

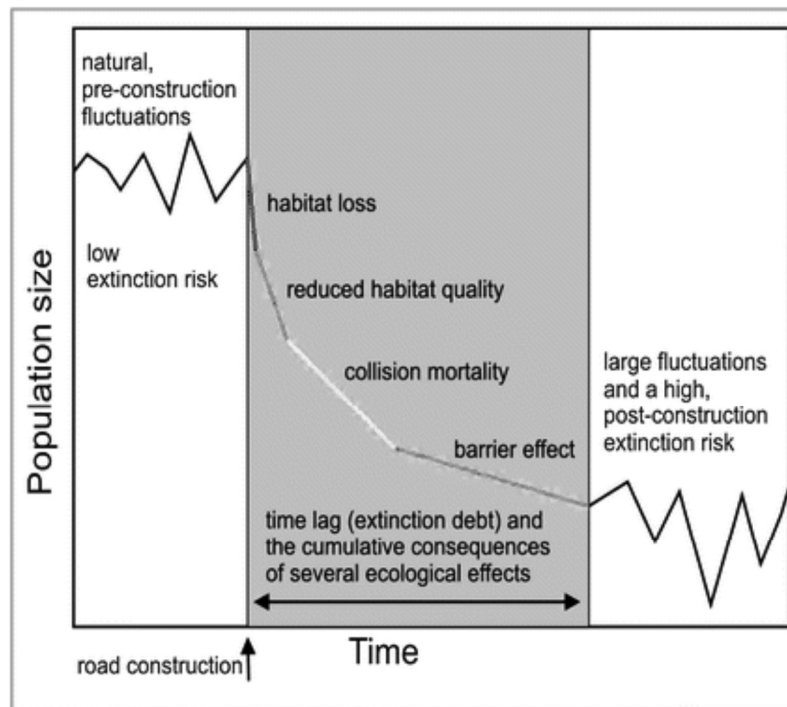


Figure 1.2 - The multiple effects of roads on bat populations (adapted from Forman et al., 2003 and Berthinussen and Altringham, 2013).

Roosts (bridges and culverts) –

Bats roosting on these structures often make use of crevices between concrete girders as they offer appropriate microclimate conditions and protection against predators (Keeley 2007). Road bridges over water are particularly used by bats because they often provide easy access to insect-rich feeding areas (Elmeros et al. 2016). Most studies investigating the use of bridges as bat roosts have been conducted in the USA and Europe; however, some data have also been published for other areas, such as Japan, Costa Rica and New Zealand (Sherwin et al. 1999; Fujioka et al. 2008). In Germany, *M. myotis* maternity colonies are often found on these structures (Walther 2002). In the USA, 24 species of bats were found roosting in bridges. One of the largest bat colonies in the world reached more than 1.5 million individuals of *Tadarida brasiliensis* roosting in a bridge in Austin (Keeley 2007). Roosting near a road poses a risk as previously mentioned. However, more studies are needed to clarify the balance between opportunities and risks provided by bridges and culverts for bat populations.

Light –

The widespread use of artificial light at night (ALAN) is commonly associated with a negative impacts on the ecosystem (see details in section 1.5.3.1 Habitat degradation), but the knowledge of the extent of its effects on bats is sparse (Hale et al. 2015; Azam et al. 2016; Da Silva 2022). At a local scale, the illuminated areas attract a large portion of the surrounding insect biomass (Nowinszky 2004) and associated with this, some bat species. However, artificial lighting increases the risk of road mortality and reduces the effectiveness of road safe crossings, for instance at lit bridges (Bhardwaj et al. 2020).

Flight corridors (verges) –

The importance of vegetated road edges for bats depends on the surrounding landscape of the road. In highly human-modified regions (e.g. intensive agricultural areas, homogeneous tree plantations), with fewer resources, such as few hedgerows, road verges often represent the last remains of natural or semi-natural vegetation strips providing corridors and enhanced foraging habitats (Abbott et al. 2012a; Laforge et al. 2019; Russo-Petrick & Root 2023). Indeed, foraging activity is facilitated close to the verges due to the greater abundance of insects (Verboom & Huitema 1997). Roadsides crossing intensive agricultural landscapes can act as habitat edges, due to the higher structural complexity of vegetation, compared to the surrounding landscape (Myczko et al. 2017). Bats can fly along such features when commuting from roosts to foraging areas because these linear vegetation elements can provide commuting paths away from predators and protect them from the wind (Bach et al. 2004). However, bats may show contrasting behaviours regarding road edges, depending on their characteristics and/or landscape context (Roemer et al. 2020). Cluttered adapted species perceive verges as an inadequate habitat (Lacoeuilhe et al. 2016). In contrast, aerial hawking species, such as *Pipistrellus spp.* and *Eptesicus spp.*, could benefit from the route and feeding opportunities provided by the road verges. Despite the potential benefits of road verges, the attraction towards the road vicinity can become an ecological trap for some species due to the higher roadkill risk (Bernes et al. 2017). Trade-offs between the risks and opportunities of road verges for bats must still be properly assessed before effective management of this infrastructure for conservation purposes can be recommended.

1.5.8 Mitigation of the effects of roads on bats: some solutions

To promote the development of a sustainable transport infrastructure and comply with stricter environmental regulation policies that protect wildlife, road agencies and national governments have developed national guidelines to minimize impacts and potentially exploit opportunities of roads. A variety of measures have been described, recommended, or implemented. Although all bats are legally protected in EU countries, and the increasingly recognized overall negative impacts of roads on bats, only half of the European countries implemented bat mitigation measures (Elmeros et al. 2016). Many of these measures have been proposed to restore habitat connectivity and reduce roadkill risk, using overpasses or underpasses, but rarely consider habitat improvement (Ward et al. 2015). Several studies recommend that passing structures should be located on commuting routes to ensure high use and should be built taking into account the need of bats to cross the roads with minimal changing of flight height and direction (Claireau et al. 2019a). These measures, in order to efficiently connect habitats, should be located adjacent to each roadside, such as hedgerows or treelines (Møller et al. 2016; Martínez-Medinas et al. 2022). However, most studies of bat mitigation measures are only descriptive and showed ambiguous results concerning their efficiency (Claireau et al. 2019a, 2019c). Furthermore, the effectiveness of mitigation measures type differs significantly between species and locations (Møller et al. 2016), and their effectiveness for conserving bat populations in the long term has not yet been proven (Martínez-Medinas et al. 2022). Further details on the effectiveness, advantages, and constraints of different mitigation measures and variations in their effectiveness for each bat species are discussed in Berthinussen et al. (2012), Elmeros et al. (2016), and Møller et al. (2016).

Underpasses/Culverts -

Underpasses are constructed specifically to facilitate safe passages for wildlife, for carrying watercourses, or can be designed for vehicles, people, or cattle to safely cross the road. Underpasses comprise culverts, tunnels, viaducts, and river bridges. Many studies confirmed that bats may regularly use underpasses that are designated for other purposes – multifunctional tunnels –, e.g., agricultural access roads, forest tracks and pedestrian paths (Bach et al. 2004; Kerth & Melber 2009; Berthinussen & Altringham

2012; Bhardwaj et al. 2017). Underpasses are used differently by different bat guilds. Clutter and edge-adapted species, such as *Myotis spp.* and *P. auritus*, usually fly under culverts rather than over the road (Abbott et al. 2012b; Bhardwaj et al. 2017; Martínez-Medinas et al. 2022). On the other hand, high-flyers and open-adapted species flew over the road more than on underpasses. Low-flying bat species are observed in both large and narrow underpasses, whereas higher-flying species (e.g. *Eptesicus spp.*) and less manoeuvrable species appear to prefer large underpasses (Elmeros et al. 2016). However, the use of underpasses is also species-specific (even among species with similar ecological requirements) and depends on their characteristics. In Germany, underpasses seem to be important to maintain unrestricted access to foraging and roosting habitat, for *B. barbastellus* (Kerth & Melber 2009). However, in the same study, *M. bechsteinii* rarely used them and showed a reduction in its home range size. Some studies indicate that screens above underpasses increase the effectiveness of the underpasses (Møller et al. 2016). Culverts associated with waterways are more effective because these habitats often act as commuting routes. Underpasses are more likely to be used if they are well connected to treelines, hedges (Abbott et al. 2012a) and/or near to the original commuting routes (Figure 1.3). Artificial light in underpasses increases the crossing rate above the road, thus increasing the vulnerability of bats to roadkill (Bhardwaj et al. 2020).



Figure 1.3 - Underpasses on the Agg Cumbria, UK, to guiding bats safely under roads. From Berthinussen and Altringham (2012)

Overpasses -

Overpasses are constructions that facilitate safe crossings of roads. Overpasses include: wildlife overpasses (e.g., green bridges) Figure 1.4a, modified overbridges, bat gantries and hop-overs (Limpens et al. 2005) Figure 1.4b. Overpasses have been built on many European and USA roads in recent years. Some of them that were built on minor roads or footpaths appear to be largely ineffective on bat crossings (Abbott et al. 2012a). On the other hand, large overpasses (e.g., wildlife overpasses) that often have been designed and built specifically for other wildlife, have high potential as bat crossing structures, particularly when they link existing bat flyways, have high potential as bat crossing structures. Overpasses can effectively guide more than 90 % of bats safely across roads if placed on bat flight corridors (Berthinussen & Altringham 2016).

Bat gantries (Figure 1.4b) are simple, narrow, and linear structures constructed specifically for bats with the objective to guide them to cross over the road at a safe height. Gantries are intended to provide echoes sufficiently strong that individuals do not decrease their flight height when crossing the road (Elmeros et al. 2016).

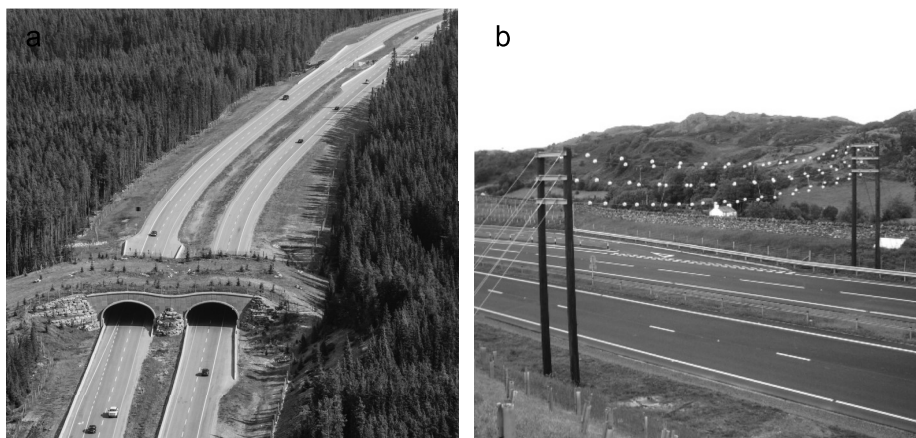


Figure 1.4 a) Green bridges over E2, Canada and b) Bat gantries over the A590(T) road, UK were used as mitigation measures to replace a hedgerow which were used as commuting and foraging corridor Photos credits: Allie Banting and Anna Berthinussen

In the UK, Berthinussen and Altringham (2012) found that few bats that approached gantries used them, and their flight paths were not raised above the risk of collision with vehicles height. Similarly in Poland, Cichocki (2015) reported that bat activity in the gantries was not greater than on the road sections next to them. Bats tended to use existing flight routes ignoring the gantries (Cichocki 2015). However, the specific design of the gantry and the location could be of importance for its efficiency. Møller et al. (2016)

mention that the use of spheres with multiple reflective surfaces with short distances among them increases the success of their use.

Finally, hop-overs consists of existing or planted tall vegetation (e.g., trees or shrubs) on either sides of a road (Limpens et al. 2005). However, hop-overs are expected to encourage bats to maintain or increase the flight height to cross the road. However, hop-overs are ineffective for *R. hipposideros*, which tend to fly along the fence or screen to cross the roads at low height at their endings (Henoux & Lombardi 2014).

Linear elements (hedgerows, trees, and screens) –

Bats use linear landscape elements such as hedgerows, treelines, stream rivers, and edges as guiding structures when commuting and in migration movements (Bach et al. 2004). Some species, such as *P. auritus*, prefer to cross roads following treelines (Abbott et al. 2012a). These linear elements can also be used to create a funnelling effect towards other passage structures (e.g., underpasses and overbridges). On a few occasions, fences have also been used to guide *R. hipposideros* and *M. daubentonii* toward safe crossing points across the road (Keeley 2007). Planting hedgerows and tree lines along the borders also reduces disturbance of traffic noise and light on adjacent land (Figure 1.5).



Figure 1.5 - Fences and Screens must be very tightly connected as even small gaps may divert the bats away from the overpass and onto the road. Photo credits: Swild and Nachtkiv

Other interventions –

Artificial noise and light warnings have been suggested to induce road avoidance behaviour in bats. However, the use of artificial light may increase insect availability, thereby attracting some bat species to the proximity of the road, increasing the risk of roadkill (Stone et al. 2015). On the other hand, artificial lights placed on crossing routes can hamper the passage of some species, such as *R. hipposideros* (Stone et al. 2009). Furthermore, combining road signs warnings or limiting vehicle speed may increase the effectiveness of other mitigation measures, which is especially important when implemented on already known bat commuting routes (Elmeros et al. 2016).

1.5.9 Research Objectives:

Bats are one of the most threatened groups of vertebrates affected by roads. However, surprisingly, there has been relatively little research on this issue and important information is still lacking for the implementation of more effective mitigation measures. Here, we combine molecular and field-based techniques to evaluate the effects of roads on bats. A large roadkill data set, collected between 2009 and 2015 in southern Portugal, Alentejo, has been used to describe bat roadkill spatiotemporal dynamics and identify the main landscape and/or weather features influencing it. Bat activity data, based on acoustic sampling, have been used to quantify the distance of the road effect for different bat guilds in different landscape contexts. Furthermore, we used fine-scale genetic relatedness models to assess the effects of roads on dispersal and gene flow of a threaten species, the lesser horseshoe bat, *R. hipposideros*, which flies close to the ground, hence being particularly vulnerable to vehicle collisions.

Specifically, the thesis main goals are:

- (i) To characterize dynamics of bat roadkill patterns through the identification of clusters of mortality (hotspots) and assessment of their spatiotemporal persistence.

- (ii) To identify the influence of road characteristics and other local environmental predictors derived from satellite imagery, such as vegetation productivity and land surface temperature, on the spatiotemporal dynamics of bat roadkill hotspots.
- (iii) To quantify the extension of the road-effect zone for different bat guilds defined on the basis of echolocation and flight characteristics.
- (iv) To assess the influence of the road-landscape context on bat activity, as well as on the extension of the road-effect zone.
- (v) To characterize, at a fine scale, the population structure and genetic relatedness of a lesser horseshoe bat population.
- (vi) To evaluate the effect of habitat quality on road surroundings on the gene flow of the lesser horseshoe population, considering the sex-biased dispersal abilities.
- (vii) Judging the existence of a road-barrier effect for the lesser horseshoe bat population studied, based on genetic data.

1.5.10 Thesis Outline:

The thesis consists of five chapters, including a general introduction (Chapter 1), three published papers (Chapters 2, 3 and 4), and a general conclusion (Chapter 5). Chapter 1 is a systematic review of the literature on impacts of roads on wildlife, strengthening the effects on bats. A short description of bat mitigation measures is also included. Moreover, this chapter contains the thesis goals and the outline of the present document.

In Chapter 2, we identify and evaluate the spatiotemporal persistence of bat roadkill hotspots in response to dynamics of habitat suitability at road surroundings and the level of bat activity. This study describes bat roadkill patterns along low-medium traffic roads for three consecutive years. The chapter also includes a short assessment of the importance and application of free available remote sensing satellite data, including

vegetation productivity, moisture content, and land surface temperature, to understand the role of fine-scale environmental dynamics on spatiotemporal variation of roadkill patterns.

Article:

Medinas, D., J. T. Marques, P. Costa, S. Santos, H. Rebelo, A. M. Barbosa, and A. Mira. 2021. Spatiotemporal persistence of bat roadkill hotspots in response to dynamics of habitat suitability and activity patterns. *Journal of Environmental Management* **277**:1–10.

In Chapter 3, we used acoustic surveys along transects perpendicular to roads to investigate the effect of distance to low-medium traffic roads, as well as the importance of road-surrounding land uses, on bat activity patterns. This assessment was done for three bat guilds: short-, mid- and long-range echolocators and the most common species in the area, *P. kuhlii*. Moreover, based on the results, we also discuss the putative importance of roads verges as bat feeding areas, accounting with road landscape-context.

Article:

Medinas, D., V. Ribeiro, J. Tiago, B. Silva, A. Márcia, H. Rebelo, and A. Mira. 2019. Road effects on bat activity depend on surrounding habitat type. *Science of the Total Environment* **660**:340–347.

In Chapter 4, we combined spatially explicit models with high-throughput genotyping data to access the fine-scale genetic relatedness within and among lesser horseshoe bat roosts for each sex. Additionally, we identified features of the contemporary landscape that may hamper or facilitate gene flow between roosts. The results are interpreted accounting for differences in behaviour and movement abilities among males and females.

Article:

Medinas, D., V. Ribeiro, S. Barbosa, F. Valerio, J. Tiago, H. Rebelo, J. Paupério, S. Santos, and A. Mira. 2023. Fine scale genetics reveals the subtle negative effects of roads on an endangered bat. *Science of the Total Environment* **869**:161705.

In Chapter 5, we summarize the main conclusions of the previous chapters and discuss the implications of the main findings for bat conservation. We also show some implications for bat populations in road-dominated landscapes and provide suggestions for future research in road ecology.



Chapter 2

Spatiotemporal persistence of bat roadkill hotspots in response to dynamics of habitat suitability and activity patterns.

Medinas, D*, J. T. Marques, P. Costa, S. Santos, H. Rebelo, A. M. Barbosa, and A. Mira. 2021. Spatiotemporal persistence of bat roadkill hotspots in response to dynamics of habitat suitability and activity patterns. *Journal of Environmental Management* **277**: 1–10. <https://doi.org/10.1016/j.jenvman.2020.111412>.

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The chapter was maintained the formatting rules of the corresponding journal in which was published.

ABSTRACT:

Wildlife roadkill hotspots are frequently used to identify priority locations for implementing mitigation measures. However, understanding the landscape-context and the spatial and temporal dynamics of these hotspots is challenging. Here, we investigate the factors that drive the spatiotemporal variation of bat mortality hotspots on roads along three years. We hypothesize that hotspot locations occur where bat activity is higher and that this activity is related to vegetation density and productivity, probably because this is associated with food availability. Statistically significant clusters of bat-vehicle collisions for each year were identified using the Kernel Density Estimation (KDE) approach. Additionally, we used a spatiotemporal analysis and generalized linear mixed models to evaluate the effect of local spatiotemporal variation of environmental indices and bat activity to predict the variation on roadkill hotspot locations and to assess hotspot strength over time. Between 2009 and 2011 we conducted daily surveys of bat casualties along a 51-km-long transect that incorporates different types of roads in southern Portugal. We found 509 casualties and we identified 86 statistically significant roadkill hotspots, which comprised 12 % of the road network length and contained 61 % of the casualties. Hotspots tended to be located in areas with higher accumulation of vegetation productivity along the three-year period, high bat activity and low temperature. Furthermore, we found that only 17 % of the road network length was consistently classified as hotspots across all years; while 43 % of hotspots vanished in consecutive years and 40 % of new road segments were classified as hotspots. Thus, non-persistent hotspots were the most frequent category. Spatiotemporal changes in hotspot location are associated with decreasing vegetation production and increasing water stress on road surroundings. This supports our hypothesis that a decline on overall vegetation productivity and increase of roadside water deficit, and the presumed lower abundance of prey, have a significant effect on the decrease of bat roadkills. To our knowledge, this is the first study demonstrating that freely available remote sensing data can be a powerful tool to quantify bat roadkill risk and assess its spatiotemporal dynamics.

2.1 INTRODUCTION

The development of transport infrastructure is one of the main human-related pressures on wildlife (Spellerrberg 1998; Fahrig & Rytwinski 2009; Benítez-López et al. 2010), mainly due to habitat fragmentation and degradation (Trombulak & Frissell 2001; Van Der Ree et al. 2015). However, the most acknowledged direct impact of roads is wildlife-vehicle collision (WVC), which often contributes to the decline of species of conservation concern (Kociolek et al. 2010; Loss et al. 2014; Visintin et al. 2016). WVCs involving bats are increasingly reported (Capo et al. 2006; Choquene 2006; Lesinsky G., Kowalsky M. 2007; Lesiński 2008; Gaisler 2009; Medinas et al. 2013; Berthinussen & Altringham 2016), and transport infrastructures may be an important factor contributing to the global decline of many bat species (Berthinussen & Altringham 2016; Fensome & Mathews 2016). Indeed, several life-history and ecological traits of bats make them particularly prone to be hit by vehicles. These include migration over considerable distances (Schofield & Mitchell-Jones 2011), large home ranges (Kelt & Van Vuren 1999), or feeding opportunities on road verges (Berthinussen & Altringham 2012; Medinas et al. 2019). Therefore, bat activity may be strongly reduced on road-dominated environments (Berthinussen & Altringham 2012; Claireau et al. 2019b). Moreover, bats are long lived, have low fecundity and late maturation, so they may be unable to withstand even moderate increases in mortality (Schorcht et al. 2009). Thus, roadkills may jeopardize the long-term viability of bat populations.

Several studies reported that patterns of bat casualties are not equally distributed in space. Higher bat mortality rates have been found where bat flyways cross roads with high quality habitats or are close to foraging locations, such as water bodies and riparian galleries (Lesinsky G., Kowalsky M. 2007; Gaisler 2009; Lesiński et al. 2011; Medinas et al. 2013; Iković et al. 2014; Secco et al. 2017). At these locations, bat casualties may be spatially clustered, being designated as roadkill hotspots. These are often computed in a static way for a defined time interval without considering how location changes across time (Malo et al. 2004; Skórka et al. 2015). For instance, bat-vehicle collision patterns are often described annually (Fensome & Mathews 2016), but substantial inter-annual variations occur and this has been scarcely analyzed. For other mammal species (e.g. mule deer, black bear, lowland tapir) these inter-annual differences are often associated

with variations in environmental factors (e.g. land cover or climate), traffic volume or population fluctuations (Seiler & Helldin 2006; Shilling & Waetjen 2015; Ascensão et al. 2019). Moreover, some species showed marked seasonality in roadkill occurrences, which were particularly evident in mating and swarming periods (Fensome & Mathews 2016). Thus, spatial and temporal changes in hotspots can give insights regarding distribution and predictability of important resources on the landscape (Fensome & Mathews 2016).

The notion of shifting hotspots relates to the concept of home range fidelity (Switzer 1993; Börger et al. 2006; Freedman & Sen Roy 2012). The consistent classification of a road section as hotspot over time, termed hotspot fidelity, may reflect the existence of permanent important landscape features for bat ecological needs, such as foraging habitats or roosts in roadsides. In contrast, when resource availability changes in space and time, bat activity will track the resources and hotspots of roadkill may shift accordingly. In these circumstances, spatiotemporal predictability of the hotspots may be low. Unfortunately, there is a dearth of information on factors driving bat roadkills hotspot dynamics, in space and time, likely due to a lack of analytical methods for capturing and characterizing spatiotemporal variability in resource availability at a fine-scale.

The current availability and development of remote sensing techniques allow them to be used for monitoring and mapping of insect outbreaks, particularly to evaluate forest damages caused by defoliators (Zhang et al. 2010). Moreover, there are many studies where remote sensing data of vegetation productivity, land surface temperature and moisture content are indicated as having greater potential for mapping the biomass at higher trophic levels (Jepsen et al. 2009; Rullan-Silva et al. 2013). These studies suggest that spatiotemporal heterogeneity of remote sensing derived indices is intrinsically linked to fluctuations in food resources availability and may provide insight about insect abundance. For instance, the Normal Deviation Vegetation Index (NDVI) has a robust positive linear correlation with ground-based measurements of net primary productivity, such as vegetation cover (Zhang et al. 2010) and, indirectly related to insect abundance in deciduous forest (Spruce et al. 2011). Furthermore, a shortage of water may increase vegetation stress and decrease foliage availability to insects. Blum et al., 2015, showed

that the use of land surface temperature resulted in satisfactory estimations of olive fly population trends (Blum et al. 2015). Thus, assuming that bats have a higher probability of being roadkilled while foraging in areas with high food resources (Medinas et al. 2013; Fensome & Mathews 2016), remote sensing data can be a powerful and cost-effective tool for gaining knowledge on spatiotemporal dynamics of bat roadkills.

