ASSESSING AND MITIGATING ROAD EFFECTS ON FUNCTIONAL LANDSCAPE CONNECTIVITY: A CASE STUDY WITH COMMON GENETS (*GENETTA GENETTA*) IN A MEDITERRANEAN CONTEXT.

Filipe Granja de Carvalho

Tese apresentada à Universidade de Évora
para obtenção do Grau de Doutor em Biologia

ORIENTADORES: *Prof. Doutor António Paulo Pereira Mira*
Doutor Pedro Rui Correia Oliveira Beja

ÉVORA, JULHO DE 2015

INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

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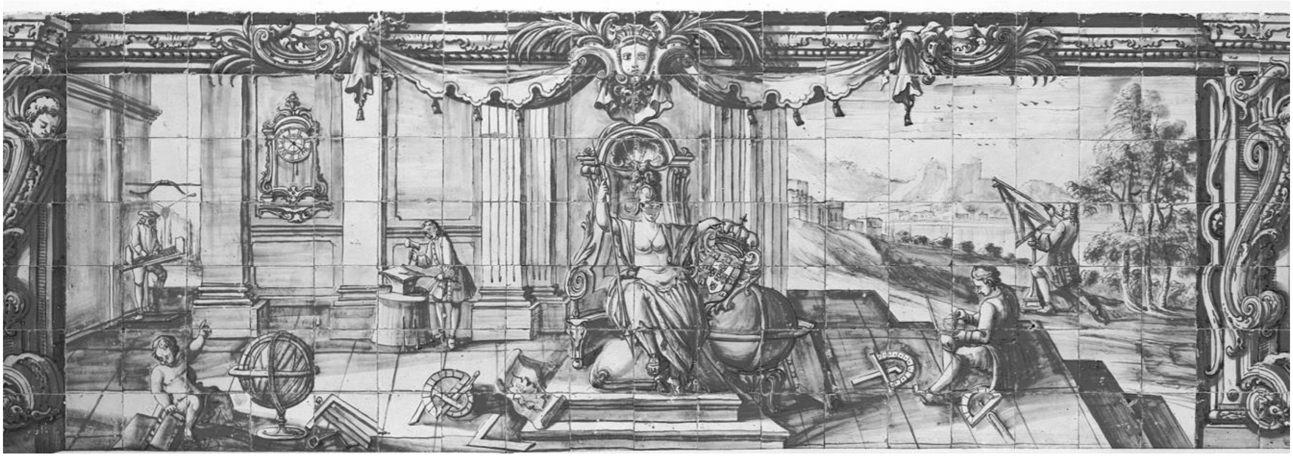

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DECLARAÇÃO DO AUTOR

Em conformidade com o Regulamento do Ciclo de Estudos conducente ao Grau de Doutor pela Universidade de Évora (Ordem de Serviço N.º18/2014), esta tese integra um conjunto de quatro trabalhos de investigação, dos quais dois estão já publicados, um submetido para publicação e outro para submeter brevemente (todos em revistas internacionais com *peer-review RiR* (ISI)). O autor esclarece que os trabalhos referidos foram realizados em colaboração, e que em todos eles liderou e participou ativamente na sua conceção, recolha, análise e discussão de resultados, bem como na escrita dos artigos. Algumas das normas relativas ao padrão de formatação de cada revista foram retidas nesta tese.

Évora, 31 de Março de 2015

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AVALIAÇÃO E MITIGAÇÃO DOS EFEITOS DAS ESTRADAS NA CONECTIVIDADE FUNCIONAL DA PAISAGEM: A GENETA (*GENETTA GENETTA*) COMO CASO DE ESTUDO EM CONTEXTO MEDITERRÂNEO.

RESUMO

A conectividade funcional da paisagem para uma espécie depende do seu comportamento e capacidade de movimento e influencia a sua persistência em paisagens humanizadas. Porém os efeitos desta componente da paisagem nos carnívoros florestais são pouco conhecidos. Esta tese usa dados de movimento, comportamento e genética para avaliar os efeitos das estradas e da heterogeneidade dos habitats na conectividade funcional da geneta numa paisagem florestal agrícola, do Sul de Portugal. Especificamente, pretende-se alcançar os seguintes objetivos: i) avaliar os fatores ambientais que influenciam o comportamento de repouso; ii) determinar a influência da intensidade da monitorização na análise do comportamento de repouso; iii) analisar trajetórias de movimentos para modelar a conectividade da paisagem e avaliar os fatores ambientais que a influenciam, a diferentes escalas; iv) combinar dados de mortalidade rodoviária, radiotelemetria, e análises genéticas para inferir atravessamentos de estradas e dispersão efetiva. As cavidades arbóreas foram o abrigo mais seguro, particularmente na época húmida, e permitiram o abrigo junto a rodovias, sempre que necessário. A monitorização diária foi a que melhor permitiu caracterizar os comportamentos de repouso. A conectividade da paisagem aumentou em grandes áreas florestais, e próximo de corredores ripícolas que permitem atravessar áreas agrícolas normalmente intransponíveis. As estradas reduziram a conectividade ao dividirem as manchas florestais, mas o efeito foi amenizado nos corredores ripícolas que frequentemente conduzem a passagens hidráulicas e viadutos. As diferentes análises sugeriram que a auto-estrada é permeável ao fluxo genético, apesar dos atropelamentos e do constrangimento ao movimento de adultos. O baixo tráfego, as inúmeras passagens hidráulicas, a agilidade das genetas em transpor barreiras, o grande efetivo populacional nos dois lados, e a ausência de tensões territoriais durante migrações terão potenciado estes resultados. Em suma, os resultados desta tese melhorarão estratégias de mitigação dos efeitos das estradas na conectividade funcional da paisagem para carnívoros florestais.

Palavras-chave: carnívoros florestais, conectividade funcional da paisagem, comportamento de repouso, estradas, fluxo genético

ASSESSING AND MITIGATING ROAD EFFECTS ON FUNCTIONAL LANDSCAPE CONNECTIVITY: A CASE STUDY WITH COMMON GENETS (*GENETTA GENETTA*) IN A MEDITERRANEAN CONTEXT.

ABSTRACT

Landscape functional connectivity of a species depends on its behaviour and movement ability and influences its persistence in human-modified landscapes. Nonetheless, the effects of functional connectivity loss by small forest carnivores are still poorly understood. This study combines movement, behaviour and genetic data to assess the effects of roads and habitat heterogeneity on the functional connectivity of the common genet in a mixed forest-agricultural landscape, in southern Portugal. Specifically, we aim to reach the following goals: i) to assess the environmental factors influencing resting behaviour; ii) to clarify the impacts of monitoring frequency on the analysis of resting behaviour; iii) to use path-level analysis to model landscape functional connectivity at different perception scales and asses the main environmental factors influencing it; iv) to combine roadkills, radiotracking and genetic analysis to infer crossing events and dispersal effectiveness. Tree hollows were the safest resting place, with prevalence on the wet season and allowed individuals to rest near roads, whenever necessary. Daily monitoring was the best sampling regime to characterize resting site behaviour. Landscape connectivity was favoured by large forest patches, and near riparian areas providing corridors within open agricultural land highly resistant to genet movement. Roads reduced connectivity by dissecting forest patches, but had less effect on riparian corridors due to the buffering offered by crossing structures. Results from the combined sources of information suggested that the motorway allowed high gene flow, despite the roadkills and the constraints to adult movements. Low traffic volume, numerous crossing structures, ability of genets to overcome obstacles, high population sizes on both sides of the motorway, and the absence of territorial constraints to effective migration potentially originated the observed results. Our results give important clues to mitigate road effects on the landscape functional connectivity of small forest carnivores.

Keywords: forest carnivores, gene flow, landscape functional connectivity, resting behaviour, roads

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1. GENERAL INTRODUCTION

Animal movement and landscape connectivity

Successful movement in animals to feed, rest, reproduce, escape predators and adapt to human disturbances and harsh climate conditions is fundamental during all life stages (Nathan et al. 2008; Morales et al. 2010; Baguette et al. 2013). Thus, any disruption of movement through landscape changes such as habitat fragmentation and loss may affect the regular individuals' life cycle and jeopardize population persistence (Fahrig 2003; Fischer and Lindenmayer 2007; Crooks et al. 2011).

Dispersal is a key ecological process that can be defined as the movement from a natal site to a new breeding site. Three main stages have been identified during natal dispersal: i) departure from the natal or breeding locations; ii) travel or transience stage; and iii) settlement in the new area (Fahrig 2007; Bonte et al. 2012; Palmer et al. 2014; Pflüger and Balkenhol 2014). In the first stage, individuals leave their current location to avoid local costs and obtain benefits in their fitness elsewhere (e.g. areas with reduced competition for resources). During the transience stage, a moving animal decides where to move (e.g. direction and distance) and how to move (e.g. path selection) (Nathan et al. 2008). In this stage, costs are linked to the movement itself, for example mortality risk or energetic expenses. Finally, an individual either stays at the new area or moves on, depending again, on its fitness in the new site (Bonte et al. 2012). Landscape composition and configuration influences these stages of dispersal in different ways, by altering movement patterns and subsequently affecting landscape connectivity according to species-specific characteristics (Pflüger and Balkenhol 2014).

In 1993, Taylor et al. defined landscape connectivity as: "…The degree to which the landscape facilitates the movement of animals, plants and other ecological flows and thus allows their successful dispersal among suitable habitat patches…" Recently, researchers and political authorities recognized the value of landscape connectivity and the concept has become critical in conservation planning. However, terms such as connectivity conservation, population connectivity, structural connectivity and functional connectivity (Taylor et al. 2006; Betts et al. 2015) are often indistinctly used, though they correspond to different concepts which need to be clarified (Taylor et al. 2006; Betts et al. 2015). Structural connectivity describes how the habitat patches are spatially distributed in the landscape (e.g. Euclidian inter-patch distances, patch configuration) and ignores the behavioural response of organism towards different patches (Taylor et al. 2006; Kadoya 2009). Functional connectivity incorporates the behavioural response of organisms across landscapes towards variable patch sizes, quality and inter-patch distances (Taylor et al. 2006; Kadoya 2009; Betts et al. 2015). High overall landscape connectivity may support or counteract the effects of habitat loss and fragmentation. Particularly, landscapes functionally connected allow individual movement and gene flow at levels compatible with population persistence (Lowe and Allendorf 2010). Instead of simply assessing isolation through Euclidean distances, functional connectivity integrates information about the spatial structure and heterogeneity of the landscape, including for instance how matrix quality impact movement routes, survival and fitness of dispersing animals (Taylor et al. 2006). Accordingly, two locations may be isolated by the same Euclidean distance, but several ecological distances depending on individual species niche requirements. This dependence on landscape matrix quality varies according to species-specific dispersal preferences or to environmental cues that either attract or repel individuals (predation risk or competition, Clobert et al. 2009; Pflüger and Balkenhol, 2014). Individual dispersal decisions may vary intra-specifically (e.g. age, sex, energetic reserves, and competitive ability), depending on how individuals balance their expected fitness between sites with different environmental conditions (Palmer et al. 2014).

In this context, the availability of local resources (e.g. resting sites), and the level of landscape fragmentation imposed for instance by human infrastructures (e.g. roads), have been referred to as major factors affecting animal movement, and hence connectivity in human-dominated landscapes (Crooks and Sanjayan 2006).

The movement of individuals among patches is the basis of functional connectivity. Nevertheless, landscape connectivity assessments are mostly based on point location (presences) or habitat use (Zeller et al. 2012; Carvalho et al. submitted; Vasudev et al. 2015) rather than movement. Some studies showed that presence data provided connectivity rankings correlated with movement data, but with several discrepancies (Bender and Fahrig 2005). Most studies assign resistance values subjectively, often relying only on expert opinion (Beier et al. 2008; Spear et al. 2010; Zeller et al. 2012), even the most recent and robust methods, such as least-cost paths or circuit theory models (Andriasen et al. 2003; Klar et al. 2012; Dickson et al. 2013). In this thesis we tried to overcome these issues by gathering an empirical robust radiotracking data. Setting functional connectivity models should rely on a more efficient tracking of dispersing individuals, currently constrained by radio and GPS collars batteries limitations (but see sections of “implications for connectivity management… and future research”). Landscape functional connectivity models should distinguish patterns of movements of residents from dispersers, i.e. regular foraging at the fine-scale from movements driven by the internal state, condition or motivation of each animal (Vasudev et al. 2015).

Effects of resting sites on landscape functional connectivity

Resting sites are physical places where animal rest after foraging periods, several different types of resting places were described such as: thickets, tree hollows, branches, nests belonging to other animals, dead trunks on the ground, boulders and either inhabited or abandoned manmade structures (e.g. Camps 2011; Brown et al. 2014). In forest carnivores, three major types of resting sites are described in literature: i) tree hollows; ii) nests; and iii) dens. Nests are structures made by carnivores using plant material, which are often located in large tree branches, and are supported by climbing plants (Palomares and Delibes, 1994). Dens are mainly located on burrows, often in riverbanks and surrounded by high shrub cover (Slauson and Zielinski, 2009). Resting sites influence fitness by: decreasing predation and parasitism risk, lessening human disturbances, reducing thermoregulatory needs; promoting social and territorial interactions and enhancing the access to food and water (Brown et al. 2014; Carvalho et al. 2014).

Proper landscape connectivity measures should thus account for resting site availability, together with daily foraging movement patterns and seasonal dispersal behaviours. However, the lack of fine-scale and direct empirical information on the importance of resting sites for most species hampers a complete understanding of the behavioural parameters affecting animal movement, and their consequences in estimating landscape connectivity (Zeller et al. 2012; Mateo-Sánchez et al. 2014). The availability of resources, in particular those that are critical for breeding success, are main factors driving dispersal (Dharmarajan et al. 2013). Most studies dealing with landscape connectivity have largely focused on feeding resources, while overlooking resting, den and maternal sites. However it has been recently recognized that the availability and location of resting sites may have a major influence on the successful movement of species across landscapes (Brown et al. 2014). Thus, reliable assessment of resting sites typology and spatial-temporal patterns of their use by animals should be crucial to properly infer landscape functional connectivity (Carvalho et al. 2014; Brown et al. 2014). However, evaluating the importance of resting sites to landscape connectivity is often precluded by the costs and difficulties of delineating accurate sampling designs.

Lower sampling efforts could increase associated bias in model estimations (Moreno and Halffter 2001; Fontaneto et al. 2012; Skalak et al 2012; Barbosa et al. 2013). Hence, in species that often use a large number of resting sites, the monitoring schedule of resting behaviour should rely on high frequency rates to avoid missing new locations and typologies of resting sites. A sparse sampling regime may fail to spot relevant variables and compromise the modelling of movement paths and habitat suitability (Roever et al. 2014).