In this study, we describe bat roadkill patterns along low, medium and high traffic roads in a Mediterranean landscape for three consecutive years. We first assess whether roadkills are spatially clustered forming “hotspots” of mortality. Then we evaluate if roadkill spatiotemporal patterns are similar along the studied years. Finally, we investigate how important are vegetation productivity, moisture content and land surface temperature, acting as a proxy of food availability in explaining roadkill hotspot patterns, taking into account local bat activity and traffic volume. We hypothesize that bat casualties will not be evenly distributed across space, and that there will be aggregations. These will tend to occur in areas with higher vegetation productivity and may vary between years, owing to variation in water availability, temperature or local land management over time. We discuss the applicability of freely available remote sensing data to describe spatial variation in roadkill patterns.

2.2 MATERIAL AND METHODS

2.2.1 Study area and bat roadkill surveys

This study was carried out in a landscape of about 400 km² in southern Portugal (38°32'24" to 38°47'33"N; -08°13'33" to -07°55'45"W; Figure 2.1). The climate is Mediterranean, with mean daily temperature ranging from 5.8 to 12.8 °C in January, and from 16.3 to 30.2 °C in August; annual rainfall averages 609.4 mm and is concentrated between October and March (Évora 1971-2000; Instituto de Metereologia, 2010). The topography is flat, with altitude ranging from 100 m to 400 m a.s.l. The landscape is dominated by savanna-like forests mainly composed of cork (*Quercus suber*) and holm-oak (*Quercus rotundifolia*), alternating with open agricultural areas for cattle grazing and

cereal crops, olive groves (*Olea europaea*), vineyards (*Vitis* spp.) and woody vegetation along streams.

Bat roadkill surveys were carried out along three national road segments (EN4, EN114 and EN370) and one municipal road segment (M529), comprising a total of 51 km. We surveyed these single-carriageway roads daily, from the 15th of March to the 15th of October, in 2009, 2010 and 2011. Surveys were conducted by a single observer driving a car at 20–40 km/h while scanning the road surface for bat carcasses (details in Santos et al., 2011). These observations started at sunrise, to reduce the impact of scavenger removal, because median persistence time for bat carcasses is one day (Slater 2002; Santos et al., 2011). Surveys were always carried out by experienced observers (more than five years conducting roadkill surveys), and most (> 80 %) were carried out by the same two experienced observers (DM, PC), thereby assuring

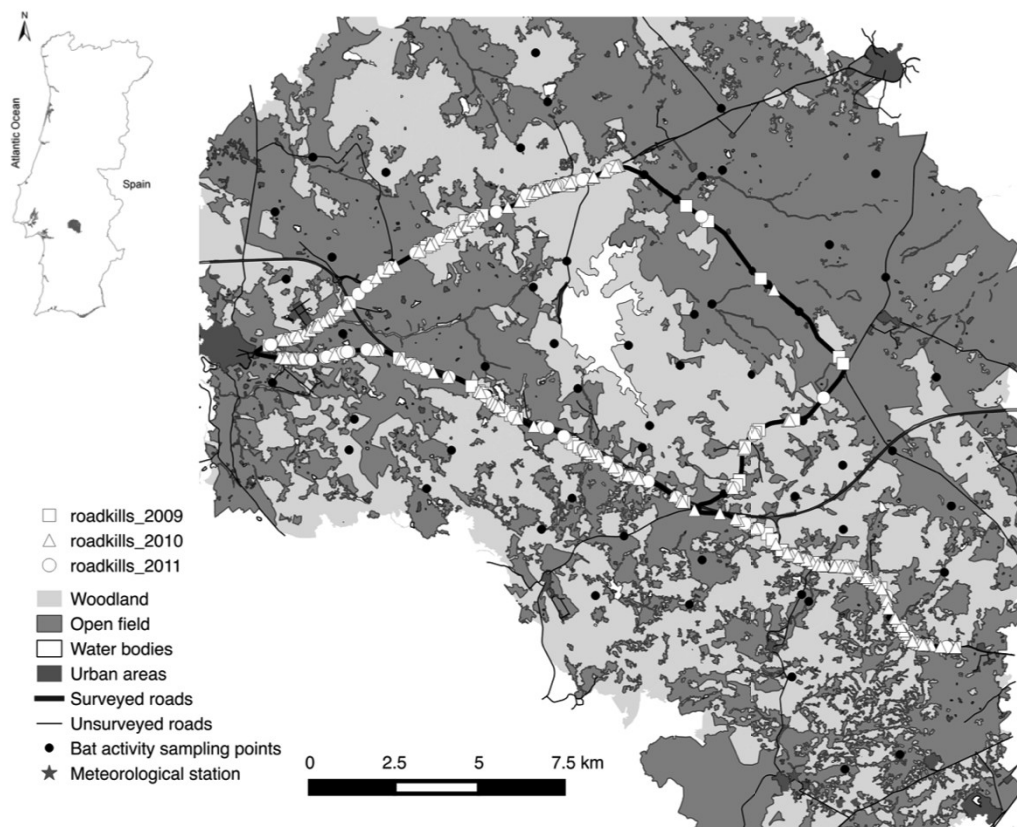


Figure 2.1 - Map of the study area in southern Portugal, showing the sections of roads that were surveyed (EN114, EN4 and EN370-M529) and the bat acoustic sampling points (black markers). The two main land uses are woodland (light grey) and open field (dark grey).

consistency of procedures and minimising errors due to variation in observer detectability skills. All bat carcasses were collected and later identified to species level using morphological keys (Palmeirim 1990; Dietz & von Helversen 2004) or genetic analysis, when the bat had no identifiable external characters (see details Medinas et al., 2013).

2.2.2 Bat activity data and traffic surveys

Bat activity was surveyed in 87 sites across the study area using 15-minute point counts at the same time period of the roadkill surveys (2009-2011). Each sampling site was visited three times each year, from April until September - the period of the highest bat activity in Mediterranean systems (Rainho 2007) - with a two-month interval between visits. The sampling sites were at least 1000 m apart and located on most representative land use categories, and at different distances from roads (Figure 2.1). We used a bat detector (D240x bat detector, Pettersson Elektronik AB) and a digital recorder (Archos AV 500 mobile digital video recorder) to record sound samples of each bat pass. The surveys were performed only on dry calm nights, which are the most favourable conditions for bat activity and detectability (Dixon 2012). We generated yearly bat activity maps for the study area using the inverse distance-weighted interpolation (IDW) of the mean number of bat passes.

Traffic volume estimates were based on, counts of all passing vehicles on 20 bat survey sites located near roads, and they were repeated three times each year. From these data we obtained an estimate of the number of vehicles per hour for every two months between April and September.

Following the classification of the Portuguese Road Institute, surveyed roads according to nocturnal traffic were categorized as: high for EN114 (1210 vehicles/night), medium for N4 (277 vehicles/night), and low for EN370 and M529 (both with <100 vehicles night), hereafter analysed together as EN370-M529 (EP, 2005; see details in Medinas et al., 2013).

2.2.3 Definition of hotspots and hotspot strength

The bat roadkill data were examined for the presence of high mortality clusters, i.e. hotspots, using the Kernel density estimation (KDE) (Okabe et al. 2009; Bíl et al. 2013; Favilli et al. 2018). This approach estimates the probability density function of the underlying data and was combined with Monte Carlo simulations with a 95 % threshold to evaluate the significance of each hotspot detected. The point pattern density method uses a moving function, controlled by a bandwidth, to weigh the importance of points within the bandwidth. In our analyses, we used a 150 m bandwidth because it was previously shown that this value is adequate to define roadkill hotspots on low-medium traffic roads (Bíl et al. 2013).

Additionally, we calculated the hotspot strength to rank the hotspots according to their hazardousness or risk. This ranking makes it possible to prioritize the most hazardous hotspots. This relative measure is directly dependent on the number of roadkills recorded in a hotspot and the length of road section, and indirectly related to the length of hotspot and the number of roadkills outside the cluster (Bíl et al. 2019). Hotspot strength estimates how much the observed distribution of mortality values on each hotspot is different from the uniform distribution (Bíl et al. 2013). Monte Carlo simulations and identification of hotspots were performed using the “sparr” package (Davies et al. 2017) and the “spatialEco” package (Evans 2018) in R software version 3.4.4 (R Development Core Team, 2018). To assess possible spatial correlations between hotspot locations, we calculated the nearest-neighbour distance between centroids of hotspots per each year of survey (D_KDE) and used a Moran’I test to evaluate potential statistical significance. Hotspots distanced up to 50 m were considered together.

2.2.4 Explanatory variables of bat roadkill hotspots

To find the best predictors that explain the spatiotemporal persistence of hotspots and their hazardousness (i.e. strength), we used three remote sensing based environmental indices reflecting local dynamics in primary production content (NDVI - Normalized Difference Vegetation Index), water content on surface and vegetation (NDWI -

Normalized Differences Water Index), and relative water stress (WETNESS - Wetness Tasseled Cap transformation Index); we also used a remotely sensed daytime land surface temperature (TEMPERATURE). The remotely sensed environmental indices and land surface temperature were obtained from a time series (from January 2006 to December 2011) of Landsat-5 satellite images, gathered from the US Geological Survey (USGS; LP DAAC - <http://lpdaac.usgs.gov>). The time frame included full calendar years of the study period and three years before its beginning. We only retained high-quality images for the whole study area (WRS-2 scene: path 203, row 33, 30 m resolution) and, among these, we only examined images with less than 20 % cloud cover. All images were pre-processed by applying the radiometric calibration to convert the pixel values to Top-of-Atmosphere reflectance (TOA) (Chander et al. 2009). Each of the remote sensing based environmental indices (Φ) was created for four time-periods (hereafter scenarios): the year of bat roadkill survey (YEAR_SURVEY; Φ_y); the year before the roadkill survey (one YEAR_BEFORE; Φ_{1y}), the whole of the two years before the roadkill survey (two YEARS_BEFORE; Φ_{2y}), and the whole of the three years before the roadkill survey (three YEARS_BEFORE; Φ_{3y}) (Figure 2.2). To derive each period composites, we first built a stack of all day-scenes for each scenario. Then, we used a 500-m buffer width around each roadkill hotspot to estimate the mean (m_Φ), sum (s_Φ) and standard deviation (sd_Φ) for each environmental indices. This buffer size was based on the foraging distances regularly covered by most roadkilled bat species in our study area, *Pipistrellus kuhlii* and *P. pygmaeus* (Dietz & von Helversen 2004; Medinas et al. 2013). Additionally, we combined remote sensing data with field-measured variables, such as traffic volume and bat activity interpolation (see details in 2.2.2 - Bat activity data and traffic surveys). Finally, we used a variable selection procedure aiming to reduce the dimensionality and eliminate highly correlated variables (see details in 2.2.5 – Statistical analyses for the evaluation of hotspot strenght).

2.2.5 Statistical analyses for the evaluation of hotspot strenght

We investigated the influence of remotely sensed environmental indices and temperature, traffic volume and bat activity on the strength of hotspots using general linear mixed models (GLMMs) with a Gaussian error distribution and an identity link function. Hotspot location (id) was included as a random factor to account for the

probable correlation between successive measurements - different years - at the same location. We also included in our models a first-order autoregressive covariance structure to account for the potential dependence in the hotspot locations over sampling years (Zuur et al. 2009).

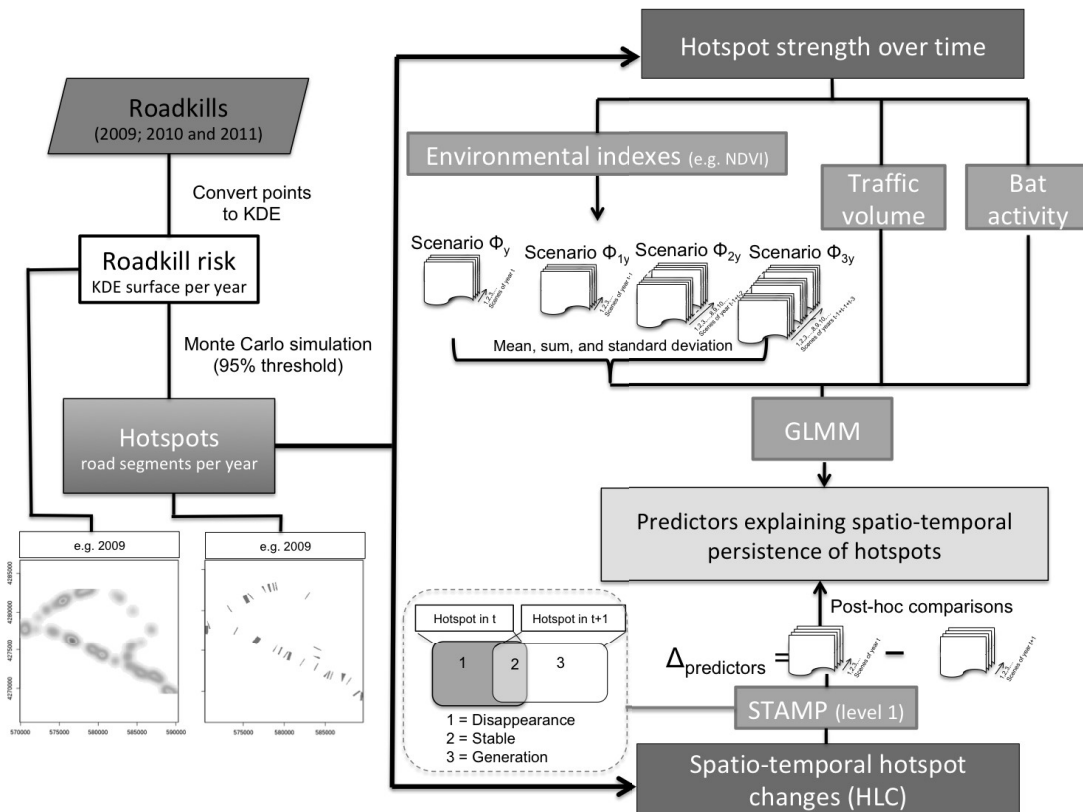


Figure 2.2 - An overview of methodology and the three methods presented. The flowchart depicts inputs (green), statistical analysis (orange), intermediate processes and preparatory analysis (no colour), partial objectives (red), interim results (blue) and for main drivers that explain the spatiotemporal variation of bat mortality hotspots (yellow). Firstly, the Kernel Density Estimation (KDE) method combined with repeated random simulations (Monte Carlo method) were used to identify clusters of bat roadkills within the roads surveyed and to determine the level of significance (threshold), selecting only significant clusters and ranking them. Then, Generalized Mixed Models (GLMM) were applied to evaluate the effects of the fine-scale spatiotemporal variation of environmental indices, traffic volume and bat activity on hotspot strength over time. Finally, we measured the hotspot location changes (HLC) on consecutive years (t and $t+1$) using Spatiotemporal Analysis of Moving Polygons (STAMP), combined with post-hoc comparisons to evaluate for environmental indices between HLC categories. Φ_y : year of bat roadkill survey; Φ_{1y} : year before the roadkill survey, Φ_{2y} : whole of the two years before the roadkill survey, and Φ_{3y} : whole of the three years before the roadkill survey.

Prior to modelling, when necessary, we log transformed the explanatory variables to approach normality, homogenise the variance and reduce the influence of outliers (Zuur et al. 2009). All explanatory variables were then standardised to zero mean and unit variance, to allow for comparison of their strength (Burnham & Anderson 2002). At a first stage, bivariate models were built for each explanatory variable. Only variables statistically significant in the bivariate phase were selected for further multiple variable modelling. To avoid collinearity, prior to modelling, we performed a Spearman correlation analysis among all remaining variables. For pairs of variables with a correlation higher than 0.70 (Dormann et al. 2013), only the variable most related to the hotspot strength was kept for further analysis. After this process, s_NDVI_y , s_NDVI_{3yB} , $s_ACTIVITY$, $TRAFFIC$, s_NDWI_y , D_KDE and $s_TEMPERATURE_y$ were the only predictors retained to build the multiple models (Supplementary Material - Table S 2.1).

All candidate models were built based on all possible subsets of selected explanatory variables, including the null and full model (Supplementary Material - Table S 2.2). The full model was structured in the following way:

$$\begin{aligned} \text{Strength of Hotspots} \sim & s_NDVI_y + s_NDVI_{3yB} + s_ACTIVITY + TRAFFIC + s_NDWI_y \\ & + D_KDE + s_TEMPERATURE_y, random = 1|id, \\ & structural\ correlation = corAR1(id/year) \end{aligned}$$

We used the AICc and the corresponding Akaike weights (w_i) to rank candidate models (Burnham & Anderson 2002). Because no single model was convincingly the most plausible ($w_i \geq 0.95$; Burnham and Anderson 2002), we performed a model averaging approach, basing the average parameters, unconditional standard errors (SE) and 95 % confidence intervals (CI) inferences on the group of models with $\Delta AICc < 2$ (Burnham & Anderson 2002). Plots of residuals were examined to check for normality, and deviance tests were done to assess the goodness of fit of the final models. All statistical tests were performed using the R software version 3.4.4 (R Development Core Team, 2018) with the packages “nlme” (Pinheiro et al. 2020) for GLMMs and ‘MuMIn’ (Barton & Barton 2020) for multi-model inference.

2.2.6 Spatiotemporal changes on roadkill hotspots patterns

To evaluate the spatiotemporal hotspot location changes (HLC), we used a GIS-based method for analysing temporally dynamic polygons (STAMP) (Robertson et al. 2007). This method adds an additional dimension to the analysis by quantifying hotspot shifts between two time periods (Nelson 2011; Smulders et al. 2012). Thus, within a paired set of hotspot locations on consecutive years (t and $t+1$), each polygon was categorized as one of the HLC categories: (i) stability or fidelity, if the area was identified as hotspot in both t and $t+1$; (ii) generation or expansion, when a new hotspot location was identified in $t+1$ but not classified in t ; and (iii) disappearance or contraction, when the hotspot location was identified in t but not in $t+1$ (Figure 2.2). To compare the changes between HLC categories among years (stable vs. expansion vs. contraction), we calculated the relative proportion of area to each HLC category per year. Additionally, we evaluated differences in explanatory variables ($\Delta\Phi$) in each roadkill polygon between every two consecutive years (t and $t+1$) and tested these differences among HLC categories. Following tests for normality and homogeneity of variance, we used PERMANOVA procedures and randomized ANOVA comparisons (Sokal & Rohlf 1995). Whenever significant differences were found, we performed post-hoc comparisons (Tukey's HSD) to test for differences between pair groups (Supplementary Material – Table S 2.3). STAMP analyses were conducted using R package “stamp” (Long et al. 2018), while the HLC analyses were performed with R package “vegan” (Oksanen 2018).

2.3 RESULTS

2.3.1 General bat roadkill patterns

Between 2009 and 2011, we recorded 509 bat carcasses belonging to 12 species, most of which were *Pipistrellus kuhlii* (34.5 %), *P. pygmaeus* (30.8 %), *P. pipistrellus* (21.9 %), *Rhinolophus hipposideros* (3.6 %) and *Eptesicus serotinus* (2.9 %) (Table 2.1).

The total number of bat carcasses per year increased along the three years of the study: 154 in 2009, 176 in 2010 and 179 in 2011 (*Figure 2.1*).

Table 2.1 - Yearly distribution of bat carcasses collected.

Species	2009	2010	2011	Total
<i>Barbastella barbastellus</i>	3	-	1	4
<i>Eptesicus isabellinus</i>	-	1	-	1
<i>Eptesicus serotinus</i>	5	3	7	15
<i>Miniopterus schreibersii</i>	1	2	-	3
<i>Myotis daubentonii</i>	2	2	4	8
<i>Myotis escaleraei</i>	1	-	-	1
<i>Nyctalus leisleri</i>	2	4	-	6
<i>Pipistrellus kuhlii</i>	66	60	53	179
<i>Pipistrellus pipistrellus</i>	21	38	54	113
<i>Pipistrellus pygmaeus</i>	45	58	55	158
<i>Rhinolophus ferrumequinum</i>	1	2	-	3
<i>Rhinolophus hipposideros</i>	7	6	5	18
<i>Total per year</i>	154	176	179	509

2.3.2 Spatiotemporal patterns of roadkill hotspots

Roadkill hotspot analyses demonstrated different aggregation patterns over the study period (*Figure 2.3A*). In total, we identified 86 bat roadkill hotspots with uneven distribution among years (27 in 2009, 33 in 2010, and 26 in 2011 – *Figure 2.3 B*). The bat mortality in hotspots accounted for more than half of the total bat casualties, 2010 being the year with the highest percentage of mortality in hotspots (2009 – 57 %; 2010 – 65 %; and, 2011 – 60 %). The total length of these hotspots amounts to, on average,

12 % of the road network (2009 – 9 %; 2010 – 13 %; 2011 – 8 %). The average hotspot length also varied among years (2009 – 175 m; 2010 – 206 m; 2011 – 162 m), but these differences were not statistically significant ($F = 0.829$, $p = 0.44$).

Concerning temporal changes in hotspots location, 24 hotspots, comprising 3 % of the road length, have remained stable along the three years of the study. On the other hand, hotspot expansion or contraction, between consecutive years had a higher representation on the road network (expansion – 6 %, $n = 46$; contraction – 7 %, $n = 51$).

2.3.3 Main drivers of bat roadkill hotspots

The most hazardous bat roadkill locations - higher hotspot strengths - were associated with higher accumulated vegetation productivity along the three-year period previous to the survey (s_NDVI_{3yB} : coef = 0.458), higher bat activity ($s_ACTIVITY$: coef = 0.314), and lower daytime land surface temperature ($s_TEMPERATURE_y$: coef = - 0.227).

Moreover, both s_NDVI_{3yB} and $s_ACTIVITY$ had higher importance ($RVI = 1.00$ and 0.5 , respectively) and were included in a higher number of models than $s_TEMPERATURE_y$ ($RVI = 0.3$) (Figure 2.4). According to the averaged models, $s_TEMPERATURE_y$ has an equivocal meaning, as zero was included in the unconditional confidence interval (Figure 2.4). Contrary to our expectations, traffic volume was not included in any of the final models of hotspot strength. All candidate models with $\Delta AICc < 2$ are presented in Table 2.2.

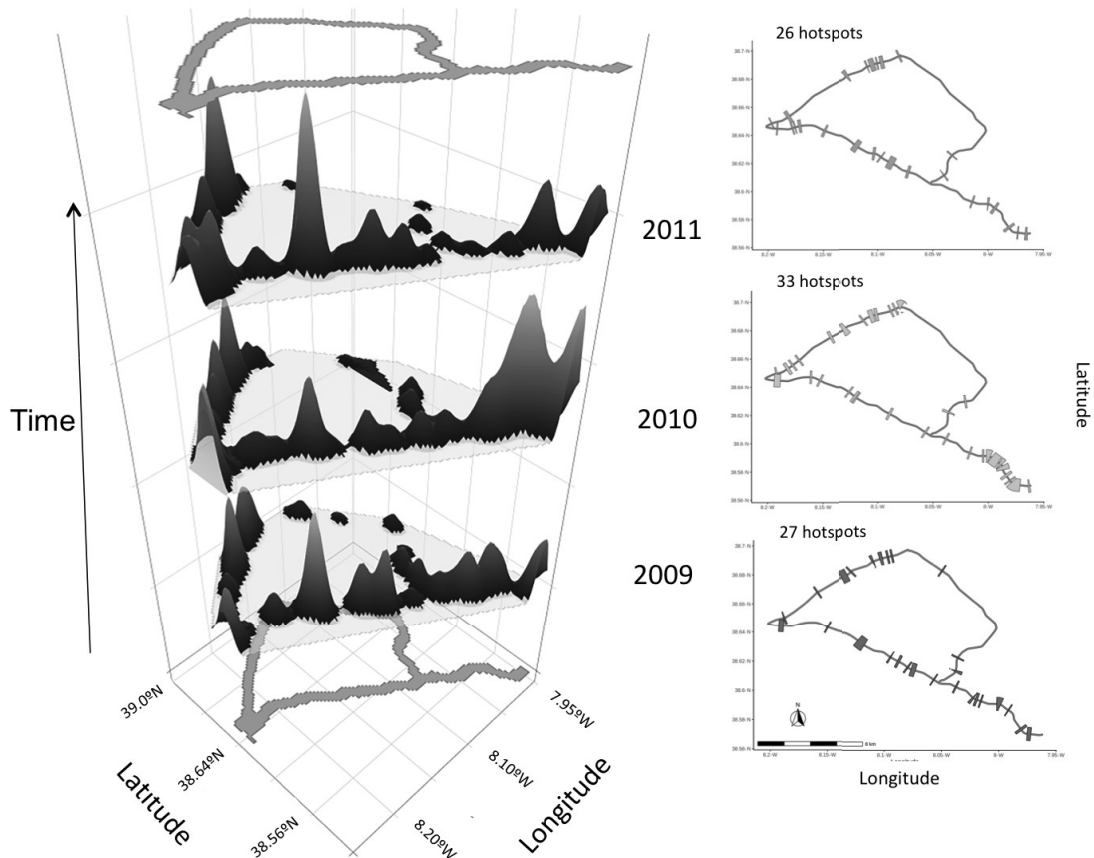


Figure 2.3 - A) Spatial and temporal distribution of bat-vehicle collision hotspots calculated with kernel-density estimation for each year surveyed. Maps depict the roads surveyed (solid line). B) Location of sections defined as hotspots per each year where the estimated probability density function rose above the threshold of 95th after Monte Carlo simulations.