Effects of roads on landscape connectivity

A myriad of negative impacts of roads and their associated traffic on wildlife (Fig. 1) are known since the early Twenty Century (e.g. Stoner 1925). A large increase in road construction associated to a strong socio-economic development occurred in the last decades, particularly in the emergent economies worldwide (Forman et al. 2003). Currently, roads cover an average of 1.2 km/km^2 in the United States (Forman 2000), and over 2 km/km^2 in some European countries (Carr et al. 2002). Consequently, a new research field named “Road Ecology” has emerged in order to offset the negative effects of roads on wildlife, which was defined by Forman et al. (2003) as: ”...the interactions of organisms and the environment linked to roads and vehicles (…) Thus road ecology explores and addresses the relationship between the natural environment and the road system…” A number of crucial publications within this field came out since its appearance, including one text book (Forman et al. 2003), several reviews on ecological effects of roads (e.g. Forman and Alexander 1998; Spellerberg 1998; Trombulak and Frissell 2000; Coffin 2007; Fahrig and Rytwinski 2009; Polak et al 2014), and also various studies on genetic consequences of roads on populations (Balkenhol and Waits 2009; Holderegger and Di Giulio 2010; Jackson and Fahrig 2011). In Portugal, road ecology studies have begun in the 1990’s, but the first published works date from the mid 2000´s, focussing on different taxonomic groups: including carnivores (Grilo et al. 2008, 2009, 2011; Mateus et al. 2011; Ascensão et al. 2013, 2014; Seronha et al. 2013; Villalva et al. 2013), bats (Medinas et al. 2013), small mammals (Sabino-Marques and Mira 2011; Ascensão et al. 2012; Mullins et al. 2015), owls (Gomes et al. 2009; Silva et al. 2012; Borda-de-Água 2014; Grilo et al. 2014), reptiles (Brito and Álvares 2004) and several groups considered jointly (Petronilho and Dias 2005; Ascensão and Mira 2007; Carvalho and Mira 2011; Santos et al. 2011; Grilo et al. 2012).

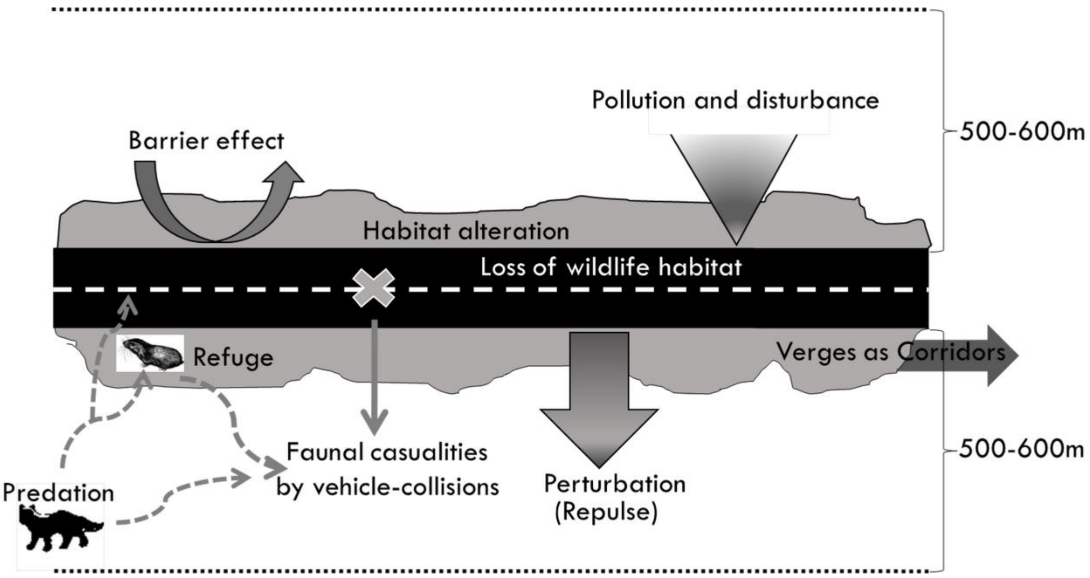

Figure 1 Overall road effects on wildlife (Adapted from Trocmé et al. 2002).

A main outcome of road ecology studies is that roads are one of the major causes of habitat loss and fragmentation worldwide (Forman et al. 2003). In addition, roads may represent barriers to movement in three different ways: physical barrier, behavioural barrier and road mortality (Forman et al. 2003; Crooks and Sanjayan 2006). Roads alter population structure in adjacent areas, with some species avoiding them and others being attracted to it (Kaczensky et al. 2003; Barrientos and Bolonio 2009). They also hamper gene flow between populations (Riley et al 2006, 2014) and enhance mortality risk (Forman et al. 2003). Roads can affect all types of organisms, from small invertebrates, such as slugs, to large animals, like moose (*Alces alces*) and brown bears (*Ursus arctos*) (Smith-Patten and Patten 2008). Indeed, several researchers have pointed out that roads are among the main causes of modern-day vertebrate population declines (Crooks and Sanjayan 2006; Ament et al. 2008). This may be especially true for small mammals, for which barrier effects include the reluctance to cross roads (behavioural barrier), thereby leading to local extinctions (Rico et al. 2007; McGregor et al. 2008; Ascensão et al. 2012). Among larger and rarer species like the Iberian lynx (*Lynx pardinus*), road kills may be the main cause of death among cubs (southwest Spain; Ferreras et al. 1992). In Great Britain, about 66% of badger (*Meles meles*) cubs and adults are roadkilled in urban areas (Clarke et al. 1998). In addition, the main non-natural causes of death in otters (*Lutra lutra*) in Britain are road fatalities, which have contributed to the general population decline of this species (Philcox et al. 1999). In Spain, barn owl (*Tyto alba*) populations decreased by 70% over a 10-year period, and road casualties are considered the major cause of these deaths (Fajardo

2001). Research by Fahrig et al. (1995) suggests that the presence of roads and the increase in traffic intensity may have contributed to the global decline among amphibians, as well. In fact, several studies have revealed a negative correlation between the anuran abundance and traffic density (Fahrig et al. 1995; Carr and Fahrig 2001; Hels and Buchwald 2001), between anuran pond occupancy and road density (Vos and Chardon 1998), and between amphibian species richness in breeding sites and paved road density (Findlay et al. 2001; Eigenbrod et al. 2008). Vertebrates remain the most studied group in road mortality research, not only because of their size but also because they comprise flagship species (Forman et al. 2003).

Of all the road impacts stated previously, direct road mortality is the most noticeable among the general public. However, the most negative impact on population persistence is probably the reduction in landscape connectivity due to the road barrier effect (Belisle 2005; Shepard et al 2008; Garriga et al. 2012; Jackson and Fahrig 2011). Such barrier effect can disrupt daily and seasonal movements, even for more vagile species like carnivores (Rondinini and Doncaster 2002; Riley et al. 2006, 2014; McGregor et al. 2008; Benitez-Lopez et al. 2010). Animals may not be able to access several resources (e.g. food, mates), or evade predators and dispersal failure may occur, thus disrupting overall population gene flow (Jakson and Fahrig 2011). Reduced gene flow due to road avoidance behaviour or road mortality can lead to a decrease in genetic diversity because genetic drift is enhanced in isolated populations. However, empirical examples showing the effects of roads on genetic diversity are mostly lacking and research has focused mainly on fragmentation effects on animal movement. Thus, little attention has been paid to the real factors causing the loss of genetic diversity: the barrier effect itself, or the depletion effects resulting from road mortality (Jakson and Fahrig 2011). Lastly, roads may increase edge effects by slicing habitats into smaller patches (Fahrig and Rytwinski 2009; Marcantonio et al. 2013).

Specialist species such as forest carnivores are highly sensitive to fragmentation and edge effects (Crooks et al. 2011; Dickson et al. 2013), and may thus be particularly affected by roads. In addition, like the other carnivores, forest species have typically wide home ranges, which are often intersected by roads. To counteract the physical barrier effects imposed by roads, carnivores regularly use culverts and other larger passages such as viaducts and bridges (Ascensão and Mira 2007; Grilo et al. 2008; Mateus et al. 2011; Seronha et al. 2013). However, their relatively high crossing rates does not always mean an increase in effective (genetic) connectivity, probably because roads act as artificial territorial boundaries for adult residents (Riley et al. 2006, 2014). If the discrepancy between road crossings and gene flow is as widespread as suggested in the literature (e.g. Riley et al. 2006), the effectiveness of fauna passages and culverts as dispersal corridors needs to be tested, in order to properly evaluate how well they meet the goal of ensuring connectivity among populations (Strasburg 2006). Besides, corridor and passage planning needs to be improved, by integrating for instance information on fine-scale environmental conditions enhancing animal movement and thus assuring that functional connectivity is maximized (Cushman et al. 2013b). In this context, forest carnivores provide a particularly useful model species to understand the scales at which animals perceives their habitat at both local and landscapes scales, while still being useful for a wider array of similar species.

Landscape genetics as a tool to measure landscape functional connectivity

Habitat fragmentation and loss decreases the connectivity among populations, directly impacting their persistence in the landscape, and the degree of gene flow. The major consequences of the reduced gene flow between increasingly smaller populations dissected by roads include inbreeding depression, loss of genetic diversity, loss of self-incompatibility alleles, mutation accumulation, and outbreeding depression, which together may constrain the ability of a population to readapt to possible environmental changes (Frankham 2006). All these negative impacts are particularly strong in human-modified landscapes, thus increasing the interest by geneticists and ecologists in assessing the consequences of landscape spatial patterns on gene flow. This interest has resulted in the development of a relatively new research field, known as landscape genetics (Manel et al. 2003). Quantitatively assessment of the relationship between gene flow and landscape structure is of paramount importance in connectivity studies grounded in landscape genetics (Manel et al. 2003; Landguth et al. 2010). Landguth et al. (2010) strengthened the need of combining different methods to infer on the genetic consequences of natural and human-made landscape barriers in a shortest time possible. Several authors have discussed the advantages and disadvantages of using empirical movement versus genetic data to assess landscape connectivity, emphasizing the need to combine both of them (Aubry et al. 2004; Fedy et al. 2008; Finnegan et al. 2012). Ecological movement data provide information on daily movements and related decisions of animals towards fine-scale aspects (microhabitat) of the landscape, which otherwise would remain undetected by using any other technique (Aubry et al. 2004). Moreover, social interactions among individuals are detected only through intensive radiotracking (e.g. a male breeding with several females; males killing subadults and other floaters during a specific season) (Aubry et al. 2004; Finnegan et al. 2012). On the other hand, landscape genetics focus on assessing the degree to which landscapes facilitate the movement of organisms by relating gene-flow patterns to landscape structure. Thus, genetic data may suggests the existence of barriers to movement at the local and landscape scale, otherwise unnoticed (Fedy et al. 2008), and may validate complex models derived by empirical ecological movement data (Manel et al. 2003; Holderegger and Wagner 2008; Manel and Holderegger 2013).

The most important methods to evaluate gene flow among populations are either based on: individual genetic distances, assignments tests, or parentage analysis. Individual genetic distances (among all possible combinations of pairs of individuals sampled) is usually related to landscape features through Mantel or partial Mantel tests (Holderegger and Wagner 2008; Cushman et al. 2013b). One of the limitations of this approach is that the landscapes features believed to influence gene flow are not known a priori. Assignment tests evaluate the degree of differentiation among populations. Initially, these tests were based on frequency statistics and used the observed allele frequencies for each of the predefined reference populations to calculate the likelihood that a particular genotype belongs to each population. Then, individuals were assigned to the population with the highest likelihood (Cegelski et al. 2003; Holderegger and Wagner 2008). Recent advances on assignment tests and the introduction of Bayesian models, eliminated the need for predefining reference populations (Holderegger and Wagner 2008). Parentage analysis is an individual-based method that evaluates gene flow by examining parental relationships among pairs of individuals, either of different populations or separated by a natural or artificial barrier (Holderegger and Wagner 2008). Parentage analysis provides evidence of gene flow (migration) on a small temporal scale (few years) (Landguth et al. 2010), which is an advantage comparing with genetic distances and assignment tests which often need large time lags to detect effects of landscape features on genetic patterns (Holderegger and Wagner 2008).

Study area

This study was carried out in a landscape of about 4572 km^2 in southern Portugal (38°21'39'' to 39°01'36'' N, 08°24'22'' to 07°34'54'' W; Fig. 2). The climate is Mediterranean, with mean daily temperatures ranging from 5.8 ºC to 12.8 ºC in winter, and from 16.3 ºC to 30.2 ºC in summer; annual rainfall averages 609.4 mm and is concentrated in October-March (Évora 1971–2000; IPMA, 2012). The relief is undulating (150m-430 m above sea level) and the landscape is dominated by agricultural land uses (≈45%), comprising mainly dry arable land and pastureland, with or without sparse oak trees, and vineyards. There is also a large area (≈43.5%) of open to closed cork oak (*Quercus suber*) and holm oak (*Q. rotundifolia*) forests, where the understory may be herbaceous or shrubby depending primarily on grazing pressure and understory management to reduce fire risk and to provide easy access to livestock. Other land cover types include intensive olive orchards, and some scattered plantations of *Pinus* spp. and *Eucalyptus* spp. (≈8.8%). This landscape is dissected by numerous roads including the A6, a controlled-access highway (hereafter highway) constructed in 1996, consisting of 2 lanes on each side separated by a central splitter, and with a mean traffic flow of 977 vehicles per night range = 330-2494; Grilo et al. 2009). National and regional roads, most of which have two lanes and paved verges, have a mean traffic flow of 873 vehicles per night (range = 439-1680; Silva et al. 2012) (Fig. 2). Overall, the study area is bisected roughly at the centre by the main transportation axis (implying several parallel linear infrastructures) linking Lisbon to Madrid. In the near future a major railway also roughly parallel to the existing main roads will be built in the region, which makes this area an important living laboratory to study the impacts of linear infrastructure on wildlife.

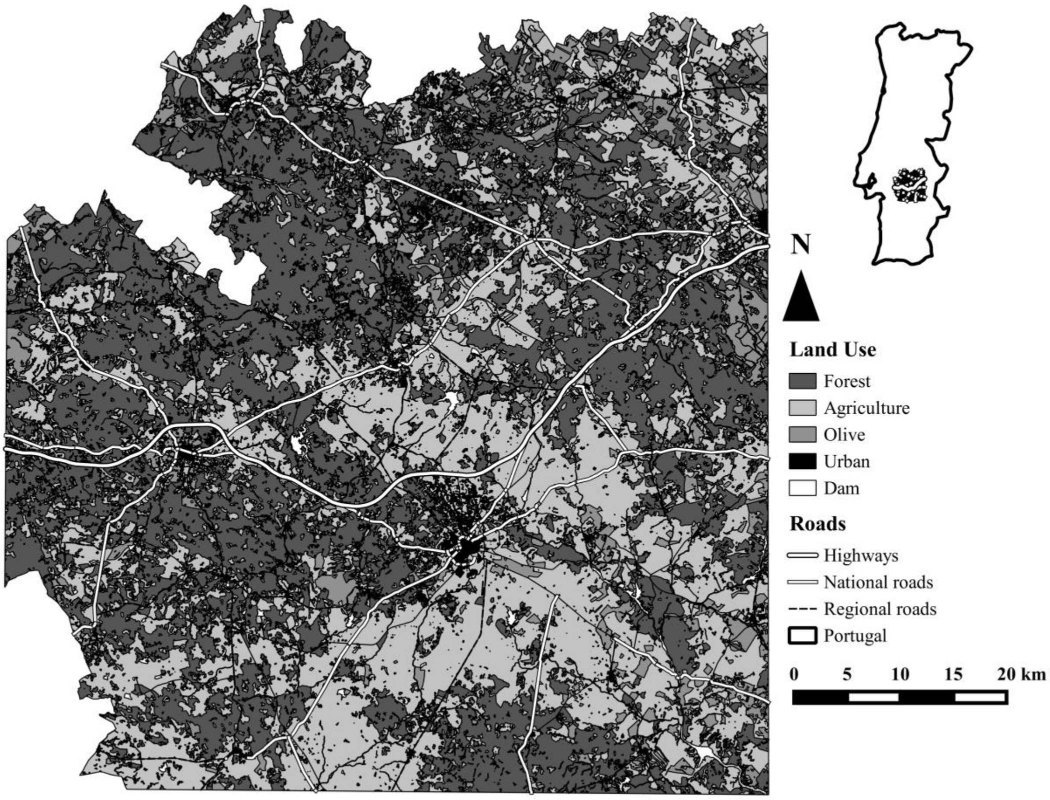

Figure 2 Map of the study area showing the dominant land cover types and the road network.

Model Species

The common genet (*Genetta genetta*) is a suitable species to evaluate the effects of roads on habitat fragmentation and loss and consequently on landscape functional connectivity, due to its habitat preference for forests and riparian galleries (Zabala et al. 2001; Galantinho and Mira 2009; Matos et al. 2009; Pereira and Rodríguez 2010; Santos et al. 2011; Camps and Alldredge 2013; Carvalho et al. 2014). This species is abundant within its range in south-western Europe, and is relatively easy to trap (Ramalho 2009), and to track using telemetry (Pereira and Rodríguez, 2010). Additionally, many biological traits and ecological needs (e.g. habitat use, diet, reproduction, and population dynamics) of genets are well know, allowing to better individualize the effects of roads on its movement and resting behaviours. Common genets (hereafter genets) are small-sized carnivores, weighting (mean ± SE) 1765.8 ± 34.8g in adults (this study). They exhibit nocturnal activity, forage both on the ground and in the tree canopy, and select dense cover for breeding and resting (Palomares and Delibes 1994; Santos-Reis et al. 2004; Camps and Llobet, 2004; Galantinho and Mira 2009; Pereira and Rodriguez 2010). Genets feed mainly on small vertebrates, mostly mammals (Virgós et al. 1999; Rosalino and Santos-Reis 2002). This species is considered of least conservation concern in Portugal (Cabral et al. 2005), and is even expanding into Eastern Europe (Gaubert et al. 2008).

Recent research in the study area showed that genets cross roads at low rates when compared with other small-sized carnivores and established adults (residents) cross roads using mainly dry underpasses (Ascensão and Mira 2007; Grilo et al. 2008; Seronha el al. 2013; MOVE Project, unpublished data). Preliminary data suggests that genets home ranges tend to be limited by major roads (Ramalho 2009), thus contrasting for instance with stone martens (*Martes foina*), which incorporate highways in their home ranges (Grilo et al. 2012; Ascensão et al. 2014). These interspecific differences in the responses towards roads may explain the higher road-related mortality of stone martens when compared to common genets (Grilo et al. 2009; MOVE Project, unpublished data), although this could also reflect differences in species abundance. Thus, knowledge on how different populations and species behave toward roads is thus needed in order to properly manage and conserve small-sized Mediterranean forest carnivores.

Research Objectives

Understanding the drivers of landscape connectivity is a major challenge in modern conservation biology. Recently, the efforts to recognize how organisms move across different landscapes contexts have increased considerably, which allowed the assessment of the landscape features that promote optimal movements for many species (Fahrig 2007).

Although most studies have focused on measuring landscapes connectivity for large carnivores such as wolves (*Canis lupus*), bears and big cats (e.g. Rodríguez-Freire and Crescente-Maseda 2008; Cushman et al. 2013a; Dickson et al. 2013), few empirical examples exist for smaller species (Grilo et al. 2011; Hartmann et al. 2013).

Here, we use a large radiotracking data set, concerning on 27 (14 females and 13 males) genets, collected along 40 months, to characterize genet´s resting and movement behaviours. These data were then combined with genetic data, to assess the effect of main roads on landscape functional connectivity at local and landscape levels.

In particular, we aim to reach the following specific goals:

1. To characterize resting site use by the common genet and identify the main factors driving it;
2. To evaluate how different monitoring frequencies influence the patterns of resting site use by genets, as well as the environmental features underlying those patterns;
3. To assess the influence of roads and habitat heterogeneity on landscape functional connectivity for the genet;
4. To assess the barrier effect of a highway on gene flow, through the combined use of roadkill, movement and fine-scale genetic data.

Thesis Structure

The thesis consists of six Chapters, including a general introduction (Chapter 1), four articles of which two are already published (Chapter 2 and 3), one is under review (Chapter 4) and another is in preparation for publication (Chapter 5), and a general conclusion (Chapter 6).

Chapter 1 includes the scope and background information underlying the research goals.

Chapter 2 focuses on the common genet resting behaviour in Mediterranean oak-managed forests in south Portugal. In this chapter, daily resting site of 21 radio-collared genets were related to environmental factors at microhabitat and home range scale through mixed-effects logistic regressions in dry and wet seasons separately.

In Chapter 3, daily radiotracking data were thinned to evaluate the impact of different sampling regimes on estimates of resting site use.

In Chapter 4, conductance surfaces in real and virtual landscapes were produced using conditional logistic regressions based on path-level analysis. These surfaces were then used to assess the effects of roads and habitat heterogeneity, at both local and landscape scale, on functional connectivity for the genet.

Chapter 5 focuses on the effects of highways on fine-scale genetic structure of the genet. We assessed the possible barrier effects of a highway on population genetic sub structuring by mainly combining radiotracking movement and microsatellite genetic data. Moreover, we also combine roadkills, radiotracking and parentage analysis to evaluate crossing events for resident and dispersers animals.

In Chapter 6, we summarize the main results of the previous Chapters and draw the overall conclusions, including guidelines to conserve and enhance landscape functional connectivity for genets. In addition, we trace several future research issues that should be addressed.

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2. USE OF TREE HOLLOWS BY A MEDITERRANEAN FOREST CARNIVORE

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Carvalho F, Carvalho R, Mira A, Beja P (2014) Use of tree hollows by a Mediterranean forest carnivore. Forest Ecology and Management, 315:54–62.

Use of tree hollows by a Mediterranean forest carnivore

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Abstract

Although tree hollows seem to be key structures for a wide range of forest mammals, their importance for Mediterranean forest carnivores remain poorly understood. Here we address this issue, by analysing daily resting site use by 21 radio-collared common genets. Tree hollows were used far more frequently during the wet season (October-April; 73.1% of daily locations) than in the dry season (May-September; 47.6%). Nests and underground dens were the second and third most frequently used resting sites, respectively, in both wet (17.5% and 9.4%) and dry (34.1% and 18.3%) seasons. Each individual reused a large percentage of its resting sites (65.7 %). Some resting sites (17.3%) were used by more than one individual, but simultaneous sharing was exceedingly rare (0.56% of daily locations). Hollow use probability during the wet season varied little in relation to environmental variables, though there was a tendency to be higher away from riparian habitats (>50m) and to be lower in sites with very high shrub cover (>80%). Environmental influences were responsible for more variability in the dry season, when hollow usage was highest in hot days, in days with precipitation, far from riparian habitats, close to sources of human disturbance, in landscapes dominated by continuous forest habitats, and in sites with low shrub cover. Results support the importance of tree hollows for Mediterranean forest carnivores, probably because they provide safe shelter against unfavourable weather, predators and human disturbance. However, results also revealed the importance of riparian trees, which offer support for building nests close to sources of water and food during the dry season. Considering home range size and the average number of hollow-bearing trees used by each genet, we recommend that management of cork and holm oak forests should strive to safeguard at least 4.6 hollow-bearing trees per 100 ha, while simultaneously maintaining large riparian trees. This will improve the resting habitat for common genets, while presumably favouring also other Mediterranean carnivores.

Keywords: Carnivores, Forest management, Genet, Large old trees, Mediterranean landscape, Tree hollows

Introduction

Tree hollows provide shelter for a range of forest mammals and thus appear to be an essential resource for the persistence of many species (Gibbons and Lindenmayer 2002; Banks et al. 2011). This resource is declining due to modern forestry, which reduces the availability of large and old trees with cavities (Gibbons et al. 2008; Ranius et al. 2009). There is thus increasing interest in understanding how mammals use tree hollows, and how the shortage of hollows can limit their populations (Lindenmayer et al. 2012a). This information is essential for the sustainable management of forests, if these are to maintain habitat conditions for hollow-dependent species (Gibbons et al. 2008; Manning et al. 2013).

Although many mammals are associated with tree hollows in natural forests, in at least some circumstances they may be able to persist despite reductions in hollow availability. For instance, den sharing in social mammals may overcome, at least partly, the shortage of hollows in managed forests (Banks et al. 2013). This strategy may be more difficult for solitary mammals such as most carnivores, where each hollow is generally used by a single individual at a time, with the exception of male and female pairs during the mating season, and of females with its cubs (Zielinski et al. 2004). In forest carnivores it is thus possible that shortage of hollows can be overcome through flexible behaviour allowing animals to use different types of resting sites. At present, however, information is still scarce on the flexibility of rest site use by forest solitary carnivores, and what factors influence such flexibility.

Forest carnivores normally resting in tree hollows can also use a variety of other structures such as underground dens, green nests, raptor nests, rock piles, and human buildings (e.g. Palomares and Delibes 1994; Zielinski et al. 2004; Birks et al. 2005; Slauson and Zielinski 2009; Camps 2011). However, three major resting site types appear to be used most regularly: i) tree hollows, ii) nests and iii) dens (Zalewski 1997b; Santos-Reis et al. 2004; Slauson and Zielinski 2009; Camps 2011). Nests are structures made by carnivores using plant material, which are often located in large tree branches, and are supported by climbing plants (Palomares and Delibes 1994; this study). Dens are mainly located on burrows, often in riverbanks and surrounded by high shrub cover (Slauson and Zielinski 2009). It is uncertain whether these three types of structures, can be used interchangeably, or whether each is associated with a specific set of environmental conditions. Clarifying this issue is important to find out under what circumstances the availability of tree hollows may be limiting for a species.

Several hypotheses may explain variation in resting site use by forest carnivores (Brainerd et al. 1995; Fernández et al. 2002; Birks et al. 2005; Purcell et al. 2009). Tree hollows may be used in more pristine habitats, whereas alternatives may be used in degraded and fragmented forests with few old and large trees (Zielinski et al. 2004, 2006; Manning et al. 2013). Variation may also be related to predation risk, because tree hollows may confer more protection than other rest-site types (Birks et al. 2005; Slauson and Zielinski 2009). For instance, hollows may be used more frequently where understory shrub cover is lower and so predation risk may be higher, especially from avian predators (Zielinski et al. 2004; Popp et al. 2007; Banks et al. 2011). Seemingly, tree hollows may be used more often where human disturbance is high (Brearley et al., 2010; Banks et al. 2011; Bryant et al. 2012). Another possibility is that variation in rest site use is influenced by their capacity to offer protection against weather extremes such as high (or low) temperatures, and heavy rain events (Taylor and Buskirk 1994). Tree hollows are judged to provide microclimate stability, and so they may be used more often under harsh conditions. On the other hand, nests may be a good alternative during hot periods, due to convective heat loss at upper canopy layers, while dens may provide fresh temperatures and insulation protection (Buskirk, 1984; Zabala et al. 2007; Lesmeister et al. 2008; Bryant et al. 2012). Finally, it is possible that rest-sites are used in relation to their proximity to critical resources such as mates, food and water, which greatly influence range use by forest carnivores (Brainerd et al. 1995; Purcell et al. 2009; Weir et al. 2012).

Here we examine factors influencing rest site use by the common genet (*Genetta genetta*) in Mediterranean oak forests. This species is considered a useful model for Mediterranean forest carnivores, because it is abundant within its range in south-western Europe, and it is relatively easy to trap and track using telemetry (Pereira and Rodriguez 2010). Moreover, their predominantly arboreal habits and climbing skills make them suitable to study the importance of tree hollows as resting sites. The genet has a slender body shape, and so it may be affected by energetic constraints under climatic stress similar to those faced by other forest carnivores (Zielinski 2000; Camps and Alldredge 2013). Also, the genet occupies landscapes with a wide range of forest composition and fragmentation levels, making it particularly suited for analysing the effects of habitat conditions on rest-site use. Therefore, we carried out a detailed examination of rest-site use based on intensive radiotracking of genets, aiming to: i) identify the main types of resting sites; ii) estimate seasonal variation in resting site use and iii) quantify variation in tree hollow use in relation to forest composition and fragmentation, resource distribution, predation risk, human disturbance, and weather conditions. Results were used to discuss Mediterranean forest management favouring the availability of resting sites for genets and other forest carnivores.

Material and Methods

Study area and species

The study was conducted in southern Portugal (38°32'24" to 38°47'33" N, 08°13'33" to -07°55'45" W), in an area of about 50,000 ha (Fig. 1). Climate is Mediterranean, with mean daily temperature ranging from 5.8 ºC to 12.8 ºC in winter, and from 16.3 ºC to 30.2 ºC in summer; annual rainfall averages 609.4 mm and is concentrated in October-March (Évora 1971–2000; IPMA, 2012). The relief is undulating (150m-430m above sea level) and the landscape is largely dominated (≈50%) by open to closed cork oak (*Quercus suber*) and holm oak (*Q. rotundifolia*) forests, where the understory may be herbaceous or shrubby depending primarily on grazing pressure. Understory shrubs are also frequently removed mechanically to reduce fire risk and for providing easy access to livestock. Agricultural areas are also important (≈45%), comprising mainly dry arable land and pastureland, with or without sparse oaks trees, and olive orchards and vineyards.

Common genets (*Genetta genetta*), (mean adult weight ± SE: 1765.8 ± 34.8g; this study) are widespread and abundant throughout the study area, though they are mainly associated with forested areas and riparian galleries (Zabala et al. 2001; Matos et al. 2009; Santos et al. 2011). They exhibit nocturnal activity, forage both on the ground and in the tree canopy, and select dense cover for breeding and resting (Palomares and Delibes 1994; Santos-Reis et al. 2004; Camps and Llobet, 2004; Galantinho and Mira, 2009; Pereira and Rodriguez, 2010). Genets feed mainly on small vertebrates, mostly mammals (Virgós et al. 1999; Rosalino and Santos-Reis 2002). The species is considered of least conservation concern in Portugal (Cabral et al. 2005).