2.3.4 Drivers of spatiotemporal changes in roadkill hotspots

Regarding temporal hotspot location changes (Figure 2.6), the Tukey post hoc test revealed that a positive ΔNDVI , an increase in primary production content between t and $t + 1$, was significantly higher in the expansion of hotspot areas ($p = 0.038$) compared with areas where hotspots contracted. Whereas no differences were found when comparing with stable hotspots areas ($p = 0.234$) (Figure 2.5). Additionally, hotspots tended to contract in areas where water stress increases ($\Delta\text{WETNESS}$), comparing with hotspots expansion areas and stable hotspots areas ($p = 0.035$; $p = 0.058$, respectively). No significant changes in ΔNDWI , $\Delta\text{TEMPERATURE}$ or $\Delta\text{ACTIVITY}$, from t to $t+1$, were detected among HLC categories (stability vs. expansion vs. contraction) (Figure 2.5).

Table 2.2 - Candidate models that were tested to access the potential influence of environmental indices, traffic volume and bat activity on the roadkill risk of the overall community of bats. Parameters: s_NDVI_{3yB} – sum of vegetation productivity of 3 years before the reference year when the hotspot was identified. $ACTIVITY$ – bat activity level on the year when hotspot was identified. $s_TEMPERATURE_y$ - daytime land surface temperature. For each model, we present the number of degrees of freedom (df), the Akaike Information Criterion corrected for small samples (AICc), AICc differences ($\Delta AICc$) and Akaike weights (w_i). The models are ranked by $\Delta AICc$.

Model	df	AICc	$\Delta AICc$	w_i
$s_NDVI_{3yB} + ACTIVITY$	6	106.75	0.00	0.35
s_NDVI_{3yB}	5	106.78	0.03	0.34
$s_NDVI_{3yB} + s_TEMPERATURE_y$	6	108.25	1.50	0.16
$s_NDVI_{3yB} + ACTIVITY + s_TEMPERATURE_y$	7	108.55	1.80	0.14

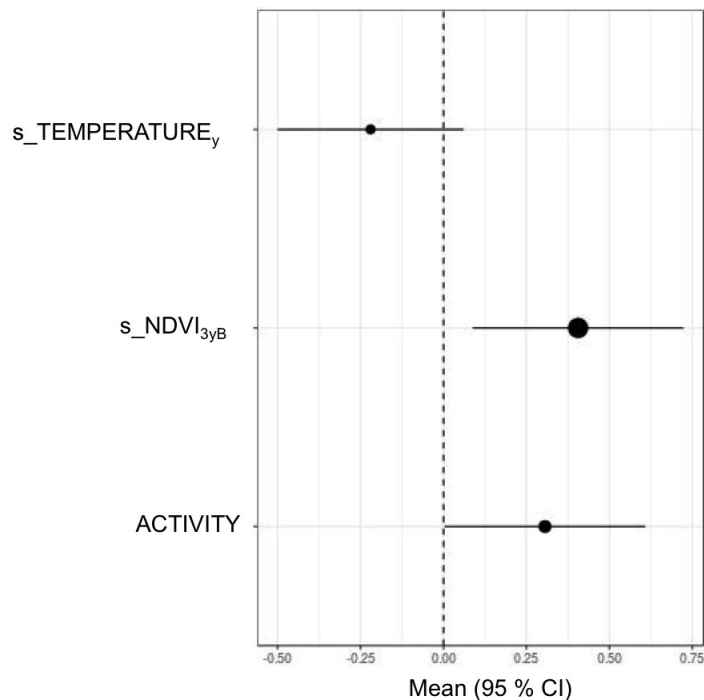


Figure 2.4 - Coefficient estimates (i.e., median values and 95 % credible intervals (bars) of the posterior distribution of the parameters from the best spatiotemporal models with $\Delta AICc < 2$. Credible intervals overlapping 0 (dotted line) indicate that the corresponding effect is not significant at the 5 % level. The dot size relates to the relative importance for each predictor on model selection approach.

2.4 DISCUSSION

We found a large number of bat roadkills (~ three bats killed/km/year) with a tendency to increase slightly along the three years of the study. Bat roadkill hotspots occupied about 12 % of the whole road length, encompassing 61 % of all roadkilled bats. However, the hotspots that remained stable along the three years of study comprised only 3 % of road length, but represented 27 % of roadkills. Our results suggest that bats are consistently more prone to be hit by a car at some specific locations across time. Thus, from the practical perspective, these results has important consequences, because in absence of significant landscape changes, it allows concentrating conservation efforts in spatially restricted areas, greatly improving the efficiency and cost-effectiveness of mitigation actions (Rytwinski et al. 2015). However, measures to reduce bat roadkills, such as overpasses, underpasses, barriers or strips to guide road crossings, are still a matter of debate, and further data are needed to assess their efficiency (Berthinussen et al. 2012; Claireau et al. 2019c, 2019a; Sołowczuk 2019). Claireau et al. (2019a) highlighted that bat overpasses should be located on intersections between roads and bat commuting routes. However, identifying these intersections remains a challenge. Other tools based on the assessment of bat flight trajectories such as acoustic flight path reconstruction (Claireau et al., 2019c), may be used to evaluate the bat roadkill risk. Nevertheless, analyses of hotspot stability and strength over time, like we applied in this study, may be a cheaper and easily implemented tool to help to find hazardous locations and decide on efficient placement of mitigation measures. Although an even longer period of sampling might increase the robustness of the study, our analysis represents an advance over other studies concerning roadkill risk locations, and one of the first that examine roadkill risk dynamics across time.

Zimmerman et al. (2017) argue that, for older roads, the rate between road mortality and population abundance in road surroundings should be the preferable, comparing with hotspot location, for informing mitigation priorities, due to the effects of past road mortality (Eberhardt et al. 2013) on long-term population abundance. These authors suggest that over time, the number of bat roadkills on high-traffic stretches with a high road mortality risk declines to the point that there are more roadkills on low-traffic stretches (Zimmermann et al. 2017). In these circumstances, the low-traffic locations

may be erroneously identified as priority sections to mitigate, because “false” roadkill hotspots only reflect the previous population depression near high-traffic stretches (Fahrig et al. 1995; Eberhardt et al. 2013). Although our study considered a relatively short period of time (three years) and an area where all roads are over 50 years old, our results do not support the Zimmerman and co-authors (2017) hypothesis. Indeed, we did not find a relationship between roadkill hotspot strength and traffic intensity, despite most roadkills having been detected on roads with higher traffic, every year.

Our results show that bat roadkill patterns are not random, although hotspot locations may shift along consecutive years. Changes in roadkill hazardousness and hotspot persistence are related to the spatiotemporal dynamics of net primary productivity (NDVI), which is a surrogate of foraging habitat suitability for bats, such as woodland areas and riparian vegetation (Rainho & Palmeirim 2011; Fonderflick et al. 2015). Thus, our results agree with previous studies which show that higher habitat quality is a key factor explaining bat roadkill patterns (Lesiński 2008; Gaisler 2009; Lesiński et al. 2011; Medinas et al. 2013; Fensome & Mathews 2016). Similarly, Ascensão and co-authors (2019) showed that roadkill risk and spatiotemporal pattern of road mortality for seven medium-large mammal species in Brazil are highly related to NDVI. A positive relationship between the cumulative NDVI for long periods – three years in our case – seems to be the strongest driver influencing roadkill hotspot strength over time. This is probably because higher values of accumulated aboveground net primary productivity (as measured by NDVI) tend to be associated with high insect availability areas that are temporally stable (Bailey et al. 2004), consistently improving the quality of foraging areas for many close-space aerial insectivorous bats, such as *Rhinolophus* spp.. High vegetation productivity is also often related to higher tree cover, which provides shelter resources for common bat species as *P. kuhlii* or *P. pipistrellus* (Kunz & Lumsden 2003; Rodríguez-Aguilar et al. 2017). Moreover, in a landscape context with few hedgerows, roadside verges often represent the last remains of dense vegetation strips providing corridors and enhanced foraging opportunities in the immediate roadside vicinity (Abbott et al. 2012a; Hale et al. 2012; Laforge et al. 2019), which increases the roadkill risk. Indeed, we also confirmed a strong positive relationship between areas of high bat activity and roadkill risk. Thus, acoustic bat activity monitoring, which is easily measured, may be a simple useful tool to assess roadkill risk (Medinas et al. 2013). We found that mortality risk tends to increase in road sections crossing areas with a slightly

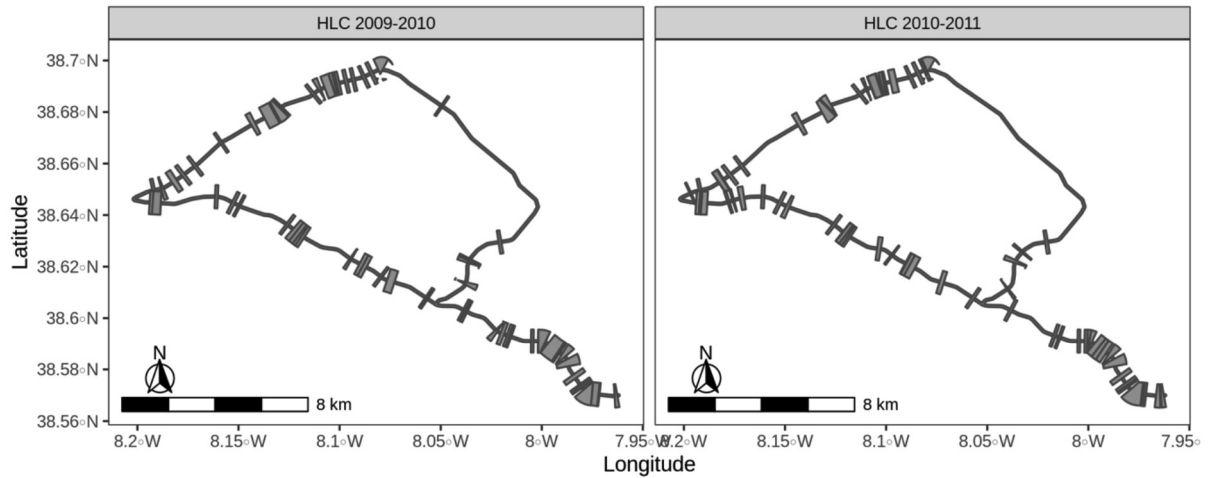


Figure 2.6 - Spatiotemporal patterns of hotspot locations in 2009 to 2010 and 2010 to 2011. Spatiotemporal patterns are defined by STAMP analysis at the broadest hierarchical level, where hotspot location changes were categorized in three types of events, stable (blue) – hotspot location detected in both times, generation (green) – hotspots in $t+1$ only, and disappearance (red) – hotspots location in t only.

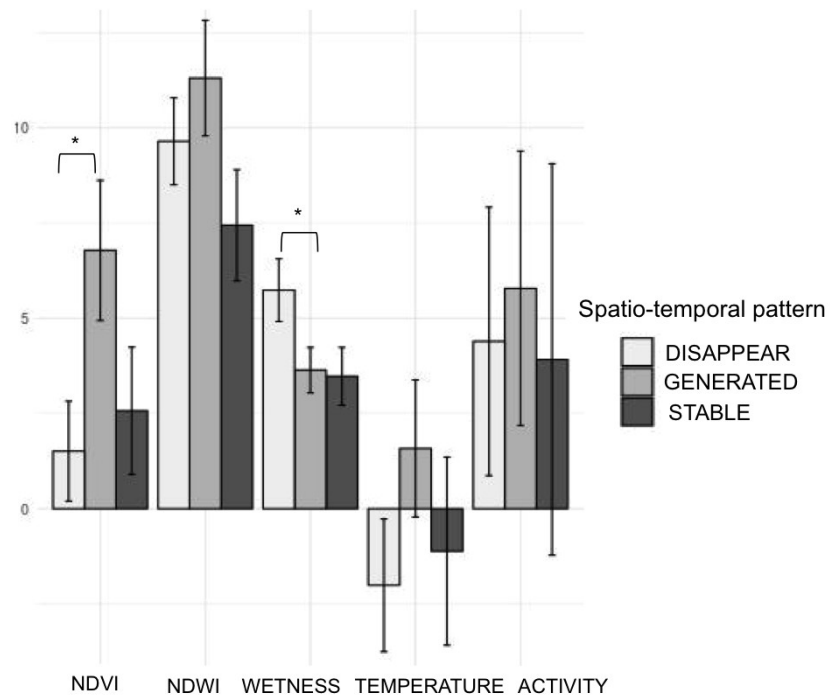


Figure 2.5 - Mean variation of predictors on hotspot change zone (stability, contraction, or expansion) over 1-year period. An asterisk indicates significant differences between zones of hotspot changes based on $p < 0.05$ using one-way ANOVA and Tukey's post hoc tests.

lower temperature. Forest areas, riparian galleries, and proximity to water bodies are the main land uses surrounding bat roadkill locations (Medinas et al. 2013). In those areas, due to the vegetation cover and/or proximity to water, the temperature tends to be lower which may explain this tendency.

The increase of vegetation productivity between consecutive years was the most important variable influencing the appearance of new roadkill hotspot locations. Conversely, the increase of water stress, reflecting lower surface moisture, was related to the contraction of hotspots in consecutive years. Our results show that even small temporal changes in habitat suitability, related with changes in moisture and vegetation productivity, influence roadkill patterns, probably due to alterations they induce in bat activity patterns. The changes in these remote-sensing descriptors should reflect mostly changes in local management actions (e.g. changes in grazing intensity, irrigation, crop harvesting, deforestation, etc.), thus linking these management actions with bat roadkill risk. Monitoring local management changes based on field work is logistically unfeasible for large areas or long periods. However, according to our results, moderately detailed freely available remote sensing imagery may adequately describe local environmental disturbance and can be used to evaluate spatiotemporal variation in bat roadkill risk. Evaluation of spatiotemporal habitat suitability changes based on remote-sensing data has already been done for other wildlife groups of different sizes and with different ecological requirements (e.g.. beetles – (Lassau & Hochuli 2008); ungulates – (Schweiger et al. 2015). However, to our knowledge, our study is among the pioneers in using these data to identify spatiotemporal changes in road sections with a higher roadkill risk.

Identifying spatially significant roadkill clusters for multiple years is a valuable tool, since it allows the identification and ranking of locations where efforts to reduce wildlife mortality should be focused. On the other hand, concentrating mitigation efforts only on overall bat roadkill hotspots may have limited benefit for some species (Clevenger & Waltho 2000). Thus, analysing roadkill at the species level or at least at habitat guild level is important, because different spatial or temporal roadkill patterns may be found,

depending on specific ecological requirements (Santos et al. 2015). However, this will require a large volume of roadkill data for single species, which is not possible to do in an efficient way for our dataset.

2.5 CONCLUSIONS

Overall, our findings confirmed previous results showing that bats are especially susceptible to roadkill in the vicinity of high quality habitats (Berthinussen & Altringham 2012; Medinas et al. 2013). We were able to demonstrate that bat roadkill hotspot locations may shift along time, accompanying spatiotemporal changes in habitat suitability, and that these can be inferred using freely available remote-sensing imagery, such as NDVI. Furthermore, using multiple year data and complex modelling, such as spatiotemporal analysis, allowed us to classify hotspots according to their strength and persistence, and thus to more precisely target and rank road stretches of interest to install temporal or permanent mitigation measures. Our results support the need of monitoring roadkills across several years to more efficiently mitigate bat casualties. Ideally, monitoring roadkills should be accompanied by monitoring bat populations on road surrounding landscapes, paying particular attention to rare and threatened species that are commonly hit by vehicles (e.g. *Rhinolophus* spp.)

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SUPPORTING INFORMATION

Table S 2.1 - Complete results from bivariate model selection according to p-value and AICc. Table shows the coefficient estimates, with standard error and p-value for each predictor. Traffic volume (TRAFFIC) and distance to minimum-neighbour hotspots (D_KDE) were not considered in bivariate selection. Each of the remote sensing based environmental indices (Φ) was analysed under different scenarios: the year of the bat roadkill survey (YEAR_SURVEY; Φ_y); the year before the roadkill survey (1 YEAR_BEFORE; Φ_{1y}), the whole of the two years before the roadkill survey (2 YEARS_BEFORE; Φ_{2y}), and the whole of the three years before the roadkill survey (3 YEARS_BEFORE; Φ_{3y})

Statistics	Period	NDVI			NDWI			WETNESS			TEMPERATURE			ACTIVITY		
		Coef.	Std. Error	p-value	Coef	Std. Error	p-value	Coef	Std. Error	p-value	Coef	Std. Error	p-value	Coef	Std. Error	p-value
Variation	Φ_y	0.05	0.16	0.76	0.08	0.15	0.60	0.14	0.16	0.38	0.06	0.15	0.70	0.28	-	0.07
	Φ_{1y}	-0.01	0.17	0.95	0.26	0.16	0.10	0.32	0.15	0.03	-	-	-	-	-	-
	Φ_{2y}	0.00	0.15	0.98	0.25	0.16	0.11	0.01	0.15	0.97	-	-	-	-	-	-
	Φ_{3y}	0.19	0.15	0.21	0.22	0.15	0.15	0.08	0.15	0.60	-	-	-	-	-	-
Sum	Φ_y	-0.30	0.16	0.05	0.01	0.01	0.03	0.10	0.14	0.46	0.27	0.14	0.05	0.04	0.15	0.01
	Φ_{1y}	-0.16	0.17	0.34	0.25	0.15	0.11	0.06	0.15	0.70	-	-	-	-	-	-
	Φ_{2y}	-0.25	0.13	0.05	0.09	0.15	0.53	0.01	0.01	0.07	-	-	-	-	-	-
	Φ_{3y}	0.30	0.14	0.04	0.10	0.14	0.46	0.01	0.01	0.03	-	-	-	-	-	-
Average	Φ_y	0.06	0.16	0.70	0.14	0.16	0.40	0.02	0.16	0.89	0.28	0.14	0.05	0.36	0.15	0.02
	Φ_{1y}	0.26	0.16	0.11	0.25	0.15	0.11	0.09	0.14	0.52	-	-	-	-	-	-
	Φ_{2y}	-0.12	0.14	0.39	0.28	0.15	0.06	0.03	0.16	0.84	-	-	-	-	-	-
	Φ_{3y}	0.39	0.16	0.02	0.28	0.15	0.06	0.03	0.16	0.84	-	-	-	-	-	-

Table S 2.2 - All candidate models that were tested to access the potential effect of environmental indices, traffic volume and bat activity on the roadkill risk of the overall community of bats. s_NDVI_{3yB} and s_NDVI_y – sum of vegetation productivity of 3 years before the reference year and on reference year when the hotspot was identified, respectively. $ACTIVITY_y$ – bat activity level on the year when hotspot was identified. $s_TEMPERATURE_y$ - daytime land surface temperature. $TRAFFIC_y$ – average of night traffic volume. For each model we show the number of degrees of freedom (df), the Akaike Information Criterion corrected for small samples (AICc), AICc differences ($\Delta AICc$) and Akaike weights (w_i). The models are ranked by $\Delta AICc$ and in bold are represented all models with $\Delta AICc < 2$.

Model	df	AICc	$\Delta AICc$	w_i
$s_NDVI_{3yB} + ACTIVITY$	6	106.75	0.00	0.35
s_NDVI_{3yB}	5	106.78	0.03	0.34
$s_NDVI_{3yB} + s_TEMPERATURE_y$	6	108.25	1.50	0.16
$s_NDVI_{3yB} + ACTIVITY + s_TEMPERATURE_y$	7	108.55	1.80	0.14
$s_NDVI_{3yB} + s_TEMPERATURE_y + D_KDE$	7	109.61	2.86	0.07
$s_NDVI_{3yB} + s_TEMPERATURE_y + D_KDE + ACTIVITY$	8	109.96	3.21	0.06
$s_NDVI_{3yB} + D_KDE + s_NDVI_y + s_NDWI_y$	8	110.11	3.36	0.03
$s_NDVI_{3yB} + D_KDE$	6	110.92	4.17	0.03
$s_NDVI_{3yB} + D_KDE + s_NDWI_y + TRAFFIC$	8	110.92	4.17	0.03
$s_NDVI_{3yB} + D_KDE + ACTIVITY$	7	110.92	4.17	0.02
$s_NDVI_{3yB} + ACTIVITY + TEMPERATURE$	7	110.92	4.17	0.02
$s_NDVI_{3yB} + s_TEMPERATURE_y + D_KDE + ACTIVITY + s_NDVI_y + s_NDWI_y$	10	111.66	4.91	0.01
$s_NDVI_{3yB} + TRAFFIC + D_KDE + ACTIVITY + s_NDVI_y + s_NDWI_y$	9	112.02	5.27	0.01
$s_NDVI_{3yB} + TRAFFIC + D_KDE + ACTIVITY + s_NDVI_y + TEMPERATURE$	10	112.22	5.47	0.01
$D_KDE + TEMPERATURE + s_NDVI_y$	7	112.24	5.49	0.01

D_KDE + ACTIVITY + TEMPERATURE	7	112.42	5.67	0.01
D_KDE + TEMPERATURE	6	112.73	5.98	0.01
s_NDVI _{3yB} + TRAFFIC + s_NDWI _y + s_NDVI _y	8	112.77	6.02	0.01
s_NDVI _{3yB} + s_NDVI _y	6	113.03	6.28	0.01
ACTIVITY + TEMPERATURE	6	113.13	6.38	0.01
TEMPERATURE	5	113.16	6.41	0.00
s_NDVI _{3yB} + TEMPERATURE + ACTIVITY + s_NDVI _y	8	113.32	6.57	0.00
s_NDVI _{3yB} + s_TEMPERATURE _y + D_KDE + ACTIVITY + s_NDVI _y + s_NDWI _y + TRAFFIC	11	113.60	6.85	0.00
ACTIVITY + s_NDVI _{3yB} + TRAFFIC	7	113.62	6.87	0.00
s_NDVI _{3yB} + TEMPERATURE + TRAFFIC	7	113.86	7.11	0.00
D_KDE + ACTIVITY + s_NDVI _y	7	114.00	7.25	0.00

*Table S 2.3 - Results from hypothesis testing for each variable: Anova and respective pairwise comparison using Tukey post-hoc tests. For Anova the following parameters are shown: degrees of freedom (df), sum of squares (Sum Sq), mean squares (Mean Sq), F values and P values (for P<0.1, * for P<0.05; and *** for P<0.001). For each variable, we show the mean value ± standard deviation calculated per hierarchical level of hotspots locations change between t and t+1 (stable, disappearance and generation). Lettering below signals plot grouping resultant from Tukey tests (results are significantly different when letters differ), only provided when Anova results were significant (P<0.05).*

Anova results					Tukey post-hoc test results				
Variable	Factors	df	Sum Sq	Mean Sq	F value	P	Stable	Disappearance	Generation
NDVI	Levels	2	1134	566	3.245	*	2.572±1.671	1.511±1.313	6.782±1.831
	Residuals	192	33535	174			a	a	a
WETNESS	Levels	2	2212	1106	2.938	.	3476±759	5739±825	3640±596
	Residuals	192	7229	3765			a	a	b
NDWI	Levels	2	395	197	1.583		7.440±1.460	9.648±1.138	11.305±1.51
	Residuals	192	23964	124			a	b	b
TEMPERATURE	Levels	2	25	12.5	0.051		2.332±0.200	1.652±0.568	1.978±0.751
	Residuals	192	47184	245			a	b	c
ACTIVITY	Levels	2	116	58.1	0.058		3.919±5.139	4.395±3.525	5.787±3.601
	Residuals	192	192060	1000.3			a	a	a



Chapter 3

Road effects on bat activity depended on surrounding habitat type.

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ABSTRACT:

The effects of roads on bats are still a poorly documented issue. Most of the available research focuses on large and high-traffic highways, while low-medium-traffic roads are often assumed to have negligible impacts. However, small roads are ubiquitous in landscapes around the world. We examined the effects of these roads, as well as habitat types, on the activity of three bat guilds (short-, mid- and long-range echolocators) and the most common bat species *Pipistrellus kuhlii*. We performed three bat acoustic surveys between May and October 2015, with these surveys being performed along twenty transects that were each 1,000 m long and perpendicular to three roads with different traffic volumes. The surveys were performed in dense Mediterranean woodland (“*montado*”) and open agricultural field habitats, which were the two dominant land uses. At each transect, bat activity was simultaneously registered at 0, 50, 100, 200, 500 and 1,000 m from the road with the use of an ultrasound recorder. According to the generalized linear mixed-effects models, the overall activity of bats and of the short- and mid-range echolocators increased with increased distance from the roads and was dependent on the surrounding habitats. In contrast, the long-range echolocators and *P. kuhlii* were more tolerant to road. Our results also show that the activity was higher in woodland areas, however road verges seem to be a significant habitat in an open agricultural landscape. The major negative effects extended to approximately 300 m from the roads in woodlands and penetrate further into the open field (more than 500 m). The management of roadside vegetation, combined with the bat habitat improvement in areas that are further from the roads, may mitigate the negative effects. To make road-dominated landscapes safer for bats, the transport agencies need to balance the trade-offs between habitat management and road kill risk.