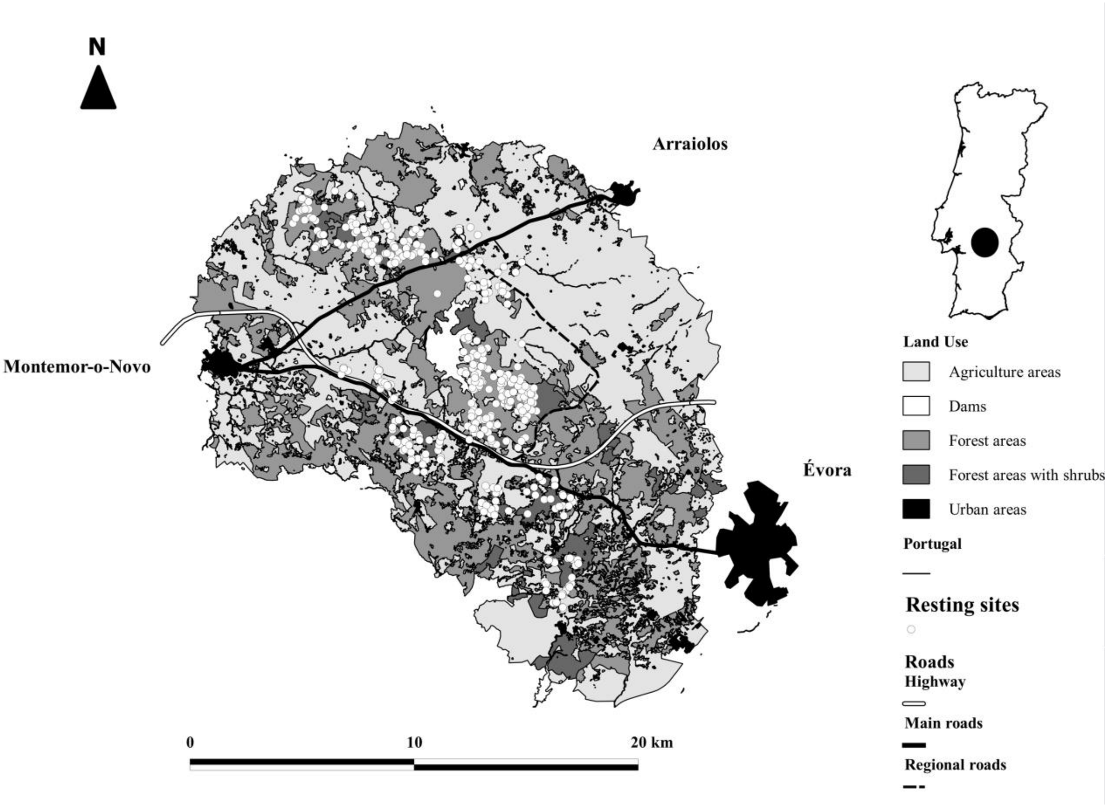


Figure 1 Location of the study area, showing the main habitat types, roads, and spatial distribution of genet resting sites.

Trapping and handling

Trapping was performed from May 2010 to December 2011, except in February-April 2011 and August-September 2011 due to logistic constraints and low capture success (Zabala et al. 2001). Trapping was carried out using 10-15 home-made box-traps (30W x 30H x 90L cm) groups, set at > 500m apart from each other, and baited with sardines in oil, fresh chicken eggs, and road-killed small mammals and passerines. Trap groups were spread all over the study area, in potential genet habitats (e.g. forest areas, riparian areas and shrubland areas; e.g. Zabala et al. 2001; Galantinho and Mira 2009; Santos et al. 2011). Traps were checked every morning after sunrise to minimize animal stress, and bait was replaced whenever needed. Mean ± SE sampling effort in the wet season (October-April) was 33.9 ± 0.8 trap nights (696 total operative trap nights), and in the dry season (May-September) it was 123.0 ± 4.4 trap nights (1190 total operative trap nights).

Once an animal was captured, it was immediately carried to a nearby veterinarian hospital, where the animal was removed from the box, immobilised, and injected intramuscularly with a mix of ketamine hydrochloride (100 mg mL-1) (Imalgene 1000, Lyon, France) and medetomidine hydrochloride (1 mg mL-1) (Domitor, Pfizer, New York, NY, USA) (ratio of 2:1 by volume) at a dosage of 0.12 mL kg-1 (Herr et al. 2010). Animals were weighted, sexed, and checked for sanitary disorders (e.g., parasites). Individuals were classified as juveniles, sub-adults or adults by analysing a combination of morphological traits such as tooth wear, body size, sexual development and overall body condition (Rodriguez-Refojos et al. 2011). All animals were marked with PIT (Passive integrated transponders) tags (model: TXP148511B, 8.5 mm x 2.12 mm, 134.2 kHz ISO, 0.067g, Biomark, Boise, USA) for individual identification. Adults and sub-adults were equipped with radio-collars (≈ 35g) (models: lpm2700A, Wildlife Materials, USA and TW-3, BioTrack, Wareham, UK), if no more than 5% of their weight were added (Sikes et al., 2011). After handling, the individuals were released in the capture location once they had completely recovered their reflexes (1–3 h). Capture procedures and animal handling were in conformity with Portuguese legal regulations.

Study animals and data collection

Overall, 32 genets were trapped 36 times in 25 different trapping places. From these, 31 were fitted with radio-collars and one juvenile female was released without radio-collar. From all animals captured, females comprised 18 (12 adults, 5 sub-adults and 1 juvenile) and males 14 (7 adults and 7 sub-adults). Mean ± SE adult weight for males and females was 1862.9 ± 56.3 g and 1709.2 ± 36.6 g, respectively. The study focused on the 21 genets tracked for at least 30 consecutive days (e.g. Zielinski et al., 2004), including 19 animals tracked in the wet season and 12 in the dry season. Each animal was monitored from the day of release until its death or when the radio-collar batteries finished, averaging (± SE) a monitoring period per animal of 183.2 ± 19.4 days (range: 36 - 389). Ten other animals could not be tracked for sufficiently long periods due to poaching (2 animals), roadkills (2), drowning (1), and radio-collar damage (5).

Resting sites were located by radiotracking through the “homing” techniques, using a BioTrack receiver (model: biotrackSika, BioTrack, Wareham, UK) and an external 3-element Yagi directional antenna (Wildlife Materials, Inc.) (e.g. Millspaugh and Marzluff 2001). Monitoring was performed on a daily basis, except on a few occasions due to logistic constraints. During an overall monitoring period of about 120 weeks, resting locations were checked in average (± SE) 6.4 ± 0.07 days per week. Although animals tend to remain on the same resting site in daytime, we checked the location of each animal at different day hours, whenever it was logistical possible. Once the animal was located in the resting structure, we took a considerable care during the approach in order to not disturb the animal. To avoid wrong locations, a snake inspection camera (model: 8802 AJ GOSCAM, TFT-LCD 2.5 colour monitor) was used several times to guarantee the correct distinction between ground nests vs. dens, and tree hollows vs. nests. All locations (coordinates) were recorded using a GPS unit (model: Garmin Map 62; 2 meter positional accuracy), and then a detailed microhabitat vegetation description was made (see below).

Explanatory variables

Analyses were based on nine explanatory variables reflecting landscape composition and configuration, resource distribution, predation risk, human disturbance, and daily weather conditions (Table 1), which were selected from previous studies reporting factors affecting resting site use by forest carnivores (e.g. Brainerd et al. 1995; Zielinski et al. 2004; Purcell et al. 2009; Slauson and Zielinski 2009; Camps 2011; Banks et al. 2011; Mergey et al. 2011). Landscape composition and configuration were estimated from the proportional cover and fragmentation of forest habitats within 1-km buffers (≈ 314 ha) of each resting site. We focused on forests because we expected the use of tree hollows to increase along with the extent and continuity of forest habitats (Santos-Reis et al. 2004). The buffer size was selected because it was close to genet mean (±SE) home range size in the study area (386.4 ± 40.1ha; minimum convex polygon with 95% of locations [MCPC95]), and it was much larger than the mean core range size (96.0 ± 12.0ha; [MCP50]) (Slauson et al. 2007). Moreover, our daily data suggests that the average distance between resting sites was about 1km (males: 1008.35 ± 95.18 meters; and females: 1022.18 ± 102.89 meters), so we believe that the buffer adopted in our study is likely to reflect a coarse spatial scale of resting site selection by common genets. Landscape composition was estimated in a Geographic Information System (Quantum GIS Development Team, 2011), based on detailed land cover maps prepared using digital aerial photographs from 2011 and field surveys. Landscape metrics were estimated using the Patch Analyst extension (Rempel and Carr 2003) for ArcView 3.2 (ESRI 1999).

Detailed information on the distribution of genet key resources (e.g. prey density) was unavailable, and so it could not be used to analyse the correlates of rest-site use. However, we used the distance to riparian habitats as a proxy for resource distribution, because they may be important sources of food and water, particularly during the hot and dry season (Matos et al., 2009). Likewise, detailed information on predation risk was unavailable, though it is likely that several predators occurring in the study area may kill or otherwise influence the behaviour of genets, including for instance domestic dogs (*Canis familiaris*), foxes (*Vulpes vulpes*), and eagle owls (*Bubo bubo*) (Larivière and Calzada 2001; Filipe Carvalho, Unpublished Data). We used as a surrogate of predation risk the proportional cover by shrubs, estimated through detailed vegetation mapping within 25 m of each rest-site (e.g. Jerosch et al. 2010). Predation risk was assumed to vary inversely with shrub cover (Popp et al. 2007; Ordiz et al. 2011; Ross et al. 2012). The distance of rest-sites to houses and roads was used as a surrogate of human disturbance. Weather conditions were described from the maximum daily temperature and daily precipitation, using data collected in the nearest weather station (Mitra; CGE 2012).

Table 1 Description and summary statistics of explanatory variables used to analyse rest site selection by the genet in Southern Portugal.

Variables (Abbreviation)	Description (transformation)	Wet Season		Dry Season	
		Mean±SE	**Range**	**Mean±SE**	**Range**
Landscape composition and configuration (within 1 km of rest site)					
Forests	Proportional cover by forests (>30% tree canopy density) (Asin[√x])	0.51±0.007	0.0-0.95	0.44±0.007	0.02-0.95
Forests with shrubs	Proportional cover by forests (>30% tree canopy density) with understory shrubs (Asin[√x])	0.18±0.003	0.0-0.76	0.30±0.006	0.0-0.76
Forest patch Size	Mean patch size (ha) of forest habitats (log10)	130.2±2.1	3.5-304.3	146.3±3.1	4.4-307.2
Forest edges	Density of (m/ha) of edges between forests and other habitats (log10)	49.8±0.4	17.4-120.5	55.4±0.7	26.4-113.0
Resource distribution					
Riparian	Distance (m) to the nearest riparian habitat (log10)	258.3±5.3	0.2-1210.4	175.0±6.9	0.1-848.2
Predation risk					
Shrub cover	Proportional cover by shrubs within a 25 m of each rest site (Asin(√x))	0.49±0.009	0.0-1.0	0.67±0.01	0.0-1.0
Human disturbance					
Human disturbance	Distance (m) of rest site to the nearest source of human disturbance (e.g., houses and roads) (log10)	846.9±12.2	15.3-2263.0	723.5±13.5	30.9-2263.0
Weather					
Maximum temperature	Maximum daily temperature (ºC) (log10)	16.1±0.1	5.7-33.4	28.8±0.1	14.5-39.5
Precipitation	Daily precipitation (mm) (Log10)	2.0±0.09	0.0-32.8	0.5±0.08	0.0-41.9

Data analysis

Prior to statistical analyses, skewed variables were transformed to approach normality and to reduce the influence of extreme values, using either angular or logarithmic transformations (Table 1). All variables were then standardised to zero mean and unit variance, to enhance comparability of effect sizes (e.g. Schielzeth 2010). Collinearity was investigated using pairwise correlations, and one variable from each pair correlated at >0.7 was eliminated from further analyses (Dormann et al. 2013). All statistical analyses were performed using the R statistical package (R Project for Statistical Computing release 2.15.3 http://www.r-project.org).

The use of hollows versus alternative resting site types was coded as a binary variable (1/0), and related to explanatory variables using generalised linear mixed effects models (GLMM), with binomial errors and logit link (Zuur et al. 2009). Wet and dry season data were modelled separately, because preliminary analysis pointed out marked seasonal differences in resting site use. On the other hand, the two sexes were considered together because no significant effects were found in exploratory univariate analyses specifying sex as a binary variable in the fixed component of the GLMM model, in both the wet ($p = 0.489$) and the dry season ($p = 0.642$). Only tree hollows were explicitly modelled, because they accounted for most rest-sites recorded (65.1% out of 497), while each of the alternative types accounted for less than 25% of the rest-site and so they did not allow for separate analysis. In GLMMs, the animals were always included as random effects, to account for lack of independence resulting from eventual individual idiosyncrasies in resting site use. Model building of the fixed component was based on the information theoretic approach, and inference was based on model averaging (Burnham and Anderson 2002). Candidate models were built based on all possible subsets of non-collinear explanatory variables, including the null (i.e., without explanatory variables) and the full (i.e., with all explanatory variables) models. Models were ranked according to their Akaike weights (w_i), and the average parameters and their unconditional standard errors (SE) were estimated based on the 95% confidence set of models (Burnham and Anderson, 2002). The relative importance of each variable was judged based on the sum of Akaike weights of models where the variable was included (w_+), and on the magnitude of the average model coefficient. GLMMs were fitted using the package lme4 (Bates et al. 2013), and multimodel inference was implemented using MuMIn (Barton 2013).

Collinearity in model building was assessed by computing the variance inflation factor (VIF) of each variable in the full model, assuming that problems may occur if VIF > 3 (Zuur et al. 2009). Model adequacy was evaluated by plotting the residuals of the average model against the fitted values and each of the variables presented in the final model (Zuur et al. 2009). Model discrimination ability was assessed using the area under the remote operating characteristic curve (AUC), with values above 0.9 taken to indicate excellent accuracy (Rapacciuolo et al. 2012). To check for eventual autocorrelation problems, we computed the Moran´s I based on spline correlograms for the raw binary response variable and the residuals from the average model, using the “spline.correlog” function with 1000 permutations from the R package “ncf” (Bjornstad 2012). Additionally we run a mantel test of overall autocorrelation between the response variable and model residuals against distance between resting sites (Urban 2003).Temporal autocorrelation was inspected by plotting the autocorrelation function (ACF) with the model average residuals vs. the lag between monitoring days (Zuur et al. 2009), using the “acf” function from the R package “nlme” (Pinheiro et al. 2012).

Results

Resting site use

A total of 497 different resting sites were used by the 21 genets tracked during 28 months. Most sites (82.7%) were used by a single individual (not shared), while the others were shared by 2 or 3 individuals. Simultaneous use of the same rest-site by two individuals occurred rarely (0.56% out of 3334 daily locations) and involved a small proportion of the rest-sites (2.8%). The simultaneous use of resting sites occurred among the following pairs: adult male/adult female, adult female/sub-adult male, adult male/sub-adult male, and two sub-adults male/female. Individuals used from 12 to 45 different resting sites, corresponding to an average (±SE) of 28.2 ± 2.3 rest-sites per individual. Resting sites reuse (percentage of resting sites used more than once by an animal) occurred quite often, both in wet (63.9 % ± 2.8) and dry (54.7 % ± 2.9) seasons. Reuse of rest-sites occurred predominately in tree hollows (60.9%), followed by nests (22.6%) and dens (16.5%).

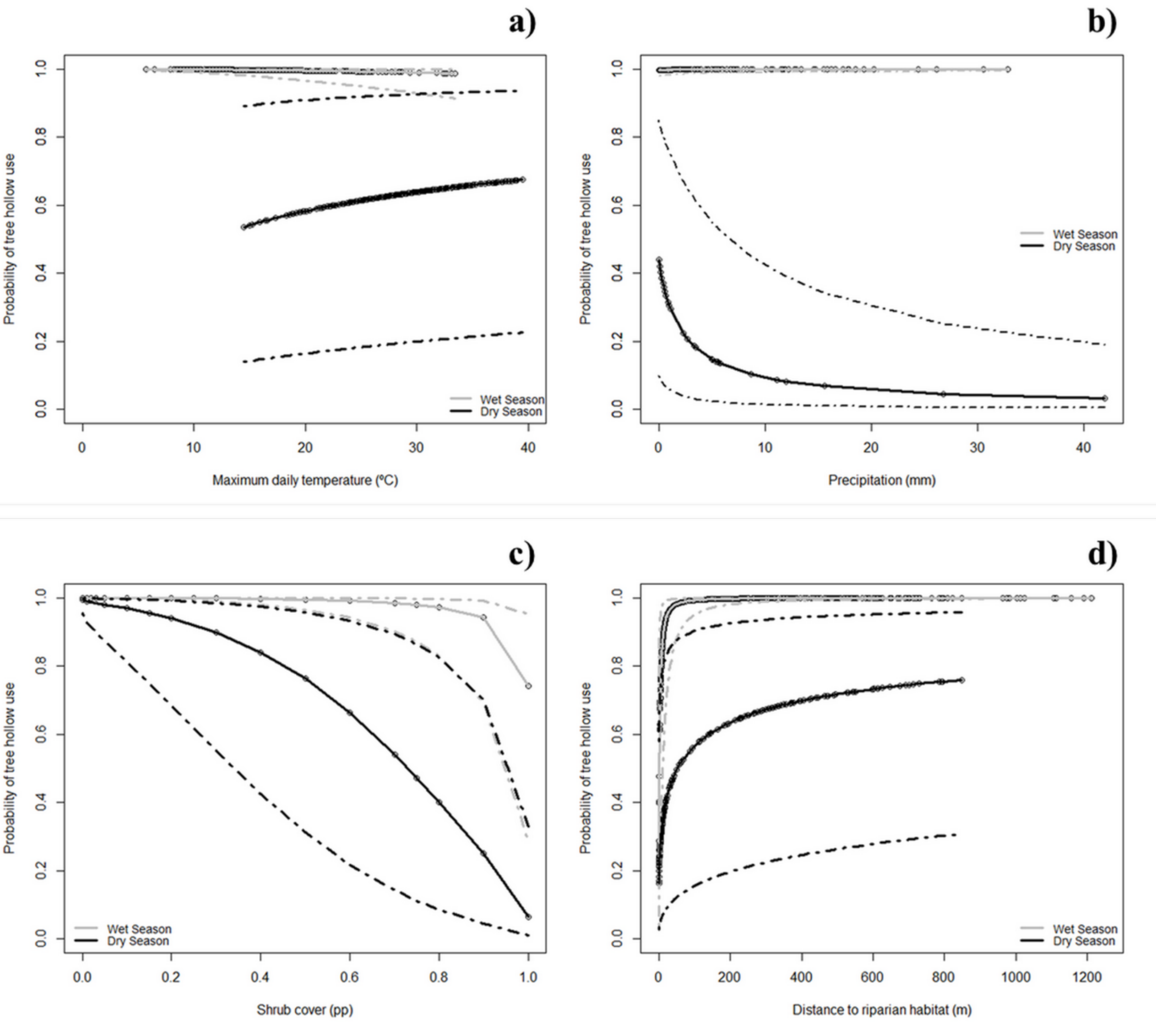
Genets rested most often in tree hollows (65.1%), which were mainly located in cork oaks (50.6%), and holm oaks (42.5%), while ash (*Fraxinus angustifolia*), poplar (*Populus* spp.), and large and old olive trees (*Olea europaea*) were used rarely (6.9%). In average (±SE), tree hollows were located 3.5 ± 0.1 meters above the ground. Nests were the second most frequent resting site (22.7%). Most nests were located above the ground (84.2%), at an average height (±SE) of 3.4 ± 0.4 meters, and they corresponded to vegetation beds made with grass and leafs supported by tree branches (most ash and poplar trees) and/or climbing plants. Nests at ground level were always under shrub cover and they were similar to grass beds. Dens (11.2 % of resting sites) were found in riverbanks under high shrub cover, and they were mainly located on burrows excavated by other animals (97.9%), or under tree roots. Most burrows had been excavated by rabbits (*Oryctolagus cuniculus*), though genets were also found in a few occasions in unoccupied red fox and badger (*Meles meles*) burrows. Other types of resting sites were used rarely (1.0%), including rock piles and one habited farmhouse. From 2291 daily locations recorded during the wet season, there were 73.1% in tree hollows, 17.5% in nests, and 9.4% in dens. In contrast, from 1043 daily locations during the dry season, there were 47.6% in tree hollows, 34.1% in nests, and 18.3% in dens.

Correlates of resting site use

The only pairwise correlations >0.7 were between forest cover and mean patch size (r = 0.88 and 0.82, using wet and dry season data, respectively), and between forest edge density and mean patch size (0.71; dry season). Therefore, mean patch size was removed from subsequent analysis to reduce collinearity problems. The GLMM model developed for the wet season indicated that the use of tree hollows prevailed across the range of variation of the explanatory variables (Fig. 2), though the probability of tree hollow use increased to some extent with distance to riparian habitats and to human disturbance, with forest cover and edge density, and with precipitation (Table 2). Hollow use declined with shrub cover around resting sites, forest with shrubby understory, and maximum daily temperature (Table 2). The strongest effects observed, were the declines in tree hollow use in areas very close (<50m) to riparian habitats and high local cover (>80%) by shrubs (Fig. 2d and c). The wet season model had high discrimination ability (AUC = 0.944) (Supplementary Figure S4a), and it did not show any problems resulting from collinearity (VIFs<3), outliers or extreme values and deviations from linearity assumptions in the logit scale. Although there was high spatial autocorrelation in the raw binary variable, this was no longer observed in the correlogram of average model residuals (Supplementary Figure S1) and in the Mantel test (p = 0.17). Temporal correlogram suggested that residuals were correlated for resting sites monitored in the same day, but correlations declined to close to zero afterwards (Supplementary Figure S3a). There were also significant correlations in the residuals for time lags of 13 and 26 days.

During the dry season, the GLMM suggested that variation in the probability of tree hollow use in relation to explanatory variables was much larger than in the wet season

(Fig. 2). The probability of genets using tree hollows increased with distance to riparian habitats and forest cover, and it declined with forest edge density, local shrub cover, distance to human disturbance, and precipitation (Table 2). Temperature and cover by forests with shrubs had low selection probabilities (w_+ <0.35), and so their effects were very little supported (Table 2). The dry season model had high discrimination ability (AUC = 0.901) (Supplementary Figure S4b), and it did not show any problems resulting from collinearity (VIFs<3), outliers or extreme values, and deviations from linearity assumptions in the logit scale. Although there was high spatial autocorrelation in the raw binary variable, this was no longer observed in the correlogram of average model residuals (Supplementary Figure S2) and in the Mantel test (p = 0.37). The temporal correlograms suggested that residuals were correlated for resting sites monitored in the same day and, to a much lesser extent, for time lags of one and two days (Supplementary Figure S3b).



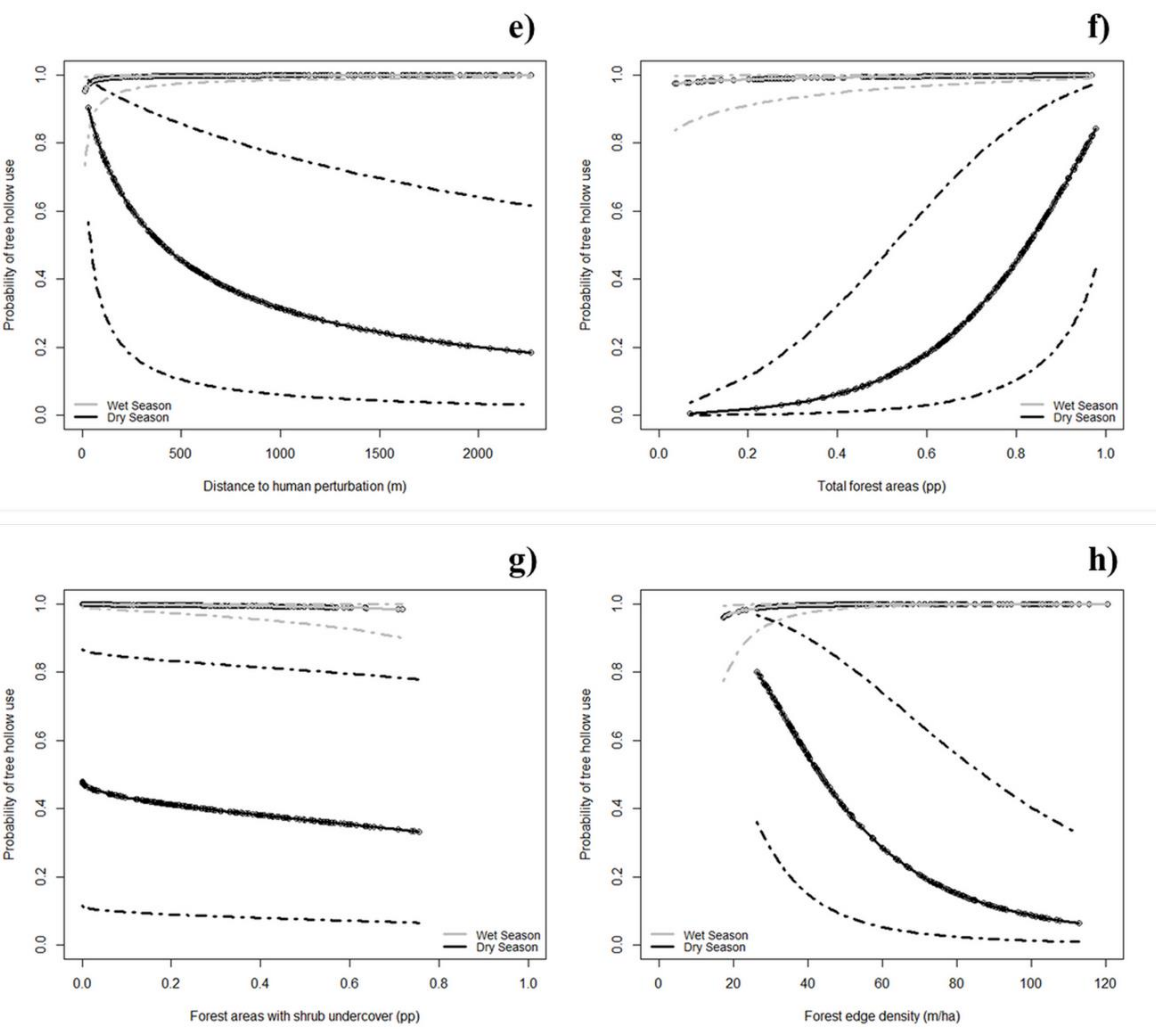


Figure 2 Probability of tree hollow use (with 95% confident limits) predicted from dry and wet season average GLMM models (see Table 2), in relation to: (a) maximum temperature, (b) precipitation, (c) shrub cover, d) distance to riparian habitat, (e) human disturbance, (f) forest cover, (g) cover by forest with shrubs, and (h) forest edge density. For each variable and season, response to one variable was extracted from the model by maintaining all the other variables at their median values. Models were fitted on transformed and standardized data, but are depicted on the original scale.

Table 2 Average models describing the estimated effects of explanatory variables on the probability of tree hollow use by genets in the wet and dry seasons. For each season, multi-model averaging was based on the 95% confidence set of models. For each variable, we show the standardised regression coefficient (β), the unconditional standard errors (*SE*), the 95% confidence interval of coefficient estimate (*CI*), and the selection probability (w_+). Variables selected in the best models are underlined and coefficient estimates whose 95%CI exclude zero are in bold.

Wet Season				
Parameters	**β**	**SE**	**CI**	w_+
Intercept	**4.469**	**0.981**	**(2.546, 6.392)**	-
Maximum temperature	**-0.562**	**0.130**	**(-0.817, -0.307)**	1.000
Precipitation	**0.298**	**0.114**	**(0.075, 0.522)**	0.940
Shrub cover	**-3.209**	**0.271**	**(-3.742, -2.678)**	1.000
Riparian	**2.315**	**0.243**	**(1.839, 2.791)**	1.000
Human disturbance	**0.662**	**0.229**	**(0.215, 1.110)**	1.000
Forests	**0.633**	**0.285**	**(0.075, 1.191)**	0.820
Forests with shrubs	**-0.649**	**0.331**	**(-1.297, -0.002)**	0.730
Forest edges	**0.988**	**0.304**	**(0.392, 1.583)**	1.000
Dry Season				
Parameters	**β**	**SE**	**CI**	w_+
Intercept	0.286	0.993	(-1.661, 2.232)	-
Maximum temperature	0.103	0.153	(-0.198, 0.403)	0.320
Precipitation	**-0.398**	**0.156**	**(-0.704, -0.092)**	0.930
Shrub cover	**-2.679**	**0.260**	**(-3.188, -2.169)**	1.000
Riparian	**0.788**	**0.179**	**(0.438, 1.138)**	1.000
Human disturbance	**-0.671**	**0.250**	**(-1.161, -0.181)**	1.000
Forests	**1.127**	**0.321**	**(0.498, 1.756)**	1.000
Forests with shrubs	-0.151	0.254	(-0.650, 0.348)	0.310
Forest edges	**-1.181**	**0.477**	**(-2.215, -0.247)**	0.890

Discussion

Our study evidenced the importance of tree hollows as key resting sites for genets inhabiting Mediterranean oak forest. Tree hollows were particularly used during the wet season, whereas alternative resting sites (nests and dens) were also heavily used during the dry season. In line with expectations, explanatory variables reflecting landscape composition and configuration, resource distribution, predation risk, human disturbance and weather affected the probability of tree hollow use, though these effects were most marked during the dry season. Overall, our daily based results support the view that tree hollows may be an important resource for Mediterranean solitary forest carnivores, in line with observations for other forest carnivores elsewhere (e.g. Brainerd et al. 1995; Zalewski 1997a, 1997b; Zielinski et al. 2004; Isaac et al. 2008; Purcell et al. 2009; Slauson and Zielinski 2009; Camps 2011; Weir et al. 2012).