3.1 INTRODUCTION

Roads are widespread features of many countries, and they form dense worldwide networks, such that there are few road-free areas remaining. In Europe, at least a quarter of the continent's surface is located within 500 m of the nearest transport infrastructure (Torres et al. 2016). The road network strongly contributes to landscape fragmentation or degradation by dissecting continuous areas that have high conservation values, and this fragmentation is considered to be a major driver for negative impacts on natural worldwide populations (Forman et al. 2003; Jaeger et al. 2005; Fahrig & Rytwinski 2009; Grilo et al. 2012). The most perceptible of such impacts is wildlife-vehicle collisions. However, the combination of road avoidance and road barrier effects may contribute to reduced gene flow between roadside populations. Roads also decrease the suitability of adjacent habitats, by altering the physical (e.g., noise and light) and chemical environments. These changes will likely increase the risk of local extinctions (Westemeier et al. 1998; Reed et al. 2007; Jackson & Fahrig 2011; Ascensão et al. 2016). Additionally, these negative effects are usually cumulative in the long-term (Balkenhol & Waits 2009) and can extend their influence to distances of several hundred metres away from roads and into the habitat matrix (e.g., road-effect zone) (Forman et al. 2003). The spatial extension of the impacts of roads on surrounding areas has been studied for many species and can range from less than 100 m to over 5000 m, depending on the road type, the crossed habitat and the traits of the species (Benítez-López et al. 2010; Bennett et al. 2013; Rotholz & Mandelik 2013). Currently, most of the studies of road effects on biodiversity focus on high-traffic transport infrastructures, highways and motorways. However, approximately 50 % of worldwide roads are low- and medium-traffic roads. Thus, it is surprising that for these types of roads, which comprise the majority of the world's roads, information about their potential impacts on wildlife is still lacking (Yanes et al. 1995; Cleverger & Waltho 2000; Dodd et al. 2004).

However, roads may also attract wildlife, as they provide a favourable habitat along roadside verges (e.g., shelter and/or movement corridors for a large number of taxa - (Davies & Pullin 2007; Penone et al. 2012) and provide foraging opportunities (e.g., carrion for scavengers – (Santos et al. 2016). For some of these species, roads and their verges can become ecological traps because the attracted individuals have a higher risk

of becoming road kill (Bernes et al. 2017). Several studies support that the role of roadside verges, either as detractors or attractants, may depend on the surrounding landscape. O'Farrell and Milton (2006) demonstrated that, when crossing high-quality habitats, road verges do not provide habitats for several threatened shrew species (O'Farrell & Milton 2006). Similarly, Galantinho et al. (2017) found no effects on the density and survival of a forest-dwelling small mammal species (*Apodemus sylvaticus*) when comparing roadside verges and roadless areas in a Mediterranean forest area, which is a high-quality habitat for this species. Conversely, in intensive agricultural landscapes, roadside verges are often the only remnants of semi-natural habitats and have an important role as corridors or refuges of biodiversity (Penone et al. 2012; Redon et al. 2015).

A primary concern for biodiversity conservation is to understand the extent to which roads affect the persistence and population of each species (Forman & Alexander 1998). Rytwinski and Fahrig (2013) showed that species with slow life histories and large home ranges can be particularly affected by roads. For example, bats have very low reproductive rates, can have long lives and need large areas for foraging. Thus, transport infrastructures are likely to induce significant negative impacts on this group of mammals.

Several studies have shown that bats suffer frequent road kills (Lesiński 2008; Russell et al. 2008; Gaisler 2009; Lesiński et al. 2011; Medinas et al. 2013; Secco et al. 2017), have reduced foraging activity near roads with street lighting (Stone et al. 2009, 2012; Hale et al. 2015) or intense traffic noise (Schaub et al. 2008; Siemers & Schaub 2011; Luo et al. 2015) and have restricted access to some habitats when these areas are dissected by roads (i.e., barrier effect) (Kerth & Melber 2009). Other studies have shown that gleaning species are less active and that species richness decreases, when approaching the major roads (Zurcher et al. 2010; Berthinussen & Altringham 2012; Kitzes & Merenlender 2014). In a study on railways, Vandeveld et al. (2014) showed that the commuting activity of aerial hawking bats is higher over verges than in the surrounding intensive agricultural habitat. However, because of the very low train traffic at night and the narrow railway corridors, the negative effects of these infrastructures on bats are potentially weaker than those of roads. Thus, bats may show contrasting behaviours, in regard to transport infrastructures, depending on their type, features and

surrounding habitats. Their response is also likely to be dependent on the functional groups of the bats (hereafter called guilds).

We investigated the effect of distance to low-medium traffic roads, as well as the importance of road-surrounding habitats, on bat activity patterns in a Mediterranean landscape. It was assumed that bats reduce their activity even in proximity to low-medium traffic roads. We also hypothesized that the characteristics of the surrounding habitats would modulate the road-effect zone, with this zone being larger in open agricultural areas, when compared with woodlands. Moreover, the road-effect zone should also be guild specific. Short- (SRE) and mid-range (MRE) echolocators, which are more adapted to fly in woodland and vegetated edges (Frey-Ehrenbold et al. 2013), would avoid roads that cross woodlands, but could use vegetated verges of roads that are imbedded in open areas. Additionally, the long-range echolocators (LRE) bats, which generally forage in wide-open spaces, as well as above the tree canopy level, should be less affected. To test these hypotheses, we identified the road-effect zone for the overall bat community, SRE, MRE and LRE bats, as well as for *Pipistrellus kuhlii* (the most common bat species). We also evaluated whether the road-surrounding habitats (woodlands or open agricultural field) had an effect on the road-effect zone.

3.2 METHODS

3.2.1 Study area and sampling design

This study was conducted in southern Portugal (38°32'24" to 38°47'33"N; -08°13'33" to -07°55'45"W) within a landscape dominated by cork (*Quercus suber*) and holm oak (*Quercus rotundifolia*) savannah-like woodlands (the Portuguese "montado"), which alternated with arable fields, olive groves and vineyards (Figure 3.1). The climate is Mediterranean, with the mean 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 season (July), as well as an annual rainfall averaging 609.4 mm (Évora 1971-2000; Instituto de Meteorologia, 2010). The terrain is flat to gently undulating and ranges from 150 m to 400 m above sea level.

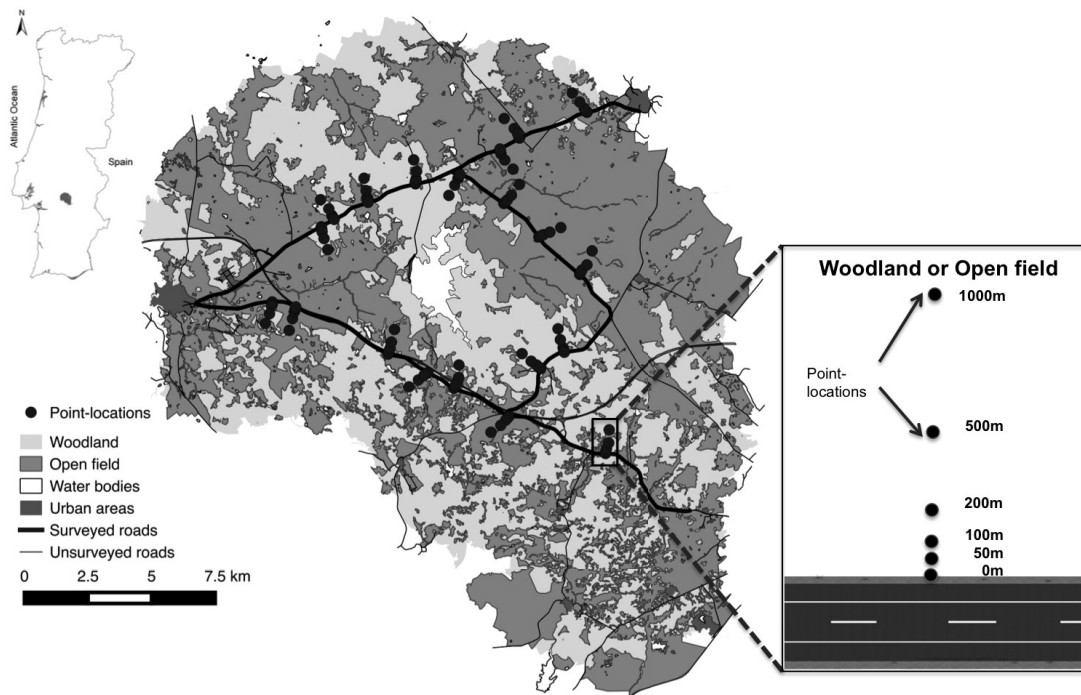


Figure 3.1 - Map of the study area in southern Portugal showing the sections of roads that were surveyed (EN114, EN4 and EN370-M529) and the bat transect routes (black markers). The two main land uses are woodland (light grey) and open field (dark grey) areas. Illustration of the layout of bat point-locations surveys at each of the six distances from the low-medium traffic roads.

We conducted point-count surveys along 20 transects, with bat activity recorded at each of six point-locations along each transect at 0, 50, 100, 200, 500 and 1,000 m (Figure 3.1) approximately perpendicular to three national roads (EN4, EN114 and EN370) and one municipal road (M529). These roads are single-carriageway roads with an average night traffic of 277 to 1210 vehicles/night (EN114 and EN4, respectively) and an average night traffic of less than 100 vehicles/night (EN370 and M529). Following the classification of the Portuguese Road Institute, these data were grouped into three nocturnal traffic categories: medium-high traffic for EN114 (1210 vehicles/night), medium traffic for N4 (277 vehicles/night) and low traffic for EN370 and M529 (both with <100 vehicles night), with the low traffic roads hereafter referred to together as EN370-M529 (EP, 2005; see details in Medinas et al., 2013). The verges of these roads vary from 5 to 10 m wide and are vegetated with dense and tall grasses, different types of shrubs and

scattered trees. When roads crosses woodland patches, the tree canopy covered most of the verges.

All transect routes were selected by their habitat homogeneity on both sides of the road within large, continuous habitat patches (of either woodland or open agricultural areas). Habitats were categorized as “woodland” when the tree canopy cover was above 30 %; otherwise, habitats were classified as “open agricultural field”. Woodland areas provide high densities of insects and shelter from aerial predators (Zimmerman & Glanz 2000; Rainho et al. 2010). These resources are much less available in open field areas, which makes them less suitable for bats. We surveyed bat activity up to 1,000 m from the roads because we expected that the road-effect zone would be smaller in low-medium traffic roads, compared to what has been reported for motorways (1,600 m road-effect zone – Berthinussen and Altringham, 2012). Moreover, this was the maximum transect length available in the homogeneous habitat patches across the study area. Half of the transects were located in woodland areas (EN-114: 3 transects; EN-4: 4 transects and EN370-M529: 3 transects), and the other half were located in open agricultural field areas (EN-114: 4 transects; EN-4: 3 transects and EN370-M529: 3 transects).

To minimize the effects of other landscape variables, we selected patches that were at least 500 m away from water bodies, human settlements or other roads. The road sections that were studied were predominantly unlit, except on a few crossings and small settlements areas, and all of the transects were conducted along unlit sections. Air temperature, wind speed and percentage of cloud cover were recorded at the road, both before and after each night-period survey, by using a digital anemometer/thermometer (WS9500 Pocket Anemometer, La Crosse Technology, USA).

3.2.2 Bat surveys and calls identification

Bat surveys took place on one transect per night (6 point-locations that were simultaneously sampled at increasing distances from the road – *Figure 3.1*), and each transect was surveyed three times between May and October of 2015, with a minimum of five weeks between the sampling sessions. Bat monitoring was performed on dry, warm and calm nights (minimum temperature > 13 °C; wind speed < 3.5 m/s). Each

survey started 30 min before sunset, in order to allow for varying emergence times of the different bat species, and ended 180 min after sunset, in order to comprise the peak of bat activity (Amorim et al. 2018). We used bat detectors (Petterson D500x, Petterson Elektronik AB, Sweden) to passively detect, record and store full-spectrum bat echolocation call sequences (Ahlén & Baagøe 2004). Bat detectors were set up on a tripod approximately 1.5 m above the ground and facing the road. Each detector was rotated between different distances to the road, in order to reduce the potential microphone sensitivity bias. Each detector's microphone was positioned at a 45° angle relative to the ground and oriented towards the tree canopy gaps, in order to increase the number and quality of ultrasound recordings.

The bat call sequences were batch-processed by using the software Kaleidoscope (Wildlife Acoustics, Inc., version 3.1.1) through scrubbing (default settings), in order to remove noise recordings or low-intensity bat calls. We measured the bat calls that remained after the post-scrubbing by using a custom R script based on the seewave package (Sueur et al. 2008). A bat pass was defined as a search-phase echolocation sequence of at least 3 consecutive echolocation call pulses of one individual bat (Fenton 2002), with the pass separated from the next pass by at least 1 second. Bat calls were analysed by measuring parameters such as maximum energy frequency (FMaxE), pulse duration (D), inter-pulse interval (IPI), pulse initial (Fi) and final frequency (Fe). The frequency parameters (FMaxE, Fi and Fe) were measured from the power spectra of the entire call and were computed with the Fast Fourier Transform, whereas the time-related variables (IPI and D) were measured from the oscillograms. After obtaining the parameters, bat calls were identified to the species level or assigned to species complexes by using dichotomous keys for Portugal and for the Iberian Peninsula (Lisón 2011; Rainho et al. 2011). Due to the high degree of overlap of the echolocation call characteristics, some species were identified to the genus level (e.g., *Eptesicus*: *E. serotinus* + *E. isabellinus*; *Plecotus*: *P. auritus* + *P. austriacus*). Species of the *Myotis* genus (most likely *Myotis daubentonni*, *M. escalerae* or *M. myotis*) were included in the same phonic group, and three *Nyctalus* species (*N. noctula*, *N. lasiopterus* and *N. leisleri*) were considered together. Other cases included the distinction between calls belonging to the *Pipistrellus* genus (mainly *P. pygmaeus*) and to *Miniopterus schreibersii*, whose echolocation calls are very similar. Thus, we opted for creating a composite category *Pipistrellus* spp./*M. schreibersii*.

To assess the influence of roads in the species with different traits, we grouped bats into guilds that reflected their echolocation ranges and clutter avoidances, namely, short-range echolocators (SRE; *Myotis* spp., *Plecotus* spp. and *Rhinolophus* spp.), mid-range echolocators (MRE; *Pipistrellus* spp. and *M. schreibersii*) and long-range echolocators (LRE; *Eptesicus* spp., *Nyctalus* spp. and *Tadarida teniotis*) (see details in Frey-Ehrenbold et al., 2013). As *P. kuhlii* comprises 57 % of the MRE guild passes, we excluded this species from the guild and bat overall community, and analysed its activity pattern separately.

3.2.3 Statistical analysis

We investigated the influence of distance to road on bat activity by using general linear mixed models (GLMM). However, because this relationship can be strongly modulated by habitat types for bat foraging, we also included the types of habitat (woodland or open agricultural field areas) as an explanatory variable in our models. Each transect and sampling session was included in the models as a random factor, in order to account for spatial and seasonal variation on bat activity, respectively. Additionally, to account for the spatial structure of the sampling point-locations along the same transect, we included an exponential correlation model that was developed with the coordinates of the point-locations (corExp) (Zuur et al. 2009). The number of overall community bat passes, the bat recordings of each guild and activity of *P. kuhlii* were log-transformed (count+1), in order to account for the occurrence of zero bat passes and to minimize the effect of possible outliers. We used the Gaussian with error distribution as an identity link function to test the effects of roads on bat activity.

Prior to the statistical analyses, the explanatory variables with skewed structures were transformed, in order to approach normality and to reduce the influence of extreme values (Zuur et al. 2009). The explanatory variables were then standardized to zero mean and unit variances, in order to allow for the comparison of their effect strengths (Burnham & Anderson 2002). Collinearity among the explanatory variables was investigated by using pairwise correlations. For correlations that were higher than 0.7, only the most biologically meaningful variables were retained for further analysis

(Dormann et al. 2013). We also did a preliminary inspection of the variation of bat activity across the traffic categories: however, we did not find significant differences ($p > 0.1$). Thus, we pooled the bat activity data from all of the roads (Figure S 3.1). Consequently, the explanatory variables that were used in the models were distance to the road and habitat type. The latter was included as a categorical variable, and the open agricultural areas were used as reference value. The distance to the roads was tested for both the linear and quadratic responses. All of the candidate models were built based on all of the possible subsets of the explanatory variables, including the null and the full models, as well as the interactions between the explanatory variables. The full models were structured in the following way:

$$\begin{aligned} \text{Bat activity} \sim & \text{Distance to road} + \text{Distance to road}^2 + \text{Habitat type} \\ & + \text{Interaction terms, } \text{random} = 1|\text{Sampling session/Transect,} \\ & \text{structural correlation} = \text{corExp}(\text{Easting} + \text{Northing}) \end{aligned}$$

We used the Akaike Information Criterion, adjusted for small sample sizes (AICc), and the corresponding Akaike weights (w_i), to rank the candidate models and to select the most parsimonious model for each case (Burnham & Anderson 2002). The residual plots of residuals were examined, in order to check for normality, and deviance tests were performed, in order to assess the goodness of fit of the final models.

All analyses were performed in R version 3.3.2 (R Development Core Team, 2006) by using the packages “nlme” (Pinheiro et al. 2020) and ‘MuMIn’ (Barton & Barton 2020).

3.2.4 Road-effect zone – identification of thresholds

Whenever we detected a non-linear pattern in the relationship between bat activity and the distance to the road, we further investigated the presence of the breakpoints by using a piecewise regression to describe this relationship. This method allows for the identification of a threshold distance for the effect of roads on bat activity. We fitted the

piecewise regression models to the transformed polynomial of the explanatory variable distance to the road by using the package “segmented” in R (Muggeo 2015). We assessed the breakpoint value and its 95 % confidence interval for each habitat type (Toms & Lesperance 2003). The breakpoint validation was performed by using the Davies’ test, which compares the regression slopes before and after each breakpoint (Muggeo 2015).

3.3 RESULTS

A total of 29,239 bat passes were recorded from 360 detector-nights (20 transects x 6 locations x 3 nights), which corresponded to over 1,080 survey hours. Of these, 24,455 (83.6 %) passes could be identified to the species level, while 4,158 (14.2 %) were assigned to a single-genus complex, 262 (0.1 %) were classified as a multi-genus complex and 364 (0.1 %) could not be identified (*Table S 3.1*).

Bat passes corresponded to eight genera, namely *Pipistrellus* ($N = 23,853$; 82.6 %), *Eptesicus* ($N = 984$; 3.4 %), *Myotis* ($N = 698$; 2.4 %), *Nyctalus* ($N = 2,386$; 8.3 %), *Barbastella* ($N = 189$; < 0.1 %), *Plecotus* ($N = 85$; < 0.1 %), *Rhinolophus* ($N = 5$; < 0.1 %), and *Tadarida* ($N = 412$; 1.4 %) (*Table S 3.1*).

The detected activity of the three acoustic guilds was uneven; MRE had much higher activity ($N = 24,115$; 83.5 %) than LRE ($N = 3,782$; 13.1 %) and SRE ($N = 977$; 3.4 %). Among the MRE, the species *P. kuhlii* accounted for more than 50 % of the guild’s activity ($N = 13,789$, 57.2 %); thus, this species was analysed separately.

3.3.2 Factors affecting bat activity and guild activity

The activity of the bat community and of each species/group was markedly higher in the woodlands than in the open agricultural areas (Supporting information - *Table S 3.2*). In fact, the overall bat activity in the woodland sites was more than three times higher,

compared with the open agricultural sites (passes per night: woodland, 810 ± 93 ; open agricultural areas, 234 ± 34).

The best models for overall bat activity, the activity of the three bat guilds (SRE, MRE and LRE) and of *P. kuhlii* explained between 28.6 to 60.9 % of the total variance. The most parsimonious models included habitat types, thus confirming that the activity of all of the groups and species tested were significantly higher in the woodlands than in the open agricultural areas (Table 3.1. and Supporting information Table S 3.3). Three models - overall bat activity, SRE and MRE activity - yielded similar outputs and included the interaction between the distance to the road and habitat type, thus suggesting that the effect of roads on bat activity is different according to the two habitats that were tested. In the woodland areas, the activity of each of these groups slightly increased until intermediate distances from the road, after which they were approximately constant after that. This pattern was particularly pronounced in the SRE and MRE guilds. Conversely, in the open agricultural field areas, the activity of each of these groups of bats was lower at intermediate distances from the road and peaked at the shortest and largest distances.

The activity of the LRE bats and *P. kuhlii* was only affected by habitat type. Both bat groups were more active in the woodland areas, which was similar to the other bat groups that were analysed (Table 3.1).

Table 3.1 - Standardized coefficients (β) and the corresponding 95 % confidence intervals (CIs) (in brackets) for the best-supported GLMMs that explain the activity of the overall community of bats (Overall), as well as for the three foraging guilds (SRE: short-range echolocators, MRE: mid-range echolocators without *P. kuhlii* (Pkuh) and LRE: long-range echolocators) and *P. kuhlii*, as functions of habitat type (HABITAT) and distance to the road (D_ROAD). The open field area was included as the reference category for the variable HABITAT. The variable distance from road (m) (D_ROAD) in quadratic form is identified with “^2”. Highlighted in bold are parameters (except for the intercept) with CIs excluding zero.

Variable	Overall		SRE		MRE	
	β	CI	β	CI	β	CI
HABITAT	1.406	(1.021; 1.791)	0.402	(0.155; 0.649)	1.430	(0.971; 1.890)
D_ROAD	1.768	(-0.644; 4.180)	1.118	(-0.948; 3.315)	2.239	(-0.527; 5.184)
D_ROAD^2	5.025	(2.666; 7.384)	2.066	(0.002; 4.110)	4.176	(1.396; 6.956)
D_ROAD*HABITAT	1.064	(-2.161; 4.291)	0.954	(-1.899; 3.807)	1.456	(-2.364; 5.275)
D_ROAD^2*HABITAT	-5.673	(-8.836; -2.510)	-2.871	(-5.610; -0.131)	-6.391	(-10.117; -2.664)
Intercept	2.464	(2.183; 2.745)	0.657	(0.473; 0.842)	1.934	(1.599; 2.270)

Variable	LRE		Pkuh	
	β	CI	β	CI
HABITAT	1.063	(0.742; 1.384)	1.360	(0.863; 1.856)
D_ROAD	-	-	-	-
D_ROAD^2	-	-	-	-
D_ROAD*HABITAT	-	-	-	-
D_ROAD^2*HABITAT	-	-	-	-
Intercept	1.263	(1.029; 1.500)	1.879	(1.280; 2.478)

3.3.3 Estimated road-effect zone

In the woodlands, the activity of the overall bats, of SRE and of MRE increased until an estimated distance of 310 m (SE = 270 - 349), 177 m (SE = 122 - 232) and 230 m (SE = 213 - 248), respectively, and remained high and approximately stable for larger distances (Figure 3.2). When regarding the open agricultural areas, the pattern was different, with a decrease in activity at approximately 300 m from the road for each of these three bat groups, and a higher activity at the maximum distance of 1,000 m. For these bat groups and for both habitat types, the slopes of each of the two regression lines differed significantly ($p < 0.001$), thus supporting the existence of a breakpoint. For the LRE guild and for *P. kuhlii*, no breakpoints and no significant effects of distance to road were identified.

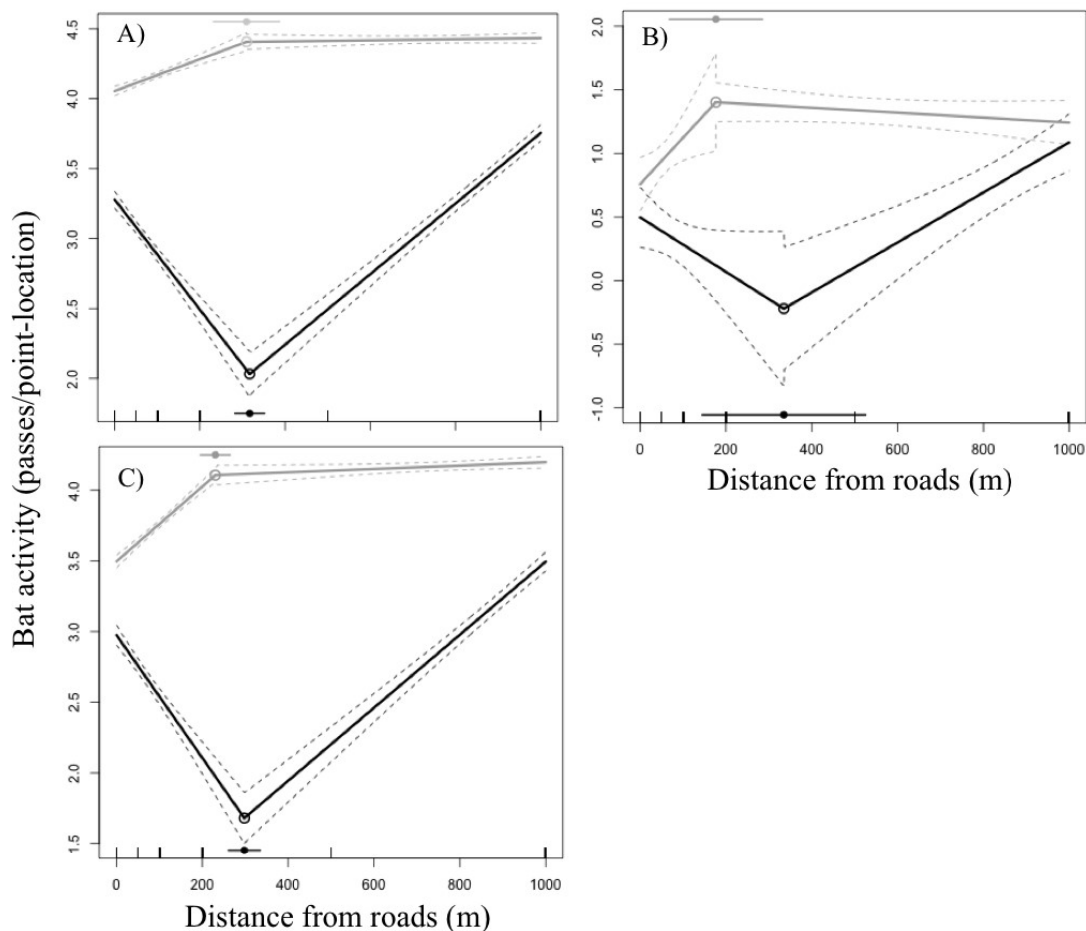


Figure 3.2 - Effect of distance from the road on the overall bat activity (A), SRE activity (B) and MRE activity (C) in two habitats: woodland (light grey) and open agricultural fields (dark grey).

Solid lines show the best fit piecewise regression models with 95 % CIs (dashed lines). Filled circles indicate breakpoints in the relationship and associated standard errors.

3.4 DISCUSSION

3.4.1 Habitat effects

We found that there is a strong association between bat activity in the vicinity of roads and habitat type. For the bat guilds and species that was individually analysed, bat activity was much higher in the woodlands. This is consistent with the well-documented high importance of this habitat for European bat species, both as foraging (e.g. (Vaughan et al. 1996; Russ & Montgomery 2002; Davidson-Watts & Jones 2006)) and roosting areas (e.g., Russo & Jones 2003; Dietz, C., Nill, D. and von Helversen 2009; Boughey et al. 2011). Food resources and roosts are much less available in open fields, which combine with low predator protection makes these areas less suitable for bats.

3.4.2 Impact of roads on bat activity pattern

The negative effects of high-traffic roads on bat activity are widely documented (Berthinussen & Altringham 2012; Kitzes & Merenlender 2014). According to Berthinussen and Altringham (2012), the avoidance of the vicinities of motorways by bats could be a response to the physical properties of large roads, such as wide gaps in the tree canopy, greater crossing distances (Fensome & Mathews 2016) and high noise levels (Schaub et al. 2008). However, previous data on the impacts of low-medium traffic roads are scarce. We assessed the effects of the distance to these roads on bat activity, especially when considering the road-surrounding habitats. We found that the relationship between bat activity and the distance to the roads is non-linear and highly dependent on the surrounding habitats. Similarly, Berthinussen and Altringham (2012) found that favourable habitats that are crossed by roads had a comparably stronger increase in bat species richness than in degraded habitats. Our results also show that, when roads cross Mediterranean woodlands, which is a habitat rich in feeding and shelter resources for bats, they tend to avoid the immediate proximity of roads. This is in

accordance with expectations because SRE bats - *Myotis* spp. and *Plecotus* spp. - consistently select woodland areas while commuting, thus avoiding movement along road edges with lower tree cover than the adjacent habitats (Fensome & Mathews 2016). In contrast, in the open agricultural areas, where resources are scarcer, we found that bat activity near the roads is similar to the activity that is registered 1,000 m away from this infrastructure but is reduced at intermediate distances. These results suggest that, in habitats with fewer resources, road vicinity (i.e., road verges) may be a valuable habitat, likely for feeding, as has been documented for other mammals (Redon et al. 2015) and birds (Laursen 1981). Furthermore, the roadsides in intensive agriculture landscape may have acted as habitat edges or corridor-like habitats, due to the higher structural complexity of vegetation, when compared with landscape-matrix at intermediate distances from the road (Bernes et al. 2016).

The pattern herein described is compatible with the hypothesis that bat activity in road-surrounding areas is determined by a compromise between road avoidance, due to disturbance, and the use of resources provided by roads, which may be relevant when these resources are limited in the adjacent habitats. Moreover, we found that this pattern describes overall, SRE and MRE bat activity, but is not universal, as no significant road distance effect was detected for LRE (i.e., *Nyctalus* spp., *Eptesicus* spp. and *T. teniotis*) (Figure S 3.2) or for *P. kuhlii* bats, which are the most common bat species in the study area. SRE and MRE guilds include the smaller bat species, and these species are likely the most prone to the deleterious effects of roads (Fensome & Mathews 2016). Kerth and Melber (2009) found strong effects of a major road on habitat use for the SRE species *Myotis bechsteinii*. Likewise, the activity of *P. pipistrellus*, an MRE bat, was also lower in proximity to a major road (Berthinussen & Altringham 2012). These results, together with ours, suggest that gleaning, edge and forest dwelling species, which forage in cluttered habitats, can be more susceptible to negative impacts from roads.

On the other hand, LRE bats, which usually fly high and fast above the tree canopy while feeding in open spaces (Jensen & Miller 1999; Marques et al. 2004), should be less affected by road disturbance (Jung & Kalko 2011; Russo & Ancillotto 2015), and may depend less on possible resources that are provided by roads (Figure S 3.1). This is supported by empirical data showing that this bat guild registered very low numbers of

road kills (Medinas et al. 2013; Fensome & Mathews 2016). *P. kuhlii* is a synanthropic and generalist species that is able to forage in a wide range of habitats (Carmel & Safriel 1998; Rainho 2007; Dietz, C., Nill, D. and von Helversen 2009; Georgiakakis et al. 2010; Lisón & Calvo 2013), including over lit and unlit road edges (Kahnonitch et al. 2018). In addition, it can roost in buildings and tree holes (Cruz et al. 2016) that can be located on road verges (DM, personal observation).

3.4.3 Spatial extent of road-effect zone

We evaluated road distance effects up to 1,000 m from roads, and we found an influence on overall bat activity up to approximately 300 m for both habitats. This is slightly shorter for the SRE (170 m) and MRE (230 m) guilds in roads that cross woodlands, when compared with open field areas (SRE -@□ 330 m; MRE - 300 m), thus suggesting that tree cover may partly buffer the negative effects of roads on surrounding areas. Other studies have shown that negative effects of roads on bats can extend to greater distances; for example, Berthinussen and Altringham (2012) reported effects at distances up to 1,600 m from a large motorway in the UK. However, those studies were performed in much larger roads with high traffic volumes that were at least six-fold higher than the registered volumes in our area, thus suggesting that traffic, road characteristics or habitat contexts need to be taken into account, in order to properly characterize the road-effect zone.

3.4.4 Implications for roadside management

This study highlights that low-medium traffic roads should be considered as a major pressure on global bat conservation, because almost 50 % of the worldwide transport infrastructure is accounted for in this traffic category (Meijer et al. 2018). Our study also shows a relatively higher bat activity for the immediate vicinity of roads when these roads cross open fields, thus suggesting that the preservation of vegetation along roads, such as strips of tall grass, shrubs and, whenever possible, treelines, may be important, in

order to increase the suitability of roadsides for bats. The important role of verges as bat feeding areas has been previously reported for railways that cross intensive agricultural areas (Vandeveldt et al. 2014). Moreover, when roads cross less suitable areas, roadsides may also act as dispersal corridors and may be the last landscape refuge for many small vertebrates and invertebrates (e.g., Forman & Alexander 1998; Trombulak & Frissell 2001; Sabino-Marques & Mira 2011; Wynhoff et al. 2011), thus offering feeding opportunities for their predators.

Nevertheless, we need to be cautious when promoting wildlife habitat use near roads because we are also increasing the risk of collision with vehicles. Medinas et al. (2013) found that approximately 3 bats/km/year are road killed on the same roads that were surveyed in our study. This is an unexpectedly large number of road kills whose impacts on the long-term viability of populations still need to be evaluated. Moreover, it is also known that the presence of trees and shrubs tends to increase the likelihood of bat road-crossing (Abbott et al. 2012a; Bennett & Zurcher 2013).

Thus, a better potential mitigation measure for reducing the effects of roads should be the creation of bat feeding habitats (e.g., planting insect friendly shrubs and trees, or creating ponds) away from roads (e.g., Wyatt 2010; Berthinussen & Altringham 2012, 2016). The recommended distance from high traffic roads to habitat improvements is 1,000 m; however according to our findings for low-medium traffic roads, we suggest that this distance should be approximately half of the previously reported distance. This new information will strengthen the road corridor planning and overall landscape management that aims to enhance bat conservation in road-dominated landscapes.

3.5 CONCLUSION

Our study provides the first insights into how low-medium traffic roads affect bat activity. Overall, the results emphasize that road-effects on bats are non-linear and depend on the functional characteristics of the species and the road-habitat contexts. We highlighted that trade-offs that are posed by roads between feeding/commuting

opportunities and road kill risk must be taken in account during road planning and in implementation of mitigation measures. This is a priority, and it represents a non-consensual and poorly addressed issue that needs to be disentangled.

We are aware that additional data (e.g. prey availability, noise, light and management of verges) are needed in order to properly understand the causes underlying of the patterns that we observed. However, our results underline that the road-surrounding habitat is a key component of this system. This study provides some important clues about the role of vegetation management in verges and surrounding areas, in order to keep road-dominated landscapes more suitable and safer for bats. Nevertheless, our results also raise key challenging questions in roadside wildlife management: (1) Are road-related habitats opportunities for bat (and other wildlife) conservation? (2) If yes, under what circumstances? These are two of the many questions that need to be considered in order to properly evaluate the pattern and intensity of road effects on wildlife and, to efficiently mitigate these effects.

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SUPPORTING INFORMATION

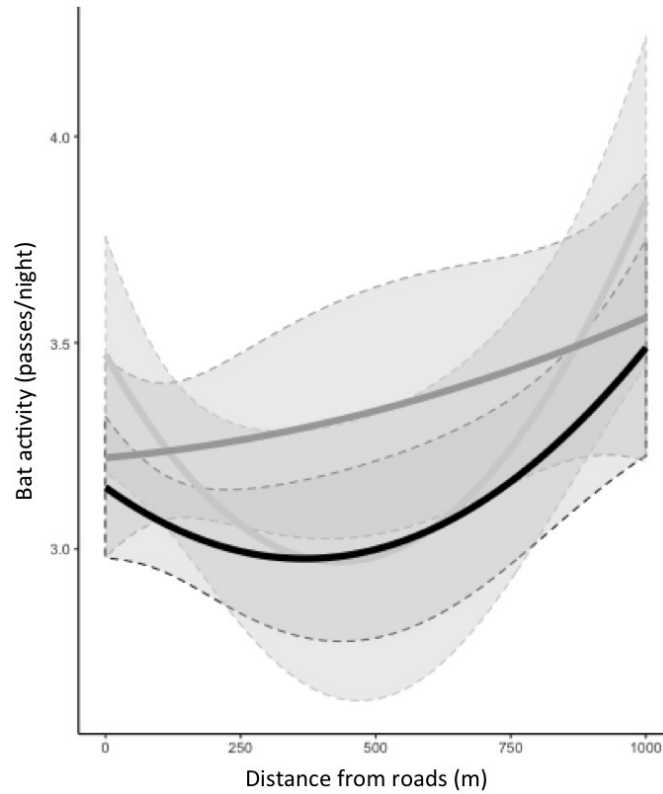


Figure S 3.1 - The effect of road distance on the number of bat passes to the overall community of bats on a log scale for each road/nocturnal traffic categories surveyed: medium-high traffic (1210 vehicles/night) (black), medium traffic (277 vehicles/night) (grey) and low traffic (<100 vehicles night) (light grey). The dashed lines show the 95 % CIs.

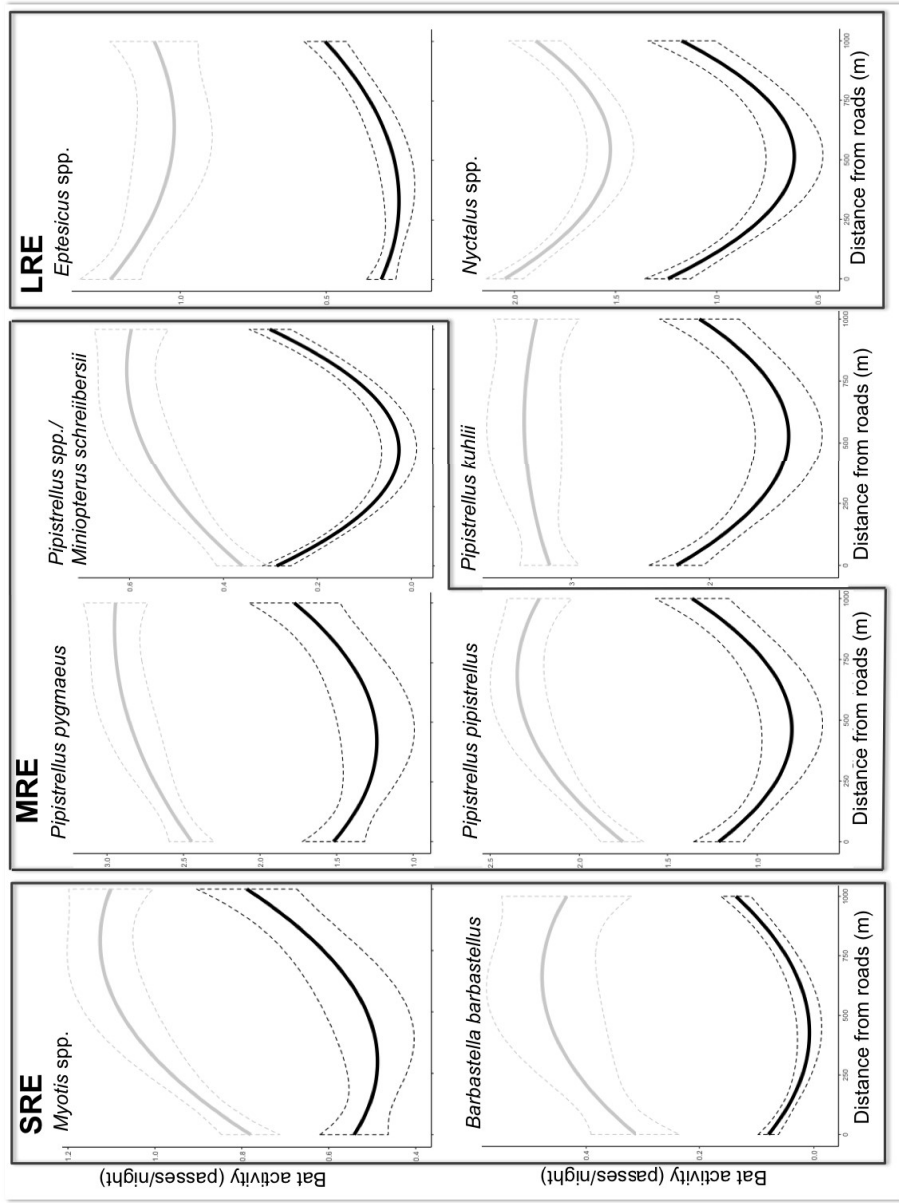


Figure S 3.2 - The effect of distance from the road on species/genus activity as predicted by the best GLMM models. The plots show the probabilities of activity levels per night with distance from the road and for the two surveyed habitats: woodland (light grey) and open agricultural areas (dark grey). The effect of distance was statistically significant ($p < 0.05$) to species/genus, belonging to SRE bats (*Myotis* spp.; *Barbastella barbastellus*), MRE bats (*Pipistrellus pygmaeus*; *Pipistrellus pipistrellus*), and non-significant for LRE bats (*Eptesicus* spp.; *Nyctalus* spp.), as well as for *Pipistrellus kuhlii*. The dashed lines show the 95 % CIs. Note that the scale of the y-axes vary among the plots, in order to assist in visualization.

Table S 3.1 - The number of total assignments of bat recordings to species, genus-complex or level guild, occurrences, number of point-locations for recorded bat passes (%) and means with standard errors of the bat passes for each sample point-location.

Species	Total bat	Occurrence on 360	Occurrence (%)	Mean of bat passes per	SE of bat passes per
Short-range echolocators (SRE)	977	218	60.56	4.86	0.02
<i>B. barbastellus</i>	189	81	22.50	2.33	0.06
<i>Myotis</i> spp.	698	217	60.56	3.20	0.01
<i>Plecotus</i> spp.	85	49	13.89	1.70	0.03
<i>Rhinolophus ferrumequinum</i>	1	1	0.01	1	-
<i>Rhinolophus</i> spp.	4	3	1.11	1	-
Mid-range echolocators (MRE)	24115	332	92.50	72.41	0.26
<i>P. kuhlii</i>	13789	332	92.50	41.40	0.23
<i>P. pipistrellus</i>	3492	296	82.50	11.76	0.08
<i>P. pygmaeus</i>	6572	312	86.94	20.99	0.15
<i>Pipistrellus</i> spp./ <i>M. schreibersii</i>	262	113	31.67	2.30	0.11
Long-range echolocators (LRE)	3782	288	80.28	13.09	0.06
<i>Eptesicus</i> spp.	984	194	54.17	5.05	0.04
<i>Nyctalus</i> spp.	2386	288	80.28	8.26	0.05
<i>T. teniotis</i>	412	31	8.89	12.87	0.71

Table S 3.2 - The number of assignments of bat recordings (mean $\pm$ SE) to species- or genus-complex or level guild at recordings per point-locations of the different distances from the roads in woodland ($n = 10$) and open field ($n = 10$) habitats.

		Mean passes per point-locations ($\pm$ SE)									
Woodland											
Distance from roads (m)		0		50		100		200		500	
Short-range echolocators		9.60	$\pm$ 2.20	7.67	$\pm$ 1.40	12.50	$\pm$ 2.61	12.10	$\pm$ 2.41	16.50	$\pm$ 5.84
(SRE)											
<i>Barbastella barbastellus</i>		4.13	$\pm$ 1.80	3.50	$\pm$ 1.19	3.00	$\pm$ 0.98	3.75	$\pm$ 0.65	7.50	$\pm$ 2.53
<i>Myotis</i> spp.		6.53	$\pm$ 1.22	5.44	$\pm$ 1.11	9.10	$\pm$ 2.03	8.00	$\pm$ 1.65	10.40	$\pm$ 3.88
<i>Plecotus</i> spp.		2.50	$\pm$ 0.65	2.00	$\pm$ 0.58	1.71	$\pm$ 0.71	2.20	$\pm$ 0.73	3.20	$\pm$ 1.20
<i>Rhinolophus</i> spp.		-		-		1.00	$\pm$ -	-		-	
Mid-range echolocators		308.20	$\pm$ 53.18	211.70	$\pm$ 24.47	316.40	$\pm$ 69.47	299.50	$\pm$ 40.12	372.30	$\pm$ 107.93
(MRE)											
<i>Pipistrellus kuhlii</i>		201.60	$\pm$ 55.53	126.80	$\pm$ 24.00	200.00	$\pm$ 64.66	150.20	$\pm$ 15.50	203.70	$\pm$ 66.30
<i>Pipistrellus pipistrellus</i>		28.10	$\pm$ 7.92	24.00	$\pm$ 4.20	36.00	$\pm$ 11.99	50.50	$\pm$ 13.19	62.80	$\pm$ 32.80
<i>Pipistrellus pygmaeus</i>		76.60	$\pm$ 21.08	58.40	$\pm$ 15.25	76.50	$\pm$ 16.93	94.70	$\pm$ 31.69	102.40	$\pm$ 43.65
<i>Pipistrellus</i> spp./ <i>M. schreibers</i>		3.17	$\pm$ 1.14	3.57	$\pm$ 0.53	4.88	$\pm$ 1.50	4.56	$\pm$ 1.57	4.25	$\pm$ 1.08
Long-range echolocators		96.80	$\pm$ 23.04	29.50	$\pm$ 5.27	40.20	$\pm$ 8.65	38.00	$\pm$ 13.61	45.10	$\pm$ 13.87
(LRE)											
<i>Eptesicus</i> spp.		25.60	$\pm$ 8.46	8.00	$\pm$ 1.18	12.50	$\pm$ 2.26	10.60	$\pm$ 3.02	13.90	$\pm$ 4.79
<i>Nyctalus</i> spp.		64.90	$\pm$ 15.82	14.90	$\pm$ 1.92	20.20	$\pm$ 4.53	16.50	$\pm$ 2.87	29.44	$\pm$ 8.20
<i>Tadarida teniotis</i>		15.75	$\pm$ 4.96	16.50	$\pm$ 7.86	18.75	$\pm$ 16.10	27.25	$\pm$ 25.29	23.50	$\pm$ 22.50
Unidentified		5.86	$\pm$ 2.57	7.00	$\pm$ 4.76	8.71	$\pm$ 3.23	8.13	$\pm$ 1.90	6.71	$\pm$ 2.68
Total woodland bat activity		418.70	$\pm$ 58.51	250.90	$\pm$ 26.92	375.20	$\pm$ 75.38	356.10	$\pm$ 54.59	438.60	$\pm$ 124.56
										418.20	$\pm$ 76.19

Mean passes per point-locations (± SE)										
Distance from roads	0	50	100	200	500	1000				
Short-range echolocators (SRE)	4.67 ± 1.07	3.67 ± 0.75	5.30 ± 1.38	3.44 ± 0.69	4.00 ± 1.20	7.30 ± 2.29				
<i>Barbastella barbastellus</i>	2.00 ±	1.00 ±	1.33 ± 0.88	1.00 ±	-	1.00 ±				
<i>Myotis</i> spp.	3.75 ± 0.88	3.00 ± 0.71	5.22 ± 1.32	3.50 ± 0.68	3.75 ± 1.08	7.33 ± 2.50				
<i>Plecotus</i> spp.	2.25 ± 1.25	1.50 ± 0.50	1.00 ±	2.00 ±	1.00 ±	1.00 ±				
<i>Rhinolophus</i>	-	1.00 ±	-	-	-	-				
<i>Rhinolophus</i> spp.	1.00 ±	-	1.00 ±	-	-	1.00 ±				
Mid-range echolocators (MRE)	148.70 ± 39.95	75.70 ± 24.79	88.00 ± 47.46	50.60 ± 15.77	61.10 ± 14.58	122.9 ± 38.12				
<i>Pipistrellus kuhlii</i>	97.00 ± 28.32	48.70 ± 23.99	53.70 ± 34.58	22.70 ± 8.03	30.80 ± 9.63	44.90 ± 14.29				
<i>Pipistrellus pipistrellus</i>	28.22 ± 7.64	9.56 ± 2.26	10.33 ± 2.72	8.33 ± 2.46	10.89 ± 2.93	30.33 ± 10.50				
<i>Pipistrellus pygmaeus</i>	24.20 ± 9.33	17.50 ± 8.05	24.50 ± 11.03	19.90 ± 8.18	22.56 ± 6.24	49.40 ± 15.84				
<i>Pipistrellus</i> spp./ <i>M. schreibersii</i>	3.5 ± 0.88	3.00 ± 1.15	2.50 ± 1.50	1.00 ±	1.00 ±	1.86 ± 0.34				
Long-range echolocators (LRE)	17.70 ± 4.83	12.20 ± 3.06	13.30 ± 2.93	20.00 ± 13.14	8.67 ± 2.51	17.10 ± 3.78				
<i>Eptesicus</i> spp.	2.78 ± 0.72	3.71 ± 1.90	4.00 ± 1.26	4.14 ± 2.33	3.38 ± 1.55	7.11 ± 3.55				
<i>Nyctalus</i> spp.	15.1 ± 4.43	9.60 ± 2.81	11.30 ± 2.17	17.10 ± 11.50	6.38 ± 1.43	10.50 ± 1.72				
<i>Tadarida teniotis</i>	1.00 ±	-	-	-	-	1.00 ±				
Unidentified	2.20 ± 0.73	3.00 ±	2.00 ± 1.00	3.75 ± 2.43	1.25 ± 0.25	2.25 ± 0.95				
Total open field bat activity	171.70 ± 39.56	91.50 ± 25.40	107.0 ± 51.03	75.20 ± 27.14	72.60 ± 16.49	148.2 ± 40.19				
Total bat activity	295.2 ± 44.54	171.2 ± 25.67	241.2 ± 53.94	215.6 ± 43.80	255.6 ± 74.17	283.2 ± 52.12				

Table S 3.3 - Candidate models that were tested to access potentially influence of habitat type and road features on the number of bat passes of the overall community of bats, as well as for the three foraging guilds (SRE: short-range echolocators, MRE: mid-range echolocators without *P. kuhlii* (Pkuh) and LRE: long-range echolocators) and for *P. kuhlii*. Parameters: HABITAT – categorization of the sampling point-locations of the habitat. The open field area was included as the reference value. D_ROAD – the distance from the road (m). The “^2” indicates a quadratic transformation. For each model, we present the Akaike Information Criterion for small samples (AICc), AICc differences ($\Delta AICc$) and Akaike weights (w_i). The models are ranked by $\Delta AICc$. The best ranked model for each species/group is highlighted in bold.