Resting site selection

The use of tree hollows versus that of alternative resting site types (i.e., nests and dens) was significantly related to the explanatory variables analysed, but the strength and shape of the relationships varied considerably across seasons, suggesting that the mechanisms of resting site selection may change across the annual cycle. During the wet season, genets were found almost exclusively in tree hollows, with variation in the explanatory variables only causing very small changes in hollow use probability. There was thus evidence for a largely inflexible resting site selection strategy, suggesting that during the wet season tree hollows may be more suitable for genets than the alternative resting site types. Reasons for this are uncertain, but they may be related to tree hollows providing relatively stable microclimates and dry conditions in a period when temperatures are relatively low, and there is high probability of precipitation. This view is supported by other studies suggesting that unfavourable climate conditions during the winter are among the key factors affecting resting site selection by forest carnivores (Taylor and Buskirk 1994; Isaac et al. 2008). Another possibility is that resting site selection was driven by human disturbance (e.g. Ordiz et al. 2011), because hunting in the region occurs through most of the wet season. Although genets are not legally hunted, it is possible that animals prefer to shelter in more secluded sites such as tree hollows when there are hunters and their dogs in the vicinity. Despite the dominant use of hollows, however, genets used alternative resting sites more frequently in areas with very high shrub cover (> 80%), and very close (< 50m) to riparian habitats. These relationships are similar to those observed during the dry season, albeit less pronounced, and may reflect predation risk avoidance and resource distribution (see below).

In contrast to the wet season, there was a high flexibility in resting site use during the dry season, with variation in several explanatory variables causing major changes in hollow use probability. Hollows were used most frequently in hot days, in days with precipitation, far from riparian areas, close to sources of human disturbance, in landscapes dominated by forest habitats and with low forest edge density, and in sites with low shrub cover. The relationships with temperature and precipitation supported the importance of climate conditions on resting site selection, suggesting that tree hollows provided insulation against extreme temperatures, and dry conditions during rainy days (Taylor and Buskirk 1994; Weir et al. 2004; Isaac et al. 2008). The higher use of tree hollows close (< 500m) to sources of human disturbance (e.g., roads, farmhouses) also supported the view that hollows may be particularly suitable to offer secure shelter from human activities (e.g. Ordiz et al. 2011). Tree hollow availability was probably another factor affecting resting site selection, as suggested by the higher usage of hollows in landscapes with more cover and less fragmentation of forests. The higher usage of alternative resting sites in areas with high shrub cover is in line with the prediction that resting site selection should reflect predation risk avoidance (Purcell et al. 2009; Slauson and Zielinski 2009; Broekhuis et al. 2013). In fact, shrub cover probably reduced predation risk due to the lower accessibility by flying raptors and owls, and poorer scent detectability for other ground carnivores such as foxes and dogs (Popp et al. 2007; Slauson et al. 2007; Mangas et al. 2008; Ordiz et al. 2011; Caryl et al. 2012), thereby relaxing the need to find safe shelter in tree hollows. Close to riparian areas genets were found resting primarily in nests and, to a much lesser extent, in underground burrows, suggesting that riparian trees may offer little shelter in hollows. The use of these trees was probably related to the distribution of key resources such as water and food, which are largely concentrated in riparian habitats during the Mediterranean dry season (Matos et al. 2009; Sabino-Marques and Mira 2011).

Forest management implications

Results from this study supported the view that the conservation of carnivores in Mediterranean forests may require the preservation of hollow-bearing trees, as it has been shown for a range of forest mammals elsewhere (e.g. Brainerd et al. 1995; Zalewski 1997b; Zielinski et al. 2004; Isaac et al. 2008; Purcell et al. 2009; Slauson and

Zielinski 2009; Camps 2011; Weir et al. 2012). In particular, the study underlined the need to maintain large and old oak trees, both cork and holm oaks, which accounted for the vast majority of genet rest sites recorded. This idea is supported by a previous study showing a strong association between genet latrines and large oak trees in Mediterranean forests (Espirito-Santo et al. 2007). Although these results derived from the study of a single species, there is some evidence that they may apply to other carnivores (e.g. Zalewski 1997b; Santos-Reis et al. 2004; Birks et al. 2005), including for instance the critically endangered Iberian lynx (*Lynx pardinus*) (Fernández et al. 2002) and the endangered wild cat (*Felis sylvestris*) (Jerosch et al. 2010).

Considering the average number (± SE) of hollow-bearing trees used by each genet (18 ± 3.4), and their average home range size (386.4 ± 40.1 ha), we suggest that a minimum of 4.6 hollow-bearing trees per 100 ha should be maintained in cork and holm oak forests, though further research is needed to assess whether the same threshold applies in other habitat types. Even in cork and holm oak forests, there may be variation in the density of tree hollows required by genets, depending on local environmental conditions and management practices. For instance, management aiming to reduce fire hazard involves the widespread clearing of undergrowth woody vegetation (Santana et al. 2011), which may reduce protection against predators and thus increase the use of tree hollows. Also, higher tree hollow densities may be required due to human disturbance, as anecdotal information suggested that some potentially suitable trees may become temporally avoided by genets due to silvicultural operations such as cork stripping and pruning. Finally, it should be stressed that a much larger density than that identified in this study is probably required to maintain the full variety of species associated with hollow-bearing trees, such as reptiles, birds and bats (e.g. Gibbons and Lindenmayer 2002; Fischer et al. 2010), though information to confirm this is largely lacking for Mediterranean forests. In fact, little is known about the typical densities of hollow-bearing trees in Mediterranean forest landscapes and the factors affecting these, and about the densities required by different species of vertebrates. Clearly, these issues require further research in order to inform the sustainable management of Mediterranean oak forests (Gibbons et al. 2008; Lindenmayer et al. 2012b).

This study also identified large trees along riparian galleries as another important resource for genets, which used them as support for nest building, particularly during the dry season. This result is in line with other studies on Mediterranean carnivores, which have shown a heavy use of riparian areas (Virgós 2001; Matos et al. 2009; Santos et al. 2011). Large riparian trees probably provided safe microclimatic refuge, and allowed the efficient exploitation of water and food resources that are concentrated along stream and river margins during the dry and hot summer season (Ruggiero et al. 1998; Zielinski et al. 2004; Pereira and Rodriguez 2010; Santos et al. 2011). Hollow-bearing oaks and large riparian trees may thus be complimentary resources, both of which are needed to assure habitat conditions for genets across the annual cycle. Maintenance of large riparian trees may thus be important for the conservation of genets and eventually other Mediterranean carnivores (Fernández et al. 2002; Birks et al. 2005; Camps 2011), further adding to the importance of riparian forests for biodiversity conservation in Mediterranean landscapes (Gibbons and Lindenmayer 2002; Pereira and Rodriguez 2010; Lindenmayer et al. 2012a).

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Supplementary Material

Use of tree hollows by a Mediterranean forest carnivore

Table S1 The 95% confidence set (wi sum ≥ 0.95) of top models for wet and dry season. Parameters: (a) maximum temperature, (b) precipitation, (c) shrub cover, d) distance to riparian habitat, (e) human disturbance, (f) forest cover, (g) cover by forest with shrubs, and (h) forest edge density.

Wet season

Model Rank	Parameters	df	logLik	AIC	ΔAIC	wi	Deviance explained (%)
1	abcdefgh	10	-284.00	588.00	0.00	0.56	60.6
2	abcdefh	9	-286.04	590.08	2.08	0.20	60.3
3	abcdegh	9	-286.40	590.79	2.79	0.14	60.2
4	abcdeh	8	-288.52	593.03	5.04	0.04	59.9
5	acdefgh	9	-287.64	593.28	5.28	0.04	60.1
6	acdefh	8	-289.18	594.35	6.35	0.02	59.8

Dry season

Model Rank	Parameters	df	logLik	AIC	ΔAIC	wi	Deviance explained (%)
1	bcdefh	8	-217.60	451.19	0.00	0.40	51.3
2	abcdefh	9	-217.40	452.80	1.61	0.18	51.3
3	bcdefgh	9	-217.44	452.88	1.69	0.17	51.3
4	abcdefgh	10	-217.26	454.53	3.33	0.08	51.4
5	bcdef	7	-220.68	455.36	4.17	0.05	50.6
6	bcdefg	8	-220.27	456.53	5.34	0.03	50.7
7	acdefh	8	-220.33	456.67	5.47	0.03	50.7
8	cdefh	7	-221.46	456.92	5.72	0.02	50.4
9	abcdef	8	-220.56	457.12	5.92	0.02	50.6
10	abcdefg	9	-220.16	458.32	7.13	0.01	50.7
11	acdefgh	9	.220.17	458.34	7.15	0.01	50.7
12	cdedgh	8	-221.22	458.44	7.25	0.01	50.5

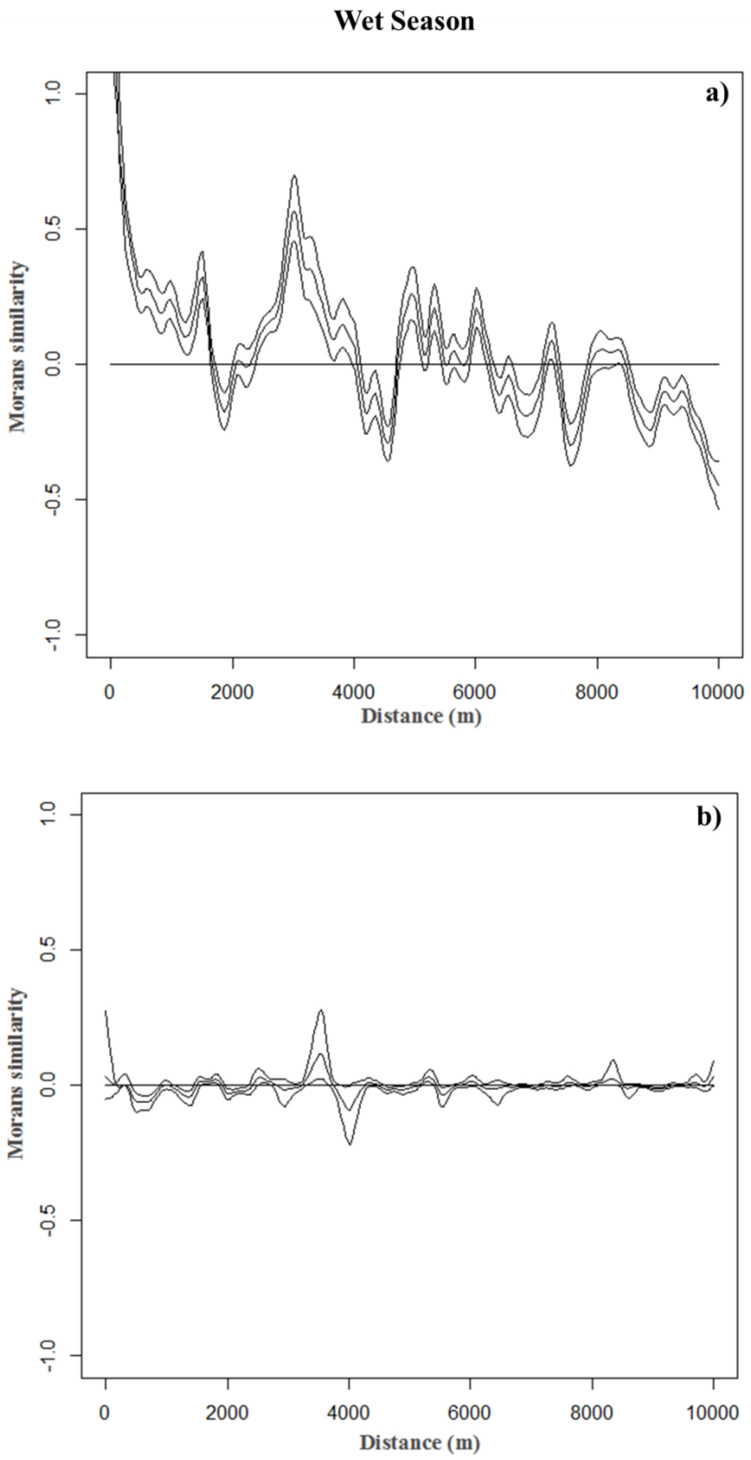

Figure S1 Wet season spline correlograms with 95% pointwise bootstrap confidence intervals, showing spatial dependency as measured by Moran's similarity index with increased distance (m) around each resting site for (a) presence/absence data (spatial points); and (b) the model average residuals.

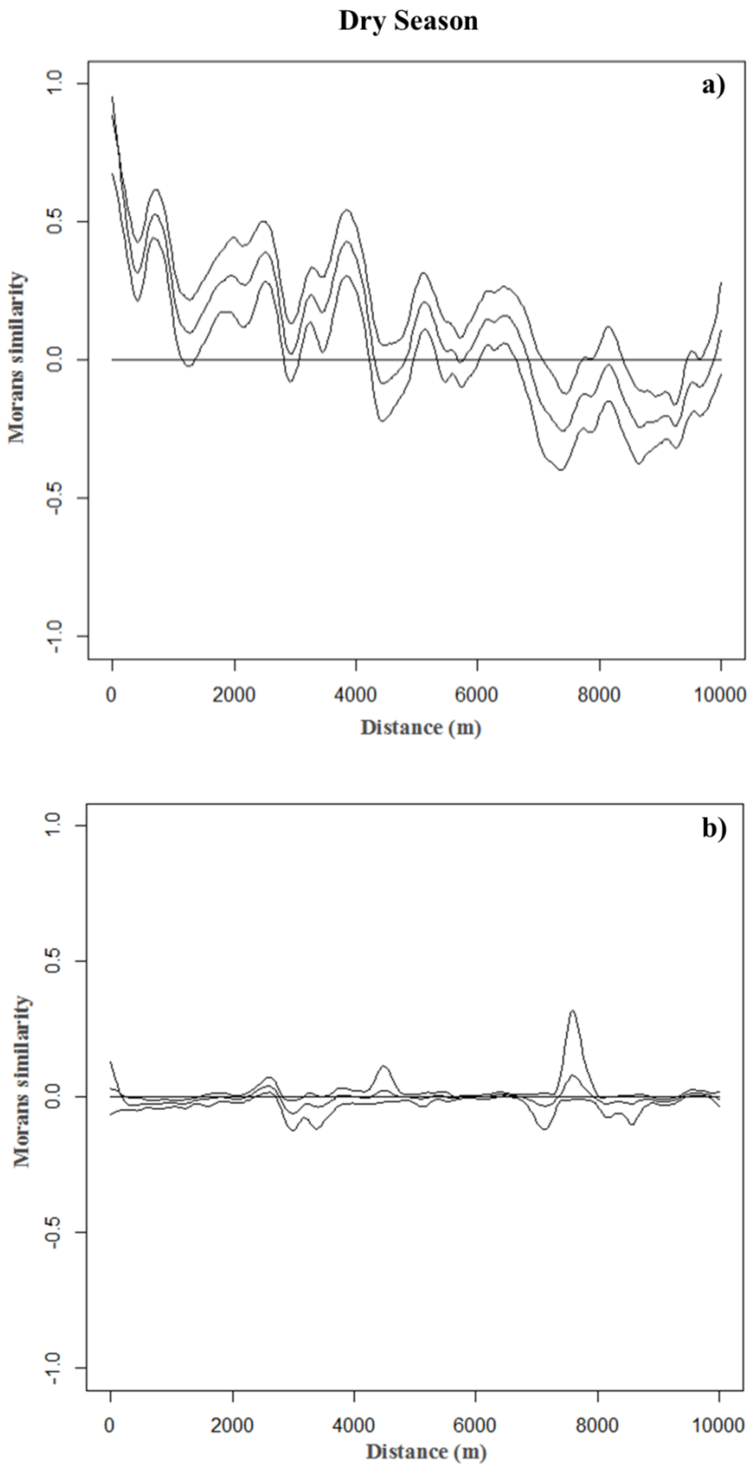

Figure S2 Dry season spline correlograms with 95% pointwise bootstrap confidence intervals, showing spatial dependency as measured by Moran's similarity index with increased distance (m) around each resting site for (a) presence/absence data (spatial points); and (b) the model average residuals.