Guilds/Species	Models	AICc	$\Delta AICc$	w_i
Overall	HABITAT+D_ROAD+HABITAT*D_ROAD^2	936.00	0.00	0.99
	HABITAT+D_ROAD^2	949.80	13.81	0.01
	HABITAT	961.70	25.67	0.00
	D_ROAD^2	985.60	49.57	0.00
	NULL	997.70	61.63	0.00
SRE	HABITAT+D_ROAD+HABITAT*D_ROAD^2	782.20	0.00	0.93
	HABITAT+D_ROAD^2	787.60	5.41	0.06
	HABITAT	792.00	9.77	0.01
	D_ROAD^2	794.10	11.89	0.01
	NULL	798.60	16.41	0.00
MRE	HABITAT+D_ROAD+HABITAT*D_ROAD^2	1047.00	0.00	0.99
	HABITAT+D_ROAD^2	1060.50	13.53	0.01
	HABITAT	1070.00	22.99	0.00
	D_ROAD^2	1088.50	41.52	0.00
	NULL	1098.10	51.12	0.00
LRE	HABITAT	931.40	0.00	0.52
	HABITAT+D_ROAD+HABITAT*D_ROAD^2	932.10	0.70	0.34
	HABITAT+D_ROAD^2	934.90	3.50	0.14
	D_ROAD^2	964.20	32.80	0.00
	NULL	985.40	54.00	0.00
Pkuh	HABITAT	1073.70	0.00	0.61
	HABITAT+D_ROAD+HABITAT*D_ROAD^2	1075.40	1.70	0.27
	HABITAT+D_ROAD^2	1077.80	4.10	0.12
	D_ROAD^2	1095.20	21.50	0.00
	NULL	1097.30	23.60	0.00



Chapter 4

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

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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 sex-biased 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 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.

4.1 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 & 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 (Frankham et al. 2002; Westekemper et al. 2021). 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 (Reed et al. 2007; Holderegger & Di Giulio 2010).

The lesser horseshoe bat (*Rhinolophus hipposideros*) is a clutter-foraging bat (Bontadina et al. 2002; Abbott et al. 2012a) 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) (Warren & Witter 2002; Van der Meij et al. 2015), 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 (Motte & Libois 2002; Dietz, C., Nill, D. and von Helversen 2009; Stone et al. 2012; Afonso et al. 2016). (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 (Medinas et al. 2013; Iković et al. 2014; Fensome & Mathews 2016). Lesser horseshoe bats often fly lower than 4 m above the ground at a low speed, particularly when crossing open spaces (Denzinger & 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 (Balkenhol & Waits 2009; Fahrig & Rytwinski 2009; Holderegger & Di Giulio 2010; Ascensão et al. 2016, 2017b).

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 (Schofield 1996; Bontadina et al. 2002; Motte & Libois 2002; Zahn et al. 2008; Ramovš et al. 2010; Tournant et al. 2013). 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 (Bontadina et al. 2002; Holzhaider et al. 2002; Boughey et al. 2011). The lesser horseshoe bat is a particularly sedentary species, usually foraging within 2 kilometres of their summer roosts (Bontadina et al. 2002; Holzhaider et al. 2002; Farcy et al. 2009) and travelling short distances (between 5 and 10 km) from winter to maternity roosts (Weiner & Zahn 2001; Crucitti & Cavalletti 2002; Hutterer Rainer et al. 2005). 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 (Manel et al. 2005; Saenz-Agudelo et al. 2009; Lowe & Allendorf 2010; Peterman et al. 2019). 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 & Waits 2009; Manel & Holderegger 2013; Wagner & Fortin 2013; Balkenhol et al. 2015). 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 (Palsbøll et al. 2010; Norman et al. 2017). 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.

4.2 MATERIAL AND METHODS

4.2.1 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; *Figure 4.1*). 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).

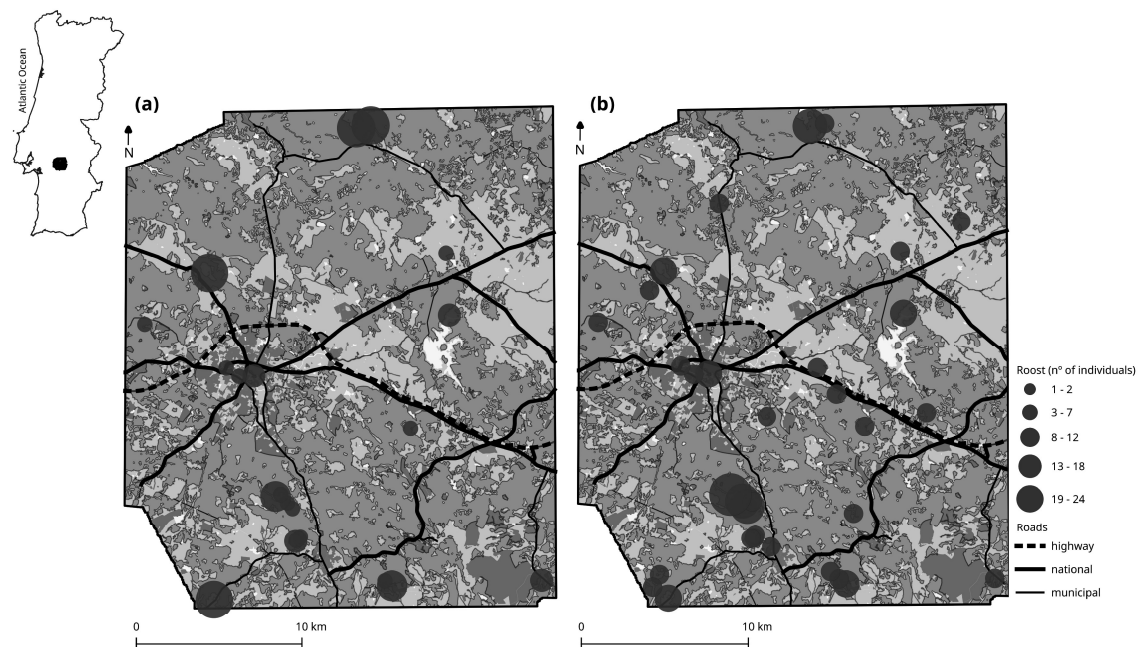


Figure 4.1 - Map of study area in southern Portugal showing roost locations occupied by (a) females ($N = 20$) and (b) males ($N = 33$). Circles represent roosts and their size represents the number of genotyped individuals per roost. The three main land uses are forest (dark grey), sparse woodland (grey) and open agricultural area (light grey) areas.

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 minutes 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).

4.2.2 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 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 *MseI* and *SbfI* 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.

4.2.3 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 1,500 m (hereafter called roost-group) and excluded roost-groups with less than 2 individuals. The adjacent roosts were clustered to reduce bias associated with small samples. The radii of 1,500 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 (g^2), which measures heterozygosity covariance across loci (David et al. 2007). We also tested a null-hypothesis of no variance in inbreeding in the sample ($H_0: g^2=0$), calculating the 95 % confidence interval (CI), via 1,000 bootstraps over individuals and the approximate P -value via 1,000 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 & 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 1,000 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 & 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).

4.2.4 Relatedness estimates

Individuals' relatedness was derived using the A_{jk} coefficient (hereafter A_{jk} -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. A_{jk} -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 A_{jk} -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 A_{jk} -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.

4.2.5 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 et al. 2013; Blangiardo & Cameletti 2015). Interpolations were calculated based on one focal roost at a time and pairwise relatedness among roosts was represented by their 95th quantile A_{jk} -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 A_{jk} . 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 x 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 greater than 0.975 or less than 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 & 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).

4.2.6 Landscape relatedness analysis

4.2.6.1 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 (*Figure 4.2*). These analyses were applied for the 95th quantile pairwise A_{jk} -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 (Bontadina et al. 2002; Tournant et al. 2013; Ducci et al. 2015). 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 1,500 and 3,000 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 (Tournant et al. 2013; Jan et al. 2017).

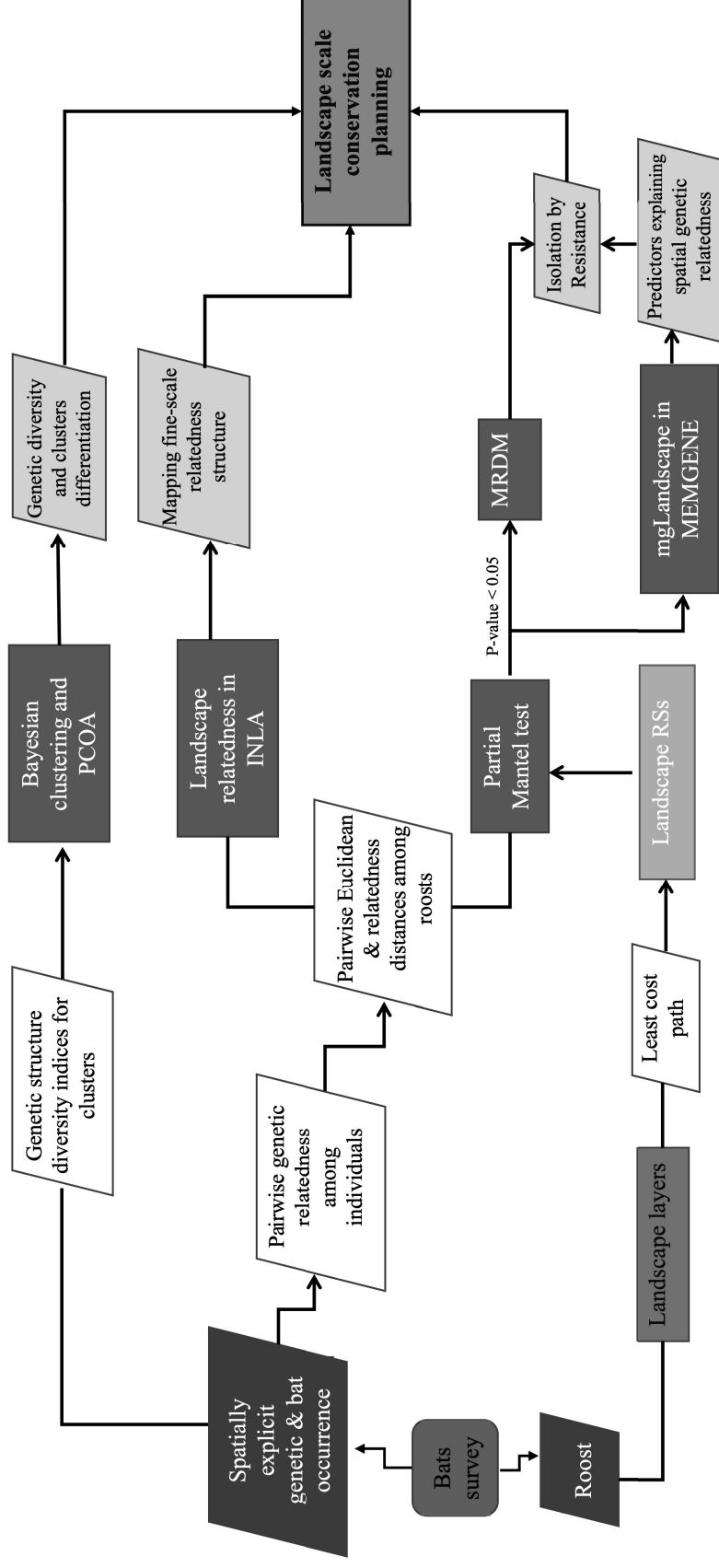


Figure 4.2 - Simplified flowchart of the methodology. The flowchart depicts inputs (green), intermediate processes and preparatory analysis (white), statistical analysis (blue), results (yellow) and intermediate objectives and conservation issues (orange). Explanation of acronyms: Resistance surfaces (RS), Principal coordinates (PCOA), Integrated Nested Laplace Approximation (INLA), Multiple regression of distance matrices (MRDM), Moran's Eigenvector Maps (MEMGENE).

Table 4.1 - 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 controlling for the effects of Euclidean distance (*ED*). MRDM model fit (*R*²) and *P*-values are also shown. *P*-values <0.05 are highlighted in bold.

Model	<i>r</i>	<i>P</i>	<i>R</i> ²	<i>P</i>
All individuals				
intercept	0.013	<0.001	0.049	<0.001
Tden250	0.002	0.016		
ALnd_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		
ALnd_FOR (1500 m)	0.001	0.726		
DRnat	0.002	0.010		
CLnd_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 250m scale; ENeig_For (1500 m) = Mean distance of nearest forest patches neighbour at distance of 1,500m scale; ALnd_FOR (1500 m) = aggregation index of forest patches at 1,500m scale; DRnat = distance to national roads; PRic (1500 m) = Patch richness at 1,500 scale and CLnd_WOOD = Contiguity Index woodland patches at 1,500 m scale.				

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 & Cushman 2012). 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.

4.2.6.2 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 *A_{jk}*-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 & Rousset 2013). Significance was assessed using 10,000 randomizations. We used a Mantel test implemented in the *ade4* R

package (Dray & Dufour 2007) and Partial Mantel tests were conducted using the *vegan* R package (Oksanen 2018).

4.2.6.3 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 (Cushman & Huettmann 2010; Wasserman et al. 2010; Koen et al. 2012). 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 & 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 (w_i) to rank candidate models (Burnham & Anderson 2002). For models with $\Delta AICc < 2$, we performed a model averaging approach, averaging parameters, unconditional standard errors (SE) and 95 % confidence intervals (CI) (Burnham & Anderson 2002). Variable coefficients whose confidence limits included zero were considered not significant (Burnham & 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 & 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 same side (0) or

opposite sides (1) of the highway that cross the study area. MRDMs were performed using the *ecodist* R package (Goslee & 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 & 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 & Fortin 2010).

4.3 RESULTS

4.3.1 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 (*Figure 4.1*).

The average number of raw RAD-tags per individual was 1,587,224 (min. 6,908; max. 8,414,170; SD 1,354,845). After filtering, we obtained a final dataset of 2,837 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.

4.3.2 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 - *Figure S 4.1*). The PCoA results also show no apparent substructuring between the sampled roosts (Supplementary Material - *Figure S 4.2*). After pooling the roosts located within a 1,500 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 S 4.2*. 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 S 4.2*). Weak but significant identity disequilibrium was detected in the genotype dataset (g_2 = 0.01, CI = 0.01 – 0.02, P -value = 0.012 based on 1,000 permutations) considering all individuals. Females also had a significant disequilibrium (g_2 = 0.01, CI = 0.01 – 0.02, P -value = 0.011 based on 1,000 permutations), while for the males were non-significant (g_2 = 0.21, CI = 0.01 – 0.02, P -value = 0.165 based on 1,000 permutations).

Pairwise F_{ST} between roost-groups were low to moderate ($F_{ST} = 0.014 \pm 0.022$) 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.

4.3.3 Relatedness estimates and interpolations

We calculated A_{jk} -values for every possible pair among the 228 individuals resulting in 25,878 A_{jk} -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 A_{jk} -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 S 4.3) are shown in Figure S 4.3 a, 4.3c and 4.3e in which the orange and light purple areas highlight levels of unrelatedness that would be expected in panmitic populations (A_{jk} -values ~ 0). On the other hand, areas with a high degree of relatedness (A_{jk} -values > 0) or unrelatedness ($A_{jk} < 0$) are represented in yellow and dark purple, respectively. Figure S 4.3b 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 - Figure S 4.3e and 4.3f; and females - Figure S 4.3c and 4.3d). 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.

4.3.4 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 A_{jk} -values) among roosts for all individuals ($R = -0.053$, $P\text{-value} = 0.819$), or considering females and males separately (females: $R = -0.100$, $P\text{-value} = 0.877$; males: $R = -0.010$, $P\text{-value} = 0.554$). 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 1,500 m radii (Supplementary Material - Table S 4.4). 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 ($P\text{-value} > 0.050$) (Supplementary Material - Table S 4.4). 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 4.1; Supplementary Material - Table S 4.5). However, the 95 % confidence interval of this variable overlapped zero and thus the coefficient was

deemed not significant (Supplementary Material - *Figure S 4.3*). 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 4.1*; Supplementary Material - *Figure S 4.3*). 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 ($R^2 = 0.016$, $P\text{-value} = 0.073$), 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: $R^2 = 0.002$, $P\text{-value} = 0.512$; males: $R^2 = < -0.001$, $P\text{-value} = 0.443$).

4.3.5 Spatial explicit mapping of genetic structure

Moran's eigenvectors (MEM) derived from Euclidean distances ([abc]) (Supplementary Material - *Table S 4.5*) explained a slightly lower proportion of spatial relatedness variation for all individuals ($R^2_{\text{adj}[abc]} = 0.147$) than the MEM eigenvector derived from the Patch richness resistance surface (with $R^2_{\text{adj}[abc]} = 0.185$). Resistance surfaces that best explain the spatial relatedness between females were highly associated with the MEM eigenvector based on distance to national roads (DRnat) ($R^2_{\text{adj}[abc]} = 0.197$) and Tree density-based resistance surface (Tden250) ($R^2_{\text{adj}[abc]} = 0.147$) (Supplementary Material - *Table S 4.5*). Spatial relatedness between males was better explained by the MEM eigenvector based on Tree density-based resistance ($R^2_{\text{adj}[abc]} = 0.281$). 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 S 4.5*).

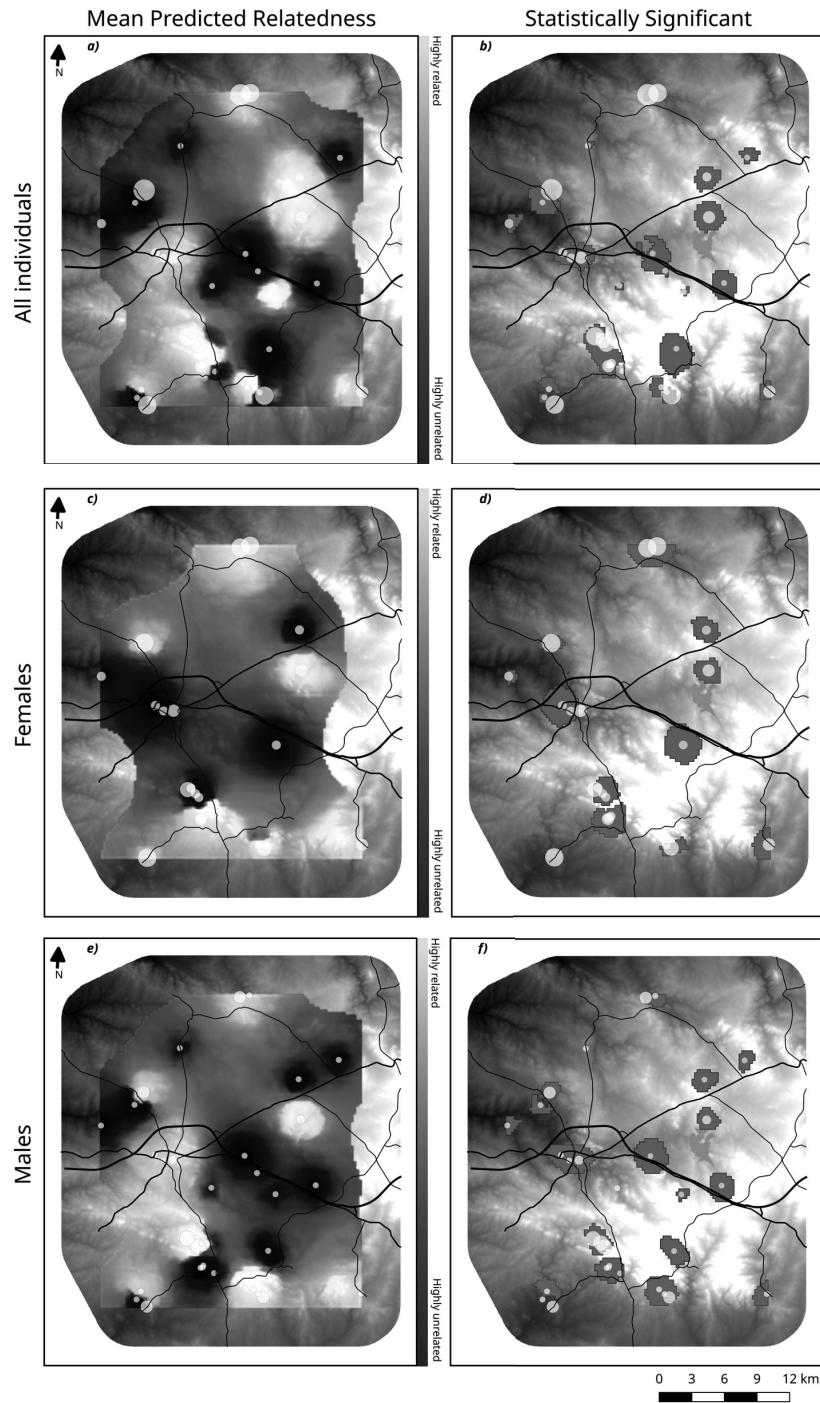


Figure 4.3 - Relatedness interpolation maps. The first column shows interpolations of the entire study area ("global") for maximum pairwise relatedness of all individuals (a), females (c), and males (e) per roost, where yellow areas represent a high degree of relatedness between individuals (A_{jk} values > 0) and purple areas represent a low degree of relatedness between individuals (A_{jk} 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.

4.4 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 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.

4.4.1 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 & 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 (Barton & Wilson 1995; Murphy et al. 2008). This suggests that many actual wildlife populations separated by roads may already have a weak genetic differentiation (Berry et al. 2004; Holderegger & Di Giulio 2010) 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. 2017b); road barrier, (Epps et al. 2005) concur simultaneously and particularly concerning species with low population sizes (Holderegger and 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. Whilst 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).

4.4.2 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. 2000, 2002), 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 (Kerth 2008; Clutton-Brock & Lukas 2012), 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 A_{jk}). 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 & Hooper 1956) also recorded that in small populations of lesser horseshoe bat, individuals rarely move more than 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 more than 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 matriline 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).

4.4.3 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 appear 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 (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) (Sachanowicz & Wower 2013; Voigt & Kingston 2016). 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.

4.4.4 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.

4.5 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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SUPPORTING INFORMATION

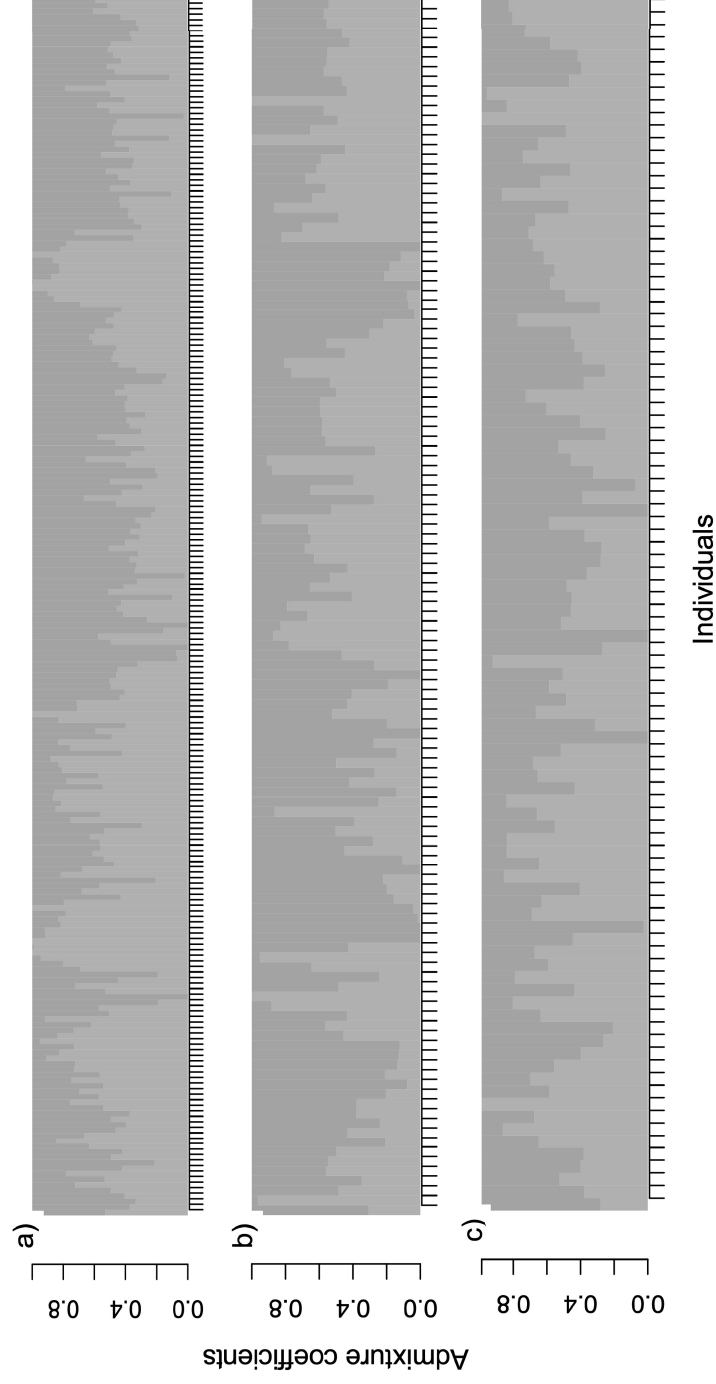


Figure S 4.1 - Graphical representation of Bayesian cluster analysis from 2.837 SNPs of *Rhinolophus hipposideros* individuals. Each bar represents the individual membership coefficient for each inferred population (K) for all individuals (a), females (b) and males (c).

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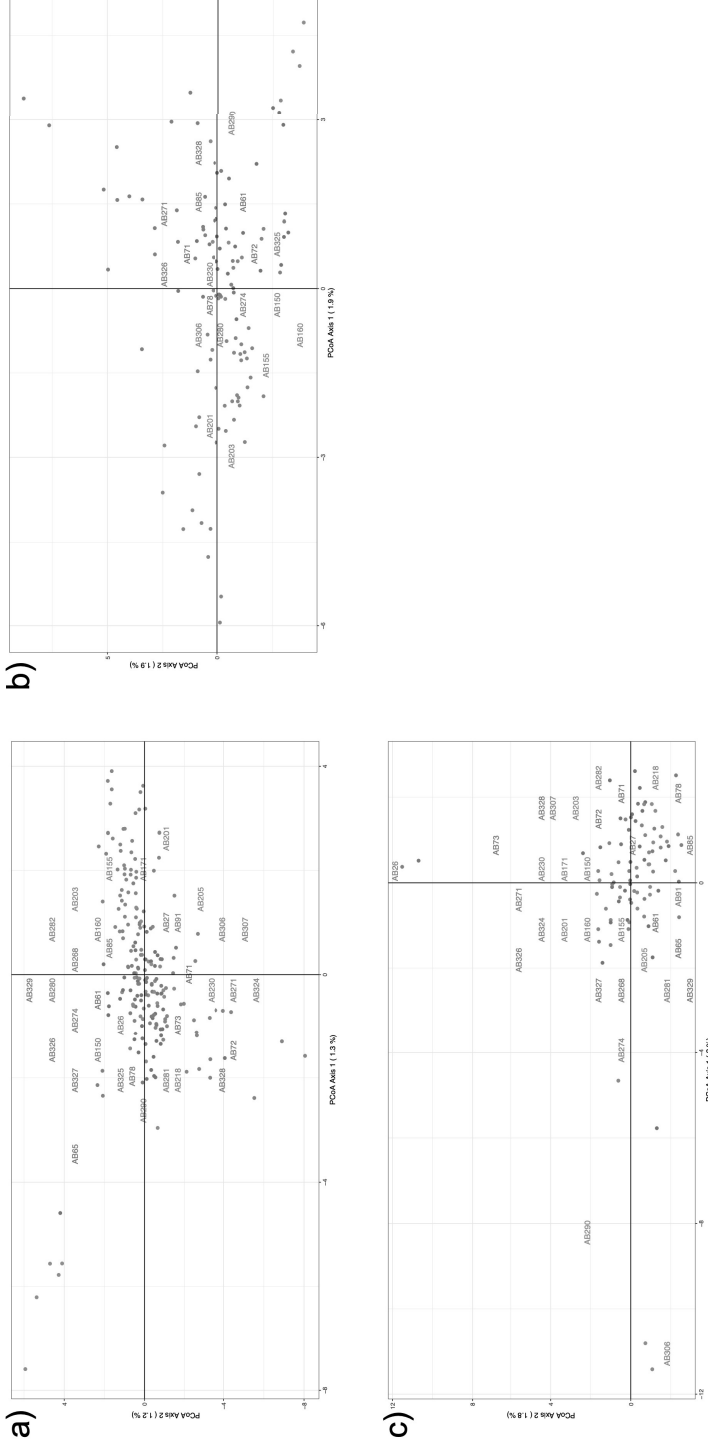


Figure S 4.2 - Results of principal coordinate analysis (PCoA) used to identify genetic differentiation among sampled individuals using F, considering all individuals (a), females (b) and males (c).

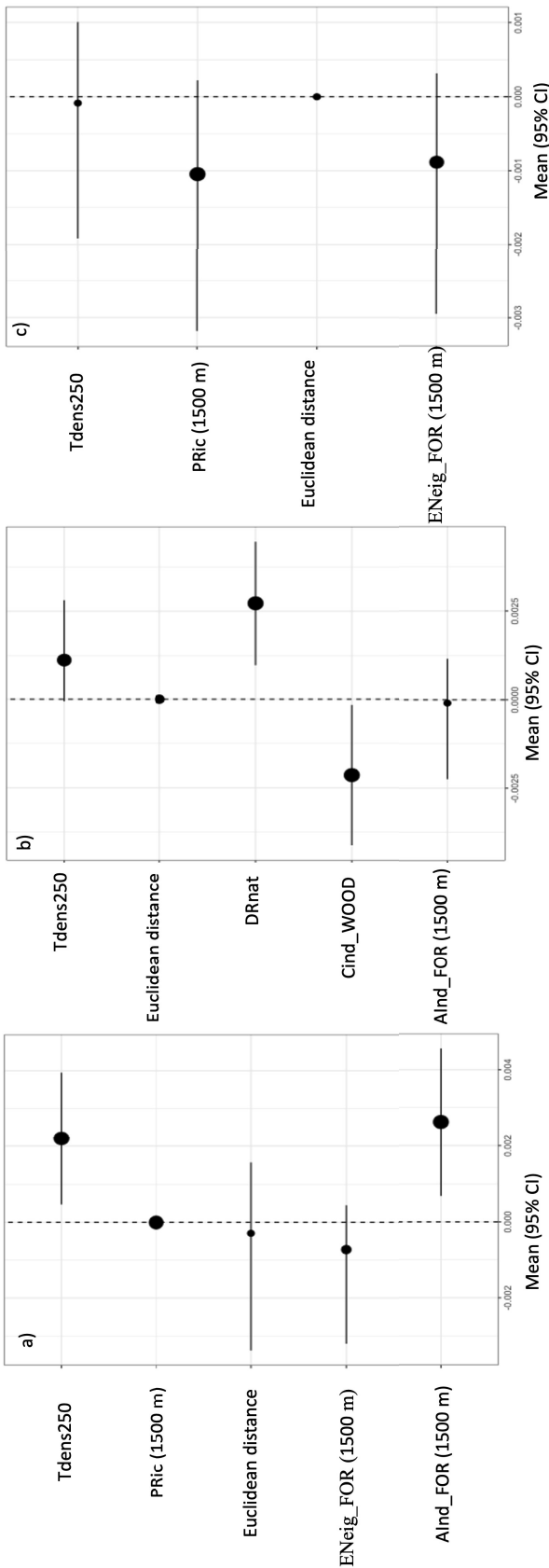


Figure S 4.3 - Coefficient estimates (i.e., median values and 95 % confidence intervals (bars) of the posterior distribution) of the parameters from the best models that explaining relatedness distance (maximum pairwise A_{jk}) to all individuals (a), females (b) and males (c) with $\Delta AICc < 2$. Confidence intervals overlapping 0 (dotted line) indicate that the corresponding effect is not significant at the 5 % level. The dot size relates to the relative importance for each variable on model selection approach.

Table S 4.1 - Description of variables used to create model landscape resistance surfaces, according to McGarical et al. 2012. All Fragstat variables were calculated using moving window of 1,500 and 3,000 m for three main land cover in study area (1) FOR - forest (i.e., dense forests of cork and holm-oak with > 30 % tree cover); (2) WOOD – sparse woodland (i.e., natural woodland with tree density < 30 %) and (3) AGRI- open agricultural area (low crops such as cereals and cut forests). For Tree density variables we used a circular window with radii of 50, 100, 150, 250 and 500 m.

Variables	Acronym	Class and Description
FRAGSTAT		
Patch Area (mean)	PArea	Measures the mean of patch area
Aggregation index.	AInd	Measures the total edge cells shared by a land cover class with itself. Equals 0 when there are no like adjacencies and 100 when the patch class type is maximally aggregated into a single, compact patch.
Euclidean Nearest Neighbour Distance (mean).	ENeig	Shortest straight-line distance between the focal patch and its neighbours of the same class.
Interspersion juxtaposition Index	IJInd	Describes the intermixing of land cover classes. Approaches 0 if a land cover class is only adjacent to a single other class and equals 100 when a class is equally adjacent to all other classes.
Configuity Index	CInd	Assesses the spatial connectedness of a land cover class. Equals 0 for a one-pixel patch and increases to a limit of 1 as patch contiguity increases
Patch richness	PRic	Number of different landcover classes in the landscape patch types
Percentage of Landscape	PLan	Quantifies the proportional abundance of each land cover class in the landscape

FOCAL CELL (mean)

Tree density 50 meters	Tden50	Tree density
Tree density 100 meters	Tden100	Tree density
Tree density 150 meters	Tden150	Tree density
Tree density 250 meters	Tden250	Tree density
Tree density 500 meters	Tden500	Tree density

DISTANCES

Distance to all roosts	Droosts	Distance of a roost to all other roosts surveyed
Distance to roosts occupied	Doccu	Distance among roosts occupied
Distance to settlements	Dset	Distance to settlements
Distance to riparian gallery	Drip	Distance to a riparian gallery
Distance to stream	Dstr	Distance to a riparian stream
Distance to urban > 3 hectares	Durb	Distance to an urban area larger than 3 ha
Distance to urban < 3 hectares	Dsmal	Distance to an urban area smaller than 3 ha
Distance to roads	DRoad	Distance to any paved road

Distance to highway	DRhigh	Distance to the highway
Distance to national roads	DRnat	Distance to national roads
Distance to regional roads	DRreg	Distance regional roads
Distance to highway + national roads	DRtraf	Distance to a national road or highway

Table S 4.2 - Genotypic characteristics of SNPs for *R. hipposideros* per each roost-group. A: number of alleles; H_E : expected heterozygosity; H_O : observed heterozygosity and F_{IS} : inbreeding index.

ALL INDIVIDUALS	Roost-group	A	Inds	H_E	H_O	F_{IS}
	1	5369	8	0.289	0.300	0.012
	2	5278	7	0.279	0.293	0.016
	3	4810	3	0.265	0.319	-0.044
	5	5666	46	0.308	0.307	0.011
	7	5651	29	0.304	0.309	-0.003
	8	4687	3	0.249	0.293	-0.009
	12	5343	7	0.295	0.337	-0.059
	13	5670	48	0.313	0.313	0.010
	14	5561	16	0.302	0.308	0.008
	16	4796	3	0.263	0.322	-0.062
	18	5523	13	0.301	0.314	-0.005
	19	5408	8	0.291	0.305	0.009
	20	5639	24	0.305	0.291	0.057
FEMALES						
	1	5272	6	0.289	0.306	0.015

2	5015	4	0.276	0.318	-0.032
4	5664	37	0.304	0.301	0.016
6	5633	22	0.305	0.317	-0.016
7	4809	3	0.262	0.314	-0.039
8	5525	13	0.300	0.309	0.001
10	5380	8	0.293	0.295	0.043
11	5227	7	0.282	0.291	0.029
12	5612	18	0.305	0.303	0.031
13	5420	9	0.296	0.314	-0.005

MALES

2	4792	3	0.261	0.321	-0.068
5	5415	9	0.293	0.307	0.002
7	5284	7	0.282	0.292	0.022
9	4399	3	0.227	0.299	-0.070
12	4843	4	0.256	0.293	-0.017
13	5326	7	0.291	0.308	0.006
15	5669	35	0.311	0.312	0.012
18	5116	5	0.283	0.320	-0.031
20	5247	6	0.289	0.314	-0.010

Table S 4.3 - Model selection for INLA models regarding different equation structures. The spatial correlation structure (Spatial) on models was defined by the SPDE approach (null model), Euclidean distance between roosts (Dist) as covariate and the identification of roost as random factor (Roost). The model selected is shown in bold.

ALL INDIVIDUALS	DIC	WAIC
$A_{ijk} \sim f$ (Spatial)	-2777.822	-2734.289
$A_{ijk} \sim Dist + f$ (Spatial)	-3288.547	-3233.473
$A_{ijk} \sim f$ (Spatial) + r(Roost)	-2777.205	-2736.233
FEMALES		
$A_{ijk} \sim f$ (Spatial)	-1586.299	-1548.894
$A_{ijk} \sim Dist + f$ (Spatial)	-1805.987	-1770.015
$A_{ijk} \sim f$ (Spatial) + r(Roost)	-1588.262	-1552.655
MALES		
$A_{ijk} \sim f$ (Spatial)	-3751.388	-3687.579
$A_{ijk} \sim Dist + f$ (Spatial)	-3894.910	-3762.534
$A_{ijk} \sim f$ (Spatial) + r(Roost)	-3756.766	-3702.748

Table S 4.4 - Results of Partial Mantel test between relatedness distance and landscape resistance surfaces while removing the effects of the isolation by distance matrix.

Variable	Distance	All individuals		Females		Males	
		Mantel r	P	Mantel r	P	Mantel r	P
Alnd_WOOD	1500	-0.071	0.855	-0.064	0.975	-0.059	0.790
Alnd_FOR	1500	0.165	0.023	0.127	0.028	0.129	0.035
Alnd_AGRI	1500	-0.036	0.560	-0.034	0.676	-0.054	0.793
PArea_WOOD	1500	0.011	0.423	-0.026	0.685	-0.029	0.697
PArea_FOR	1500	0.088	0.853	-0.012	0.825	0.093	0.853
PArea_AGRI	1500	-0.001	0.481	-0.040	0.750	-0.035	0.718
Clnd_WOOD	1500	0.005	0.479	-0.123	0.032	-0.033	0.746
Clnd_FOR	1500	0.117	0.024	0.075	0.863	-0.106	0.021
Clnd_AGRI	1500	0.001	0.636	-0.034	0.697	-0.010	0.578
ENeig_WOOD	1500	-0.026	0.660	-0.087	0.910	0.013	0.445
ENeig_FOR	1500	-0.127	0.012	0.102	0.035	-0.112	0.023

ENeig_AGRI	1500	-0.002	0.489	-0.064	0.867	-0.020	0.618
IJInd_WOOD	1500	0.093	0.021	0.123	0.042	0.094	0.032
IJInd_FOR	1500	0.014	0.419	0.053	0.877	0.101	0.029
IJInd_AGRI	1500	-0.009	0.112	0.043	0.312	0.022	0.499
PLan_WOOD	1500	0.071	0.374	-0.027	0.621	0.099	0.064
PLan_FOR	1500	-0.064	0.777	0.005	0.428	-0.095	0.927
PLan_AGRI	1500	0.006	0.470	-0.017	0.623	0.001	0.527
<u>PRic</u>	1500	-0.102	0.013	-0.097	0.013	-0.096	0.032
ALnd_WOOD	3000	-0.043	0.233	-0.037	0.353	-0.047	0.199
ALnd_FOR	3000	0.101	0.013	0.113	0.016	-0.063	0.691
ALnd_AGRI	3000	-0.013	0.703	-0.034	0.852	0.105	0.037
PArea_WOOD	3000	-0.073	0.834	0.053	0.234	0.095	0.068
PArea_FOR	3000	0.131	0.035	-0.083	0.901	-0.073	0.474
PArea_AGRI	3000	-0.006	0.535	0.032	0.752	-0.064	0.715
CLnd_WOOD	3000	0.005	0.468	-0.075	0.886	0.062	0.142

CInd_FOR	3000	0.113	0.003	0.041	0.532	-0.043	0.77
CInd_AGRI	3000	-0.022	0.435	-0.027	0.632	0.029	0.668
ENeig_WOOD	3000	0.001	0.504	-0.034	0.725	-0.044	0.762
ENeig_FOR	3000	0.097	0.013	0.029	0.733	-0.105	0.038
ENeig_AGRI	3000	0.003	0.503	-0.017	0.573	0.016	0.352
IJInd_WOOD	3000	0.104	0.002	0.095	0.034	0.134	0.013
IJInd_FOR	3000	0.121	0.005	0.058	0.843	0.022	0.365
IJInd_AGRI	3000	-0.042	0.243	0.032	0.634	0.098	0.041
PLan_WOOD	3000	0.051	0.234	-0.030	0.674	0.078	0.099
PLan_FOR	3000	0.050	0.196	-0.041	0.765	0.060	0.160
PLan_AGRI	3000	-0.010	0.498	-0.082	0.855	-0.010	0.427
PRic	3000	-0.031	0.332	-0.001	0.634	-0.043	0.764
Droads	-	-0.032	0.660	-0.027	0.605	-0.036	0.661
DRhigh	-	0.028	0.357	0.098	0.081	0.022	0.387
DRnat	-	0.051	0.426	0.181	0.016	0.062	0.195

DRreg	-	0.021	0.395	-0.057	0.751	0.008	0.405
Dtraf	-	0.042	0.277	0.020	0.402	0.061	0.215
Droosts	-	-0.008	0.511	0.043	0.291	0.008	0.432
Doccu	-	-0.039	0.217	-0.051	0.273	-0.018	0.345
Dset	-	-0.021	0.432	0.073	0.342	-0.033	0.613
Drip	-	0.090	0.313	0.004	0.496	0.096	0.077
Dstr	-	0.103	0.023	0.141	0.017	0.085	0.128
Durb	-	-0.103	0.973	-0.066	0.887	-0.077	0.940
Dsmal	-	-0.020	0.594	-0.033	0.706	0.003	0.467
Tden50	-	0.103	0.008	0.112	0.013	0.085	0.128
Tden100	-	0.113	0.004	0.101	0.012	0.084	0.122
Tden150	-	0.098	0.012	0.115	0.012	0.080	0.154
Tden250	-	0.124	0.001	0.121	0.025	0.104	0.018
Tden500	-	0.107	0.001	0.112	0.011	0.060	0.196

Table S 4.4 - Results of Partial Mantel test between relatedness distance and Euclidean distance while removing the effects of the landscape resistance surfaces.

Variable	Distance	All individuals		Females		Males	
		Mantel r	P	Mantel r	P	Mantel r	P
Alnd_WOOD	1500	-0.009	0.539	-0.006	0.329	-0.037	0.552
Alnd_FOR	1500	-0.098	0.962	-0.092	0.934	-0.101	0.974
Alnd_AGRI	1500	0.006	0.556	0.006	0.439	-0.011	0.574
PArea_WOOD	1500	-0.016	0.568	0.008	0.417	-0.013	0.551
PArea_FOR	1500	-0.034	0.401	0.021	0.392	0.043	0.623
PArea_AGRI	1500	-0.008	0.547	0.016	0.385	-0.011	0.541
Clnd_WOOD	1500	-0.012	0.539	0.102	0.927	-0.012	0.566
Clnd_FOR	1500	0.093	0.937	0.030	0.286	-0.123	0.952
Clnd_AGRI	1500	-0.017	0.543	0.012	0.405	-0.028	0.659
ENeig_WOOD	1500	-0.014	0.603	-0.021	0.515	-0.012	0.646

ENeig_FOR	1500	-0.035	0.837	-0.109	0.903	0.112	0.937
ENeig_AGRI	1500	-0.007	0.561	0.037	0.274	-0.015	0.578
IJInd_WOOD	1500	-0.015	0.839	-0.104	0.907	-0.103	0.982
IJInd_FOR	1500	0.022	0.553	-0.082	0.153	-0.083	0.838
IJInd_AGRI	1500	-0.031	0.753	-0.045	0.853	-0.056	0.823
PLan_WOOD	1500	-0.018	0.601	-0.007	0.539	-0.049	0.797
PLan_FOR	1500	0.001	0.490	-0.010	0.581	-0.021	0.529
PLan_AGRI	1500	-0.008	0.533	0.015	0.393	-0.010	0.542
PRic	1500	0.063	0.912	-0.103	0.913	-0.069	0.894
ALnd_WOOD	3000	0.033	0.855	0.047	0.822	0.06	0.871
ALnd_FOR	3000	-0.053	0.948	-0.094	0.853	0.025	0.429
ALnd_AGRI	3000	0.005	0.589	0.023	0.473	-0.126	0.926
PArea_WOOD	3000	-0.175	0.181	-0.093	0.833	-0.132	0.982
PArea_FOR	3000	-0.031	0.916	0.029	0.317	-0.047	0.545
PArea_AGRI	3000	-0.007	0.542	-0.037	0.373	0.016	0.577

CInd_WOOD	3000	-0.012	0.548	0.030	0.305	-0.065	0.828
CInd_FOR	3000	-0.073	0.953	-0.173	0.713	0.025	0.371
CInd_AGRI	3000	0.072	0.583	0.021	0.327	-0.018	0.604
ENeig_WOOD	3000	-0.011	0.554	0.006	0.446	-0.016	0.577
ENeig_FOR	3000	-0.015	0.915	-0.022	0.401	-0.141	0.919
ENeig_AGRI	3000	0.013	0.577	0.011	0.353	-0.031	0.716
IJInd_WOOD	3000	-0.023	0.853	-0.103	0.912	-0.102	0.823
IJInd_FOR	3000	-0.003	0.887	0.016	0.396	-0.044	0.734
IJInd_AGRI	3000	-0.028	0.642	-0.093	0.435	-0.102	0.905
PLan_WOOD	3000	-0.023	0.662	-0.002	0.510	-0.056	0.841
PLan_FOR	3000	-0.051	0.790	0.039	0.280	-0.065	0.851
PLan_AGRI	3000	-0.012	0.572	-0.018	0.633	-0.037	0.736
PRic	3000	0.021	0.803	-0.002	0.606	0.016	0.523
Droads	-	-0.008	0.539	-0.007	0.575	-0.027	0.636
DRhigh	-	-0.027	0.679	-0.074	0.886	-0.043	0.748

DRnat	-	-0.031	0.789	-0.113	0.943	-0.045	0.781
DRreg	-	-0.013	0.563	-0.003	0.495	-0.039	0.746
Dtraf	-	-0.028	0.671	-0.017	0.584	-0.061	0.863
Droosts	-	-0.001	0.501	-0.038	0.735	-0.036	0.734
Doccu	-	-0.030	0.665	-0.066	0.732	-0.042	0.762
Dset	-	0.043	0.753	-0.053	0.802	-0.034	0.759
Drip	-	-0.034	0.709	-0.010	0.578	-0.062	0.857
Dstr	-	-0.073	0.913	-0.094	0.853	-0.055	0.825
Durb	-	0.071	0.105	0.044	0.239	0.033	0.267
Dsmal	-	0.012	0.455	0.024	0.363	-0.021	0.634
Tden50	-	-0.073	0.973	-0.106	0.952	-0.030	0.700
Tden100	-	-0.084	0.899	0.102	0.905	-0.031	0.709
Tden150	-	-0.061	0.893	0.105	0.969	-0.032	0.708
Tden250	-	-0.075	0.973	-0.095	0.875	-0.105	0.849
Tden500	-	-0.053	0.972	-0.107	0.949	-0.038	0.757

Table S 4.5 - Candidate models that were tested to access the influence of landscape resistance surfaces on the genetic relatedness among all individuals, as well as for the females and males separately. For each model, we present the Akaike Information Criterion for small samples (AICc), AICc differences ($\Delta AICc$) and Akaike weights (w_i). The models are ranked by $\Delta AICc$. The best ranked model for dataset is highlighted in bold.