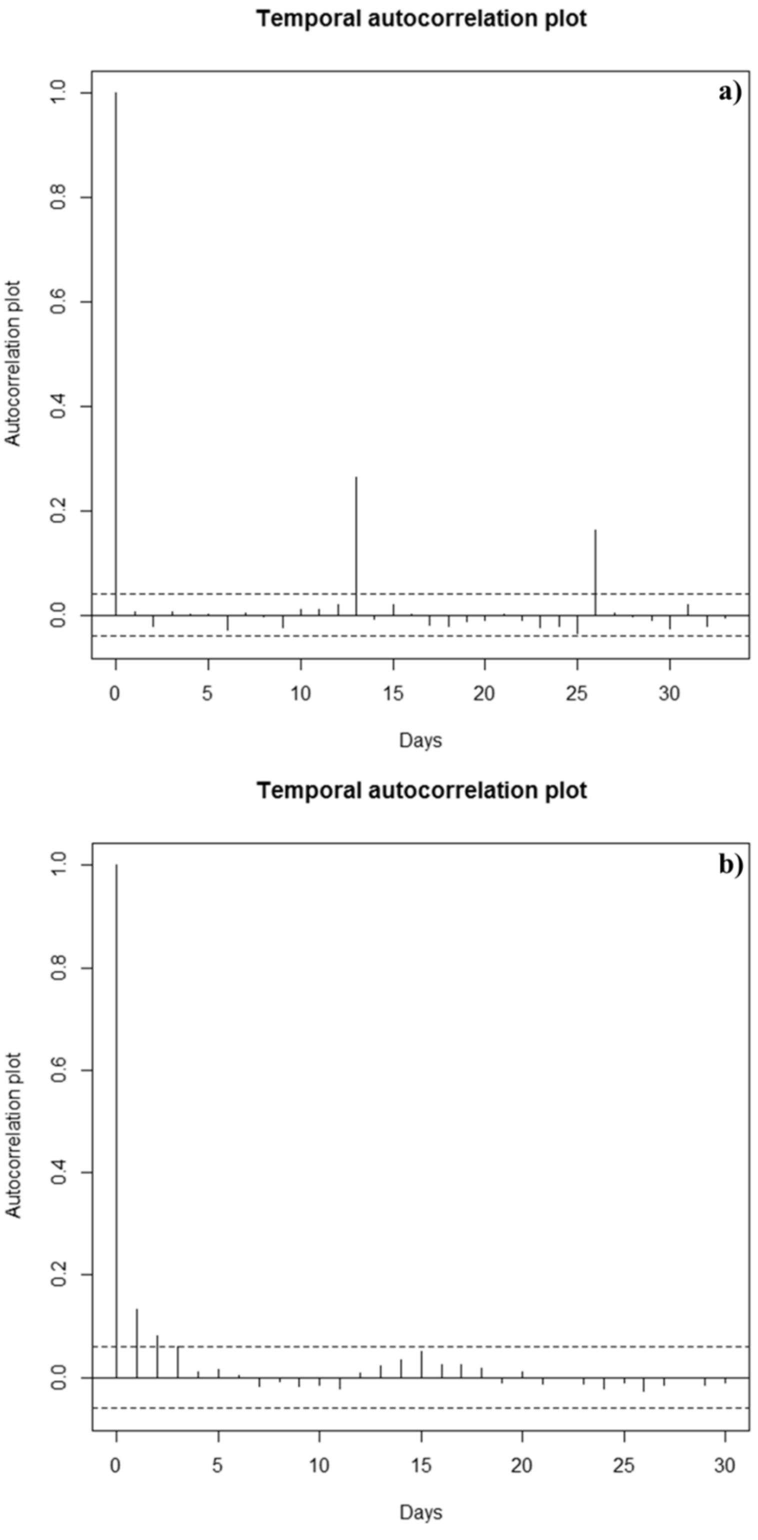

Figure S3 Auto-correlation function (ACF) of the common genet daily resting site monitoring. The horizontal axis shows the time lags and the vertical axis the correlation. The dotted line represents the 95% confidence bands. a) Model average residuals for wet season; b) Model average residuals for dry season.

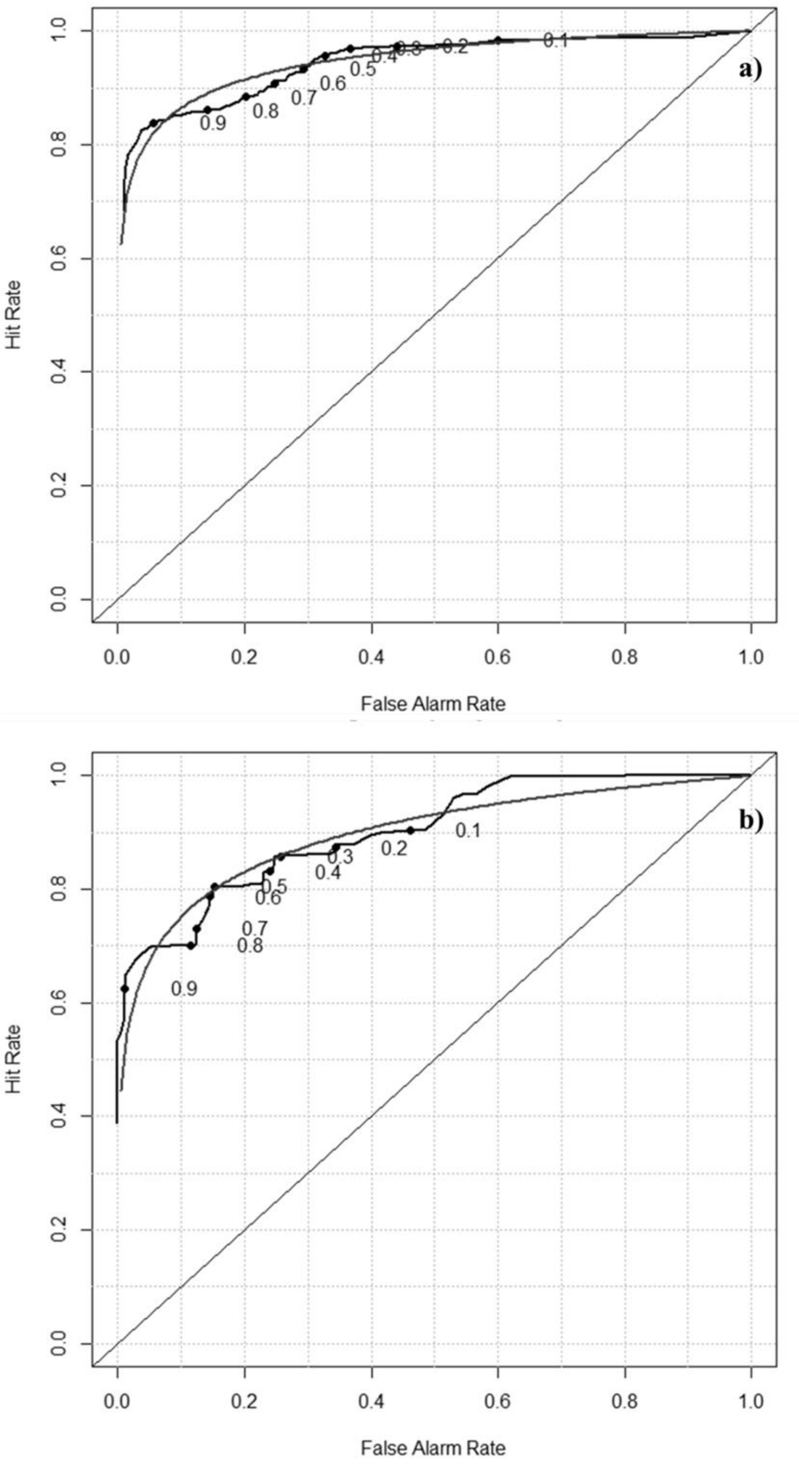

Figure S4 Area under the curve (AUC) obtained from the model average residuals for (a) wet season 0.944: and (b) dry season 0.901.

3. MONITORING FREQUENCY INFLUENCES ANALYSIS OF RESTING BEHAVIOUR IN A FOREST CARNIVORE

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Monitoring frequency influences the analysis of resting behaviour in a forest carnivore

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Abstract

Resting sites are key structures for many mammalian species, which can affect reproduction, survival, population density, and even species persistence in human-modified landscapes. As a consequence, an increasing number of studies has estimated patterns of resting site use by mammals, as well as the processes underlying these patterns, though the impact of sampling design on such estimates remain poorly understood. Here we address this issue empirically, based on data from 21 common genets radiotracked during 28 months in Mediterranean forest landscapes. Daily radiotracking data was thinned to simulate every other day and weekly monitoring frequencies, and then used to evaluate the impact of sampling regime on estimates of resting site use. Results showed that lower monitoring frequencies were associated with major underestimates of the average number of resting sites per animal, and of site reuse rates and sharing frequency, though no effect was detected on the percentage use of resting site types. Monitoring frequency also had a major impact on estimates of environmental effects on resting site selection, with decreasing monitoring frequencies resulting in higher model uncertainty and reduced power to identify significant explanatory variables. Our results suggest that variation in monitoring frequency may have had a strong impact on intra- and interspecific differences in resting site use patterns detected in previous studies. Given the errors and uncertainties associated with low monitoring frequencies, we recommend that daily or at least every other day monitoring should be used whenever possible in studies estimating resting site use patterns by mammals.

Keywords: carnivores, data thinning, forest ecology, resting sites, sampling regime

Introduction

Resting sites are key structures for many mammalian species, by providing shelter against predators, thermoregulatory stability and secure access to resources (Brown et al. 2014; Carvalho et al. 2014). The spatial distribution of resting sites influences important aspects of mammalian biology, including the use of foraging habitats, reproduction success, social behaviour and population size (Banks et al. 2011; Weir et al. 2012). In recent years it has become apparent that human activities often reduce the availability of adequate resting sites, which in turn can affect the ability of species to persist in human-modified landscapes (Lindenmayer et al. 2012a; Manning et al. 2013). As a consequence, there has been increasing interest in characterising the resting ecology of mammals, in order to identify species most at risk and devising appropriate management solutions (Birks et al. 2005).

A key aspect of mammalian resting ecology is the typology of resting sites used, including for instance tree hollows, underground dens, beds in dense shrubby vegetation, and arboreal nests, which may influence species vulnerability to human activities. For instance, species requiring tree hollows are vulnerable to silvicultural practices reducing the abundance of large and old trees (Gibbons and Lindenmayer 2002; Lindenmayer et al. 2014), while species building their own resting sites or otherwise using a wide range of structures may be more tolerant to human activities (Zalewski 1997; Herr et al. 2010). Flexibility in the use of resting site typologies is another important aspect, because some types may be limiting in one region but not in another. For instance, stone martens (*Martes foina*) in central Europe often rest inside buildings near urban areas (Herr et al. 2010), while in southwestern Europe they select tree hollows and dens away from human settlements (Santos-Reis et al. 2004). Also, within any given population the use of different typologies may vary in relation to local habitat context and climatic conditions, which imply that resting site resources may be limiting only in certain locations or at certain times of the year (Carvalho et al. 2014).

Other important aspects characterising mammalian resting ecology are the reuse rate of sites by each animal, and the level of sequential or simultaneous sharing of sites. If each animal reuses each resting site rarely, then a large number of adequate sites may be needed for a species to persist in a landscape (Purcell et al. 2009; Carvalho et al. 2014). Also, if each resting site is used exclusively by a single animal, then a larger number of sites may be needed to support a population than if the same site can be shared by different animals. Reuse rate and site sharing may in turn vary with resting site availability, which may provide a mechanism for species to persist in a landscape under moderate declines of resting site resources (Banks et al. 2011). It is thus important to accurately estimate these parameters and the factors affecting their variability in time and space, though this is hindered by a limited understanding of the potential errors and shortcomings of sampling designs normally used to analyse mammalian resting ecology.

Studies of resting site use by mammals are normally based on the tracking of individuals using VHF transmitters, GPS telemetry, 3D accelerometers and PIT (Passive Integrated Transponder) tags detectors (e.g. Banks et al. 2011; Brown et al. 2014; Carvalho et al. 2014). Typically, animals are followed at regular intervals for several weeks or months, and their resting sites detected during the inactivity periods. Resting site typologies are then identified and their frequency of use are estimated and analysed in relation to environmental and anthropogenic factors (Carvalho et al. 2014). Also, continuous tracking of the same animal allows the estimate of resting site reuse rates, while the simultaneous tracking of several individuals allow the estimation of site sharing. Despite this common approach, however, methodological details tend to vary significantly across studies, which may affect their key conclusions to an unknown extent. The frequency of animal monitoring is particularly relevant in this respect, as sampling schedule may range from daily intervals (Carvalho et al. 2014), to five times a week (Herr et al. 2010), every other day (e.g. Zalewski 1997; Zielinski 2004), and once a week (Camps 2011; Gess et al. 2013). Longer monitoring intervals correspond to lower sample sizes, thereby reducing the precision of estimates and the statistical power to detect significant relationships (Krebs 1989). However, the consequences of these problems to resting ecology studies remain uncertain (Purcell et al. 2009).

In this study we addressed these issues empirically, based on a dataset of daytime resting site use by the common genet (*Genetta genetta*), collected through daily monitoring of 21 animals radiotracked during 28 months. Focusing on a carnivore species was considered adequate, because they play a key role in ecosystems, they are often threatened, and some species are strongly influenced by resting site availability (Pereira and Rodriguez 2010; Crooks et al. 2011). The common genet was considered a suitable species, because it is relatively easy to trap and radiotrack, its resting ecology has been recently described, and it often uses tree hollows in forests habitats (Camps 2011; Carvalho et al. 2014). We used a data thinning procedure (Lahoz-Monfort et al. 2014) to evaluate the influence of monitoring frequencies (daily, every other day and weekly) on estimates of: (i) average number of resting sites per animal; (ii) reuse rates; (iii) sharing frequency; (iv) proportional use of resting site typologies; and (v) environmental correlates of resting site use. Results were then used to discuss the most cost-effective monitoring frequencies to study resting site ecology.