	Models	AICc	$\Delta AICc$	w_i
All individuals	Alnd_FOR (1500 m) + ENeig_FOR (1500 m) + PRic (1500 m) + Tden250	-3002.5	0.00	0.34
	Alnd_FOR (1500 m) + PRic (1500 m) + Tden250	-3002.5	0.02	0.34
	Alnd_FOR (1500 m) + ENeig_FOR (1500 m) + Euclidean distance + PRic (1500 m) + Tden250	-3001.3	1.24	0.18
	Alnd_FOR (1500 m) + Euclidean distance + PRic (1500 m) + Tden250	-3000.7	1.79	0.44
Females	Tden250 + DRnat + Clnd_WOOD	-3160.4	0.00	0.392
	Tden250 + DRnat + Clnd_WOOD + Euclidean distance	-3159.5	0.86	0.255
	DRnat + Clnd_WOOD + Euclidean distance	-3158.8	1.55	0.180
	Alnd_FOR (1500 m) + Tden250 + DRnat + Clnd_WOOD	-3158.7	1.65	0.172
Males	PRic (1500 m)	-3097.9	0.00	0.229
	ENeig_FOR (1500 m) + PRic (1500 m)	-3097.6	0.27	0.200
	ENeig_FOR (1500 m) + Euclidean distance	-3097.2	0.68	0.163
	ENeig_FOR (1500 m)	-3096.8	1.13	0.130
	Tden250 + PRic (1500 m)	-3096.2	1.10	0.098
	Tden250 + ENeig_FOR (1500 m) + PRic (1500 m)	-3096.0	1.87	0.090
	ENeig_FOR (1500 m) + PRic (1500 m) + Euclidean distance	-3096.0	1.87	0.090

Table S 4.6 - Comparison of the proportion of spatial genetic variation (R^2_{Adj}) among all individuals and subsets of males and females explained by Moran's eigenvector maps derived from different models.

Model	[abc]	P[abc]	[a]	P[a]	[c]	P[c]	[b]	[d]
All individuals								
Euclidean	0.147	0.010	0.103	0.020	0.037	0.040	0.008	0.853
Tden250	0.080	0.010	0.035	0.010	0.075	0.010	-0.030	0.920
Alnd_FOR (1500 m)	0.106	0.010	0.061	0.010	0.074	0.010	-0.029	0.894
PRic (1500 m)	0.185	0.010	0.143	0.010	0.053	0.030	-0.008	0.812
ENeig_FOR (1500 m)	0.124	0.010	0.079	0.010	0.071	0.010	-0.026	0.876
Females								
Euclidean	0.123	0.010	0.086	0.030	0.105	0.020	-0.069	0.877
Tden250	0.147	0.010	0.111	0.010	0.083	0.030	-0.046	0.853
Alnd_FOR (1500 m)	0.118	0.010	0.082	0.010	0.097	0.010	-0.061	0.882
DRnat	0.197	0.010	0.161	0.010	0.100	0.010	-0.063	0.803
Clnd_WOOD	0.096	0.010	0.059	0.010	0.099	0.020	-0.063	0.904
Males								
Euclidean	0.244	0.001	0.184	0.010	0.020	0.140	0.040	0.756
Tden250	0.281	0.001	0.221	0.020	0.027	0.120	0.033	0.719
ENeig_FOR (1500 m)	0.148	0.001	0.087	0.010	0.047	0.050	0.014	0.852
PRic (1500 m)	0.231	0.003	0.170	0.010	0.042	0.090	0.018	0.769

Note: The table describes the proportion of variation in genetic distance that can be explained by spatial variables [abc], spatial pattern in the landscape resistance surface [a], coordinates [c], spatial and confounded patterns in the landscape resistance surface and coordinates [b]. Lastly, we included the residual not explained by spatial variables [d]. The resistance surface with highest (R^2_{Adj}) values explained by spatial variables and selected pattern in the model [a] is identified in bold. P[abc], P[a] and P[b] represent the significance (*P-value*) of each calculated proportion.

Chapter 5

Final Considerations

5.1 Overview – research questions

In this thesis, we assessed the threats posed to bats by roads. At the community level, we analysed spatiotemporal changes in bat roadkill patterns and the main factors influencing them, and the effects of road surrounding habitats and distance to roads on the activity of different bat guilds. At the species level, we studied the effects of roads on the relatedness structure and gene flow in a population of the lesser horseshoe bat (*Rhinolophus hipposideros*).

Our results showed that bats tend to avoid the immediate proximity of roads when those cross high-quality habitats; and that bats are consistently more prone to being hit by a car in locations with consistently higher vegetation productivity, where surface temperature is lower and where bat activity is higher. In addition, we found evidence that roads may act as partial a barrier to bat movements and seem to play a role in genetic structuring of a lesser horse population living in road-dominated environments.

The pattern described for bat roadkill is consistent with the hypothesis that bats are especially susceptible to being killed in the vicinity of high-quality habitats and that the locations of roadkill hotspots follow changes in habitat quality over time (Chapter 2). The persistence of roadkill hotspots is related to the spatiotemporal dynamics of the net primary productivity, which can be interpreted as a surrogate of foraging habitat suitability (i.e., prey availability). This result shows that changes in bat roadkill risk can be inferred using freely available remote sensing data (Chapter 2), which is promising for predicting roadkill concentration in other existing roads and for helping to avoid risky areas in future road planning.

The effect of distance to roads on bat activity is nonlinear and depends on the surrounding habitat, as well as on the functional characteristics of bat species (Chapter 3). In high-quality habitats, such as Mediterranean woodlands, bats tend to avoid the immediate proximity of roads, with bat activity remaining higher beyond 300 m from the road. In contrast, in open agricultural areas, the level of bat activity near roads is similar

to the activity peak that is registered 1000 m away from the road (Chapter 3). This pattern is more evident for short- and mid-range echolocators, which are more adapted to flying in woodlands and vegetated edges. The effect of roads on long-range echolocators and the most common species, *P. kuhlii*, is less pronounced (Chapter 3).

Roads seem to limit connectivity among lesser horseshoe roosts and to be a main shaper of contemporary population structure, particularly for females (Chapter 4). Indeed, relatedness analysis suggests that a long-term structuring of population on opposite sides of roads may be ongoing, although it may only be fully noticed further ahead. Based on genetic data, we recorded a low number of inter-roost individual exchanges, and matings mainly occur between males and related breeding females (Chapter 4). Furthermore, the detected relatedness patterns suggested that forest areas with a high tree density among roosts promote the relatedness among individuals, probably by facilitating movements among roosts (Chapter 4).

5.1.1 Main Findings

5.1.1.1 Spatial distribution of bat roadkill

We demonstrated that bat roadkill hotspots pattern is not randomly distributed and that their locations shift for consecutive years. Bat roadkill hotspot persistence can be predicted by the temporal dynamics of habitat suitability proxies, such as the net primary vegetation productivity (NDVI). This is probably because higher values of vegetation productivity tend to be associated with high resource availability (e.g. insects) (Haskell 2000; Bailey et al. 2004). Therefore, our results agree with previous studies that show that bat roadkill pattern is highly related to higher suitable habitats (Gaisler 2009; Lesiński et al. 2011; Medinas et al. 2013). Other vertebrate groups show similar patterns, such as carnivores (Ascensão et al. 2017a), rodents (Duarte et al. 2021), amphibians (Arevalo et al. 2017; Pinto et al. 2023) and even invertebrates, such as butterflies (Kantola et al. 2019) or dragonflies (Soluk et al. 2011). Unfortunately, there is a dearth of information about the temporal variability in habitat suitability at a fine-scale, hampering explanations on how roadkill risk changes over time. Additionally, monitoring local changes in land

use and management is logistically unfeasible for large areas or long periods. Concerning this, this work provide a novel and important evidence, that freely and globally available spatiotemporal high-resolution satellite data, as a proxy of habitat quality, can be used to predict temporal changes in bat casualty risk across multiple years. This approach allows researchers and road managers to easily predict roadkill hotspots and their persistence in time, indicating a higher reliability in using high-risk locations as mitigation targets. In fact, the differentiation between persistent and non-persistent roadkill hotspots will allow one to target and rank the road stretches where temporal or permanent mitigation measures should be installed more precisely. In addition, remote sensing information can help road planners minimize risk when designing new road corridors on a global scale. For the future, this, combined with other autonomous devices such as sensors that detect and identify in real time the presence of animals near or on the road (Animal Detection System, ADS, or Roadside Animal Detection System, RADS) or that assess, continuously, spatiotemporal changes in road traffic, will be a powerful tool to decrease bat and other fauna roadkill risk and thus, to promote safer roads for both wildlife and people (Nandutu et al. 2022). Moreover, open-source biodiversity metrics (i.e., citizen science data) can allow modelling and predicting spatiotemporal patterns over large extents (Ascensão et al. 2019; Valerio et al. 2021). Although we were unable to use Artificial Intelligence (AI) (i.e., machine learning algorithms) to predict hotspot locations, AI is one of the most promising areas to be applied to this topic (González-Vélez et al. 2021). In fact, the use of techniques that improve the modelling framework and that reduce time consuming analysis, can provide more accurate roadkill risk predictions, eventually in near real time, for any part of the globe (Nandutu et al. 2022).

Our study showed that the roadkill hotspots tended to be located in areas with high bat activity. Therefore, autonomous acoustic methods can also be used to monitor bats on the landscape scale and assess roadkill risk (Medinas et al. 2013). The use of this method allows bat activity monitorization and does not require the attendance of specialized staff, which drastically reduces costs. Moreover, some of the software and scripts for automatic bat call identification are open source and freely available. In roads that cross particularly important areas for bats, acoustic bat monitoring could be included in Environmental Impact Assessment (EIA) procedures to better assess the risk of roadkill. Bat activity surveys should be performed before and after road construction

(Fensome & Mathews 2016) to better assess reliability of predictions based on remote sensing data. The before phase would provide an initial state against which it would be possible to identify the main existing bat flight areas, bisected by the future road, and to evaluate the putative location of mitigation measures (Elmeros et al. 2016; Møller et al. 2016). Post-construction monitoring would allow an assessment of possible changes in activity areas induced by the road and would be used mainly to evaluate the efficacy of mitigation measures.

5.1.1.2 Road-effect distance and landscape context

The ecological effects of roads are not restricted to the site of the road itself and often extend far into the surrounding habitat (Forman & Alexander 1998). In general, roads are expected to have a negative impact on wildlife, with species richness, abundance, and activity tending to decline with proximity of roads (Forman & Alexander 1998; Jaeger et al. 2005; Cushman et al. 2013; Kitzes & Merenlender 2014). Several studies have shown that bats reduce foraging activity near roads. In the UK, bat activity was 3.5 times higher at a distance of 1600 m than near the major roads (Berthinussen & Altringham 2012), and Kitzes and Merelender (2014) reported that activity was twice as high at 300 m from a highway in the USA. However, a study on railways with low traffic found that bat activity was higher on verges than in the surrounding intensive agricultural landscape (Vandeveldt et al. 2014). Thus, it seems that bats can show contrasting behaviours regarding the type of transport infrastructure and surrounding habitat. This distinction is of paramount importance for designing road bat mitigation measures. Few studies on the effects of roads on bat activity were often conducted along major high traffic roads or highways (Berthinussen & Altringham 2012; Kitzes & Merenlender 2014). Information for low- and medium-traffic roads, which represent about 50% of the worldwide transportation infrastructure and are the most widespread within natural and protected areas (Meijer et al. 2018), are virtually non-existent (Clevenger et al. 2001; Westekemper et al. 2021).

In Chapter 3, we showed that the overall pattern of bat activity regarding the distance to low- and medium-traffic roads depends on the type of habitat in the roads surrounding. In high-quality habitats, bat activity is reduced near the road, while the opposite happens

in the low-quality habitat. In high-quality habitat (for example, woodlands), the negative effects extended to approximately 300 m from the roads and penetrated further into the open agricultural field (> 500 m). Other studies have shown that the negative effects of roads on bats can extend to greater distances. For example, Berthinussen and Altringham (2012) reported effects at distances up to 1600 m from large motorways in the UK. In France, Claireau et al. (2019) showed an avoidance of major roads by bats up to 5 km from the infrastructure. However, those studies were carried out on high-traffic roads where the traffic volume was at least six times higher than the volumes registered in our study area. Despite this, our results suggest that low-traffic roads (e.g., single-carriageways) should be taken into account when considering future road planning or mitigation measures to already existing roads. We emphasize that road practitioners should also consider the construction of crossing structures on low- and medium-traffic roads to reduce the risk of roadkill and as an important part of the conservation of connectivity. In fact, animal activity near roads is often considered a good proxy of roadkill risk and is often used to decide about mitigation measure location (Jack et al. 2015; Skórka et al. 2015; Arevalo et al. 2017; Duarte et al. 2021).

Furthermore, our results are in agreement with those of Claireau et al. (2019), who found, in France, that low-flying species are more affected than high-flying species. These contrasting behaviours among species may be linked to their specific foraging requirements (Kerth & Melber 2009). In fact, low-flying bats that include gleaning and clutter-adapted species, which forage in clutter habitats and fly less in open spaces, are least likely to road crossings. It is suggested that this is a possible behavioural response, as these species could perceive roads as an inadequate habitat. Studies suggest that *Plecotus spp.* and *Rhinolophus spp.* avoid areas with high disturbance (light and noise from traffic) (Bonsen et al. 2015; Stone et al. 2015), which reduces their foraging performance (Luo et al. 2015) and may increase the energy expenditure that reflects a long-stress response, increasing the incidence of diseases (Song et al. 2019). In contrast, according to our results, high flying bats that generally forage in more open habitats seem to be less affected by road disturbance and may depend less on possible resources that are provided by roads (Vandeveldt et al. 2014; Claireau et al. 2019b). This is supported by empirical data showing that these bats registered low numbers of roadkill (Medinas et al. 2013; Fensome & Mathews 2016). This result is also important for designing appropriate mitigation measures that must comply with bat guild behaviours

and characteristics, prioritizing measures that aim to reduce impacts on low-flying species.

Our study also shows that road verges seem to provide a significant resource (probably as feeding areas) in open agricultural landscapes. Road verges can increase the availability of insects in habitats where there is less competition from other bats, creating an attractive foraging resource. However, in Australia, Bhardwaj et al. (2018) found that the biomass of nocturnal flying insects does not change with distance from the road. Thus, the characteristics of habitat and management of vegetation on roadsides may be crucial for bats that occur in these areas. Up till now, the recommendations are to avoid clear vegetation cutting and to keep some shrub cover (Bhardwaj et al. 2018). However, increased activity near roads also means a higher risk of roadkill, and the trade-offs between the two must be clarified in further studies, before recommending general vegetation promotion along road verges as a positive measure for bats that occur in open agricultural landscapes. More studies are also required to assess the relative importance of these landscape elements in explaining the reduction in distance from the effect of roads on other wildlife.

5.1.1.3 Genetic relatedness of the lesser horseshoe population: roads lead to resistance

Roads can strongly affect the degree of landscape permeability to animal movement and may have a significant impact on genetic patterns between and within populations (Riley et al. 2006; Di Giulio et al. 2009; Holderegger & Di Giulio 2010; Frantz et al. 2012). In the last two decades, several studies have indicated that roads can shape the gene flow of highly mobile mammals, such as carnivores and bats (Kerth & Petit 2005; Jackson & Fahrig 2011; Tournant et al. 2013; Dorland et al. 2014; Dool et al. 2016; Voigt & Kingston 2016; Murphy et al. 2022). Although clear genetic isolation as a direct result of roads has not been proven in bats, this group of bats is particularly vulnerable to road developments, as they use large areas of the landscape for foraging and their large dispersal movements are also likely to increase their encounter rates with roads (Fensome & Mathews 2016). In addition, many species live at low densities and have a patchy distribution, making them more vulnerable to local extinctions. For example, the

clutter-adapted lesser horseshoe bat, which has suffered a wide population decline in recent years, is particularly susceptible to roadkill (Medinas et al. 2013) and road barrier effects, avoiding areas with high traffic noise and artificial lighting (Russo & Ancillotto 2015; Stone et al. 2015). Thus, the combination of direct mortality and road avoidance behaviour can quickly reduce connectivity among populations on opposite sides of the road (Balkenhol & Waits 2009; Fahrig & Rytwinski 2009; Holderegger & Di Giulio 2010). However, information is still lacking to assess the potential impact of connectivity loss among lesser horseshoe bat populations, or to identify how road characteristics and landscape features shape their genetic relatedness structure.

In Chapter 4, we identified a slight genetic structure on the lesser horseshoe population: The females from the roosts closer to the roads were less genetically related compared to those located away from the roads. This is very interesting from a management and conservation viewpoint, as genetic effects of roads are expected to only become detectable after dozens of generations (at least 15) (Murphy et al. 2008). Furthermore, our analysis of fine-scale genetic relatedness revealed a decrease in parentage level among roosts on opposite sides of the highway, although there is no clear genetic differentiation in the overall population. Indeed, the results of relatedness analysis suggest that a long-term structuring of population on opposite sides of the roads may be ongoing but may only be fully noticed further ahead. This suggests that many wildlife populations currently separated by roads, even recently constructed ones, may already have a weak genetic differentiation, although not yet translated into a formal genetic structuring in subpopulations (Holderegger & Di Giulio 2010). Studies exploring genetic discontinuities linked to linear barriers have documented the relevant effects of infrastructures 40 years after their construction (Epps et al. 2005; Hepenstrick et al. 2012). This time span is almost twice that elapsed from the enlargement of main roads and the construction of the motorway (1997 to 1999) in the study area. It is therefore perhaps surprising that we detected the genetic structure in the studied population, particularly in the case of a long-lived species. Several studies highlight that genetic effects of roads on wildlife can be significantly accelerated when they occur simultaneously with other strong impacts, such as mortality (Jackson & Fahrig 2011; Grilo et al. 2020) and road-avoidance (Epps et al. 2005). Roads are often assumed to be the main source of nonnatural mortality in the case of lesser horseshoe bats (Medinas et al. 2013; Iković et al. 2014; Fensome & Mathews 2016) and road avoidance has been

documented in several bat species (Kerth & Melber 2009). Therefore, it is possible that these two factors, coupled with forest density patches, drive together the movement capabilities, and thus, genetic structuring, of the bats of the studied population. Only further genetic monitoring of the studied population would allow to confirm the genetic differentiation pinpointed in our study. This could include roadkill monitoring of the lesser horseshoe bat over several years and the genetic analysis of the carcasses, which could allow us to infer about the proportion of the effective population size that is killed and forecast in a more consistent way population viability in the long-term. Within the same context, the role of traffic volume on road avoidance behaviour and implications for gene flow among populations on either side of the road could be evaluated, to develop guidelines to reduce behavioural avoidance. In fact, to ensure the implementation of appropriate and effective mitigation measures, a global approach that includes ecological, behavioural, and genetic components is essential to fully understand the impacts of roads and properly mitigate them.

Our results are in agreement with previous studies which highlight that forested areas favour bat movements (Kerth & Melber 2009; Reiter et al. 2013) and, thus tend to counteract genetic differentiation. For example, Tournant et al. (2013), argue that the low genetic differentiation in lesser horseshoe bats in the Franche-Comté region was due to the large amounts of forest patches with high tree density among bat roosts. Therefore, the presence of suitable habitats can effectively facilitate movement, which contributes the most to connectivity within bat populations. In contrast, our results also showed that greater landscape heterogeneity, representing intermixed open agricultural fields and small forest patches, seems to restrict bat movements, particularly for females. Thus, besides road mitigation measures, the protection and restoration of landscape elements that promote landscape connectivity could also help prevent genetic structuring of the population. High-quality habitats must be conserved and, where possible, new structural elements must be installed to promote habitat connectivity. The presence of trees and hedgerows on roadsides also tends to increase the likelihood of bat road-crossing (Abbott et al. 2012a; Bennett & Zurcher 2013).

5.2 Implications for bat populations in road-dominated landscapes

Bat populations living in road-dominated landscapes are affected by road disturbance associated with traffic noise or artificial light, and their long-term population viability may be hampered by additional non-natural mortality and constraints to their movements. However, the risk to populations may be variable depending on other environmental conditions, such as habitat quality in surrounding areas, which tends to attenuate the barrier effect (Chapters 2 and 4); and ecological attributes, such as species flight height, with species flying at lower heights, having increased risk of being hit by a car (Chapter 3). In Chapter 4, we suggest that a threatened and protected species, with a small population size (< 500 individuals), is already facing genetic problems despite the short time lag since the enlargement of a road and motorway construction in the study area. Those findings are expected to be extended to other wildlife species, with the road acting as a semi-permeable filter that, over long periods of time, can lead to significant population structure and isolation, increasing its risk of local extinction. Therefore, the impacts of roads on bats should be considered a major issue in bat conservation and population management. However, determining the likely impacts on the population is difficult in the absence of good data on population density.

Despite rare examples of rigorous population-level road effect research, some long-term studies have already shown that road mortality trends can reduce population densities (Baker et al. 2004; George et al. 2011). Thus, roadkill monitoring, which is easily measured, may be a simple useful tool to assess the long-term survival of populations. A meta-analysis of bat roadkill confirms that, despite low-flying species being the most common casualties, high-flying bats are hit more frequently than would be expected from their relative population sizes (Fensome & Mathews 2016). Moreover, although males may have a higher casualty risk, even low mortality in females can significantly reduce the effective size of the population. Importantly, females in roosts bounded by a high traffic road had lower reproductive success (Kerth & Melber 2009). Our results do not allow us to confirm these conclusions. However, they show that roads negatively impact bats, and that those impacts are guild, sex and landscape specific. This highlights the importance of studying bat populations in different contexts and

considering bat community and population traits. Roads seem to restrict bat movements, which may contribute to the low number of inter-roost individual exchange recorded (Chapter 4). Unfortunately, it is difficult to draw firm conclusions about the effects of dispersal movements and mating patterns on local populations, as they are rarely quantified.

5.3 Future prospective

The impacts of roads on population connectivity and abundance are one of the main concerns of conservation biology. Therefore, a key challenge for wildlife conservation is resolving how to increase the permeability of roads to wildlife movements without increasing the likelihood of vehicle collisions. One of the main problems facing road agencies is the accessibility of evidence for conservation interventions and the time required to assess this evidence. Bats, are among vertebrates the group for which road mitigation measures are still in their infancy and still need a proper assessment of their effectiveness. Some measures to facilitate bat movement across roads (e.g., overpasses) or reduce roadkill risk (e.g., flight elevation walls) have already been essayed. However, results are scarce or contradictory, and it is still unknown whether these devices are truly effective in reducing road barrier effects and roadkill, and thus how they contribute to the long-term viability of the population. This lack of knowledge has often been the argument for avoiding implementing expensive road mitigation measures for bats, causing misunderstandings among conservation researchers and road practitioners. Therefore, it is urgently necessary to provide a framework for evaluating the efficiency of road interventions to reduce the impact of roads on bats. In particular, researchers must communicate their findings in ways that help audiences (e.g., road practitioners) maintain a sense of urgency and inspire long-term engagement and action (de Lange et al. 2022).

Moreover, as demonstrated in this thesis, the assessment of the impacts of roads, should be a multidisciplinary approach, including landscape ecology, (Chapters 2 and 3) population ecology (Chapter 4), remote sensing (Chapter 2), and genetics (Chapter 4). Remote sensing data can be a powerful and cost-effective tool for gaining knowledge on

the dynamics of hazardousness of specific disturbance to population viability, and the vast amounts of information available can facilitate the understanding of fine-grain habitat suitability for different species. High-throughput genotyping techniques (with high-resolution genomic markers, such as SNPs) can allow a large improvement in the ability to quantify fine-scale population genetic variation and provide better insights into the contemporary subpopulation structure. This information can be associated with landscape characteristics (Chapter 3) and population traits to gain insight into factors that affect population trends and species behaviour. This would provide a better understanding of road ecology and positive insights for new conservation strategies to effectively mitigate road effects. Although our focus is on bats and roads, the issues and recommendations discussed can be applied to the conservation of other wildlife.

5.4 Concluding remarks

Roads are a widespread feature of the landscape in most countries, and only a few road-free areas remain in the inhabitable part of the Earth. In the upcoming decades, a large expansion of the road network is expected to happen mostly in high-biodiversity countries. This makes even more critical the development and proper assessment of the efficacy of mitigation measures, particularly for poorly studied taxonomic groups or for which contrasting information has been produced, such as bats. It is time for researchers to become more innovative and for practitioners to be receptive to testing new ideas. Effective results and solutions are urgently needed if we do not want wildlife to be suffocated by 'the giant that embraces us', the road network.

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