Material and Methods

Study area and species

The study was conducted in southern Portugal (38°32'24'' to 38°47'33'' N, 08°13'33'' to 07°55'45'' W), in an area of about 50,000 ha. Climate is Mediterranean, with minimum and maximum mean temperatures of 5.8 ºC and 12.8 ºC during the winter, and 16.3 ºC and 30.2 ºC in the summer; annual rainfall averages 609.4 mm and is concentrated in October-March (Évora 1971–2000, IPMA 2012). The relief is undulating (150m-430m a.s.l.) and the landscape is dominated (~50%) by open to closed oak (*Quercus suber* and *Q.rotundifolia*) woodlands, where the understory may be herbaceous or shrubby depending primarily on grazing pressure. Understory shrubs are also frequently removed mechanically to reduce fire risk and for providing easy access to livestock. Agricultural areas are also important (~45.0%), comprising mainly dry arable land and pastureland, with or without sparse oaks trees, olive orchards and vineyards.

Common genets (*Genetta genetta*) (mean adult weight (±SE): 1765.8 ± 34.8g; this study) are widespread and abundant throughout the study area and elsewhere in south-western Europe, and they are mainly associated with forested areas and riparian galleries (Zabala et al. 2001; Matos et al. 2009; Santos et al. 2011). Genets exhibit nocturnal activity, forage both on the ground and in the tree canopy and select dense cover for breeding and resting (Palomares et al. 1994; Galantinho and Mira 2009; Pereira and Rodríguez 2010). They feed mainly upon small vertebrates, mostly mammals (Virgós et al. 1999; Rosalino and Santos-Reis 2002). Previous research in the study area indicated the genets often use tree hollows as daytime resting sites, though they also use nests located in large riparian trees and underground dens (Carvalho et al. 2014). The frequency of tree hollow use varies across seasons, and is affected by weather conditions, human disturbance and characteristics of the surrounding habitats (Carvalho et al. 2014).

Trapping and handling

Trapping and handling procedures are described in detail in Carvalho et al. (2014). Briefly, trapping was performed from May 2010 to December 2011, using groups of 10-15 home-made box-traps (30W x 30H x 90L cm), set at an average (± SE) distance of 4148.31 m ± 421.71 from each other, and baited with sardines in oil, fresh chicken eggs, and road-killed small mammals and passerines. Once an animal was captured, it was immediately carried to the veterinarian hospital at Évora University. Animals were then removed from the box, immobilised, and injected intramuscularly with a mix of ketamine hydrochloride (100 mg mL-1) (Imalgene 1000, Lyon, France) and medetomidine hydrochloride (1 mg mL-1) (Domitor, Pfizer, New York, NY, USA) (ratio of 2:1 by volume) at a dosage of 0.12 mL kg^{-1} (Herr et al., 2010). All animals were marked with PIT (Passive integrated transponders) tags (model: TXP148511B, 8.5 mm x 2.12 mm, 134.2 kHz ISO, 0.067g, Biomark, Boise, USA). Adults and sub-adults were equipped with radio-collars (≈ 35g) (models: Ipm2700A, Wildlife Materials, USA and TW-3, BioTrack, Wareham, UK), if no more than 5% of their weight were added (Sikes et al. 2011). After handling, the individuals were released in the capture location once they had completely recovered their reflexes (1–3 h). Capture procedures and animal handling was in conformity with Portuguese legal regulations.

Resting sites monitoring

The study focused on the 21 genets tracked for at least 30 consecutive days (e.g. Zielinski et al. 2004), of which 19 animals were tracked in the rainy season (October-April) and 12 in the dry season (May-September). Each animal was tracked until its death or failure of the radio-collar, using a Biotrack receiver (model: Biotrack Sika, BioTrack, Wareham, UK) and an external 3-element Yagi directional antenna (Wildlife Materials, Inc.). Each tracking day, the location of the daytime resting site of each animal was assessed through “homing” techniques (e.g. Millspaugh and Marzluff 2001), during day light hours when the animals are inactive and tend to remain in the same site until dusk. Once we detect a radiotracking signal coming from a resting structure (e.g., tree, shrubs, underground burrow), we approached carefully to confirm its location without disturbing the animal. A snake inspection camera (model: 8802 AJ GOSCAM, TFT-LCD 2.5 colour monitor) was often used to guarantee the correct distinction between ground nests vs. dens, and tree hollows vs. nests. All resting site locations (coordinates) were recorded using a GPS unit (model: Garmin Map 62; 2 meters positional accuracy), and then a detailed microhabitat vegetation description was made (details in Carvalho et al. 2014). Daytime resting sites were located on a daily basis, except on a few occasions where we could not track the animals due to logistic constraints. In total, during the approximately 120 study weeks, we located resting sites on average (± SE) 6.4 ± 0.15 days a week, and 183.2 ± 19.4 days per animal.

Data analysis

We compared resting site use parameters estimated from daily monitoring schedules, with estimates based on every other day and weekly monitoring schedules. These sampling regimes were selected because they are often used by researchers, as assessed through a comprehensive review of recent radiotracking studies (> 1995) on the resting ecology of mammalian carnivores. For each sampling schedule, we assumed that one daytime resting site per animal was always detected in each tracking day, which is equivalent to what was actually observed during the field study (Carvalho et al. 2014). Analysis were made for the overall data set, and separately for the wet and dry seasons, because previous information suggested that there was significant seasonal variation in resting site use (Carvalho et al. 2014). We combined all data irrespective of animal gender and age (adults and subadults), because these factors did not affect the patterns of resting site use (Carvalho et al. 2014). However, females with cubs (n=5) were excluded from analysis of rest-site reuse, because they tend to remain in the same rest site for extended periods during the rearing season (Carvalho et al. 2014).

Analysis started by estimating the effects of sampling effort on the number of resting sites identified, using accumulation curves in relation to the number of sampling days (e.g. Fontaneto et al. et al. 2012 for similar application with species richness). Accumulation curves were built for each tracked animal, in both the wet and the dry seasons, computing mean values among one hundred permutations of the sampling data. We then thinned at appropriate time intervals the daily monitoring data series of each tracked animal, so as to obtain simulated data series corresponding to monitoring at weekly and every other day intervals. For each sampling schedule (daily, every other day, and weekly), we then estimated (i) the average (± 95%CI) number of rest sites used per animal, (ii) the average percentage (± 95%CI) use of the three main resting site typologies (hollows, nests and dens), (iii) the average (± 95%CI) of resting site reuse index, and (iv) the average percentage (± 95%CI) of resting site sharing. Resting site reuse index was estimated as $RI = 1 – (Ds / Ts)$, where Ds is the number of different resting sites identified, and Ts is the total number of days that the animal was found in a resting site (Zalewski 1997). Resting site sharing was estimated as the average percentage of resting sites from one animal that were used at least once by at least another animal. We tested for differences in parameter estimates among the three sampling schedules using Kruskal-Wallis tests, followed by post-hoc tests based on multiple comparisons between treatments (Siegel and Castellan 1988).

In a second set of analyses, we assessed the influence of monitoring schedule on the detection of significant effects of explanatory variables on resting site use patterns. We followed the approach of Carvalho et al. (2014), relating a dichotomous variable coding whether a genet in a given day used a tree hollow (1) or other resting site type (0), to nine explanatory variables reflecting landscape composition and configuration, resource distribution, predation risk, human disturbance, and daily weather conditions (Supplementary Table S1; Carvalho et al. 2014). Modelling used generalised linear mixed effects models (GLMM), with binomial errors and logit link, with animal identity as the random effect (Zuur et al., 2009). We used the information-theoretic approach in model building and multimodel inference (Burnham and Anderson, 2002). Candidate models were built based on all possible subsets of non-collinear explanatory variables and ranked according to their Akaike weights (wi). In multimodel inference, the average parameters and their unconditional standard errors (SE) were estimated using the 95% confidence set of models (Burnham and Anderson, 2002). The relative importance of predictors in each average model was estimated from model selection probabilities (w_+) (Burnham and Anderson 2002). The models were not affected by spatial or temporal autocorrelation problems, as assessed through the methods outlined in Carvalho et al. (2014). We compared the models produced using data from each of the three sampling schedules, considering the number of models included in the 95% confidence set of models, the Akaike weights of the most supported models, the selection probabilities of the variables included in the best models, and the size and type (positive or negative) of effects supported by the average model.

All statistical analysis were performed using the R statistical package (R Project for Statistical Computing release 2.15.3 http://www.r-project.org). We used the specaccum function of the *vegan* package to estimate accumulation curves (Oksanen et al. 2015), the *pgirmes*s package for the Krukal-Wallis and post hoc tests (Giraudoux 2013), *lme4* (Bates et al. 2013) for mixed modelling analysis and *MuMIn* (Barton 2013) for multi-model inference.

Results

The accumulation of daytime resting sites with the number of sampling days varied greatly among tracked individuals, though there was an overall tendency for the number of sites identified accumulating rapidly during the first 20-30 sampling days, then tending to slowly level-off after about 50 days (Fig. 1). However, a clear asymptote was never reached, suggesting that animals progressively use new resting sites over time.

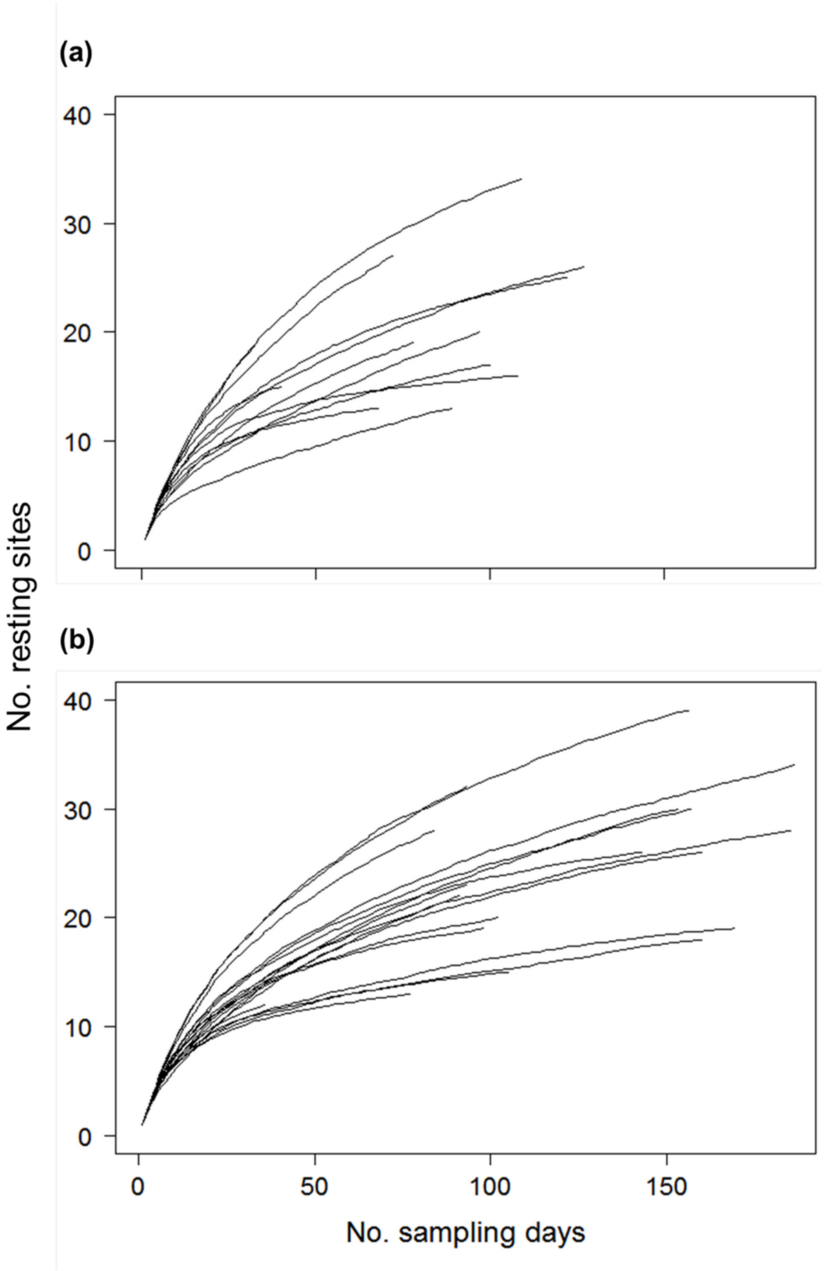

Figure 1 Accumulation curves of resting sites identified for each tracked animal, in the dry and wet seasons, in relation to the number of sampling days. Curves were obtained through one-hundred random permutations of sampling data.

Monitoring frequency showed a marked effect on estimates of the number of resting sites used per animal during the tracking period, for which values estimated using weekly monitoring were always less than half those based on daily monitoring (Fig. 2; Supplementary Table S2a). These estimates did not vary significantly between daily and every other day sampling schedules, though the latter always showed smaller values (Fig. 2; Supplementary Table S2a).

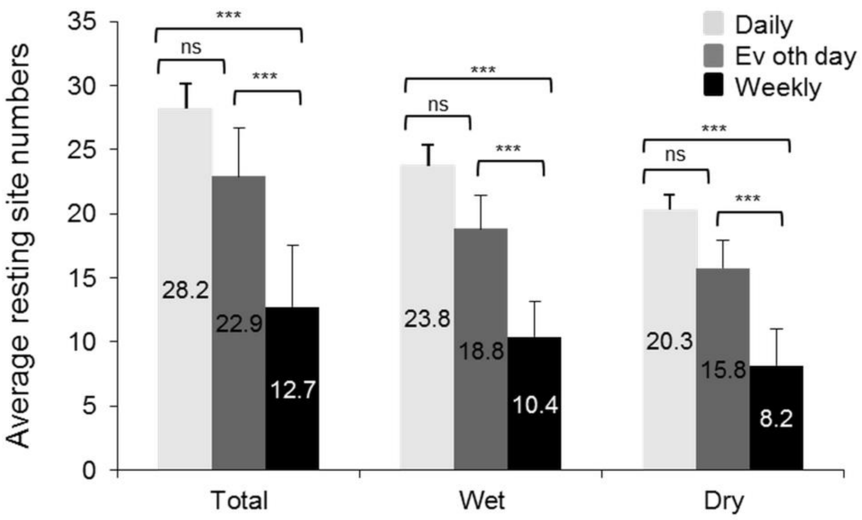

Figure 2 Comparison of the average number (±95%CI) of resting sites pooled across seasons, wet and dry season, under three monitoring frequencies schedules. Daily (every day); Ev Oth Day (every other day); Weekly (once a week). In cases where statistical differences between monitoring schedules were detected using Kruskal-Wallis tests ($P < 0.05$), we represent the results of post-hoc pairwise tests: ns – not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Estimates of percentage use of the three main resting site typologies did not vary between sampling regimes (Fig. 3; Table S2b). Estimates of resting site reuse rates were virtually identical using data collected daily or every other day, but these were always much larger than estimates based on weekly surveys (Fig. 4; Supplementary Table S2c). Estimates of resting site sharing tended to decline with decreasing sampling frequency, but significant differences were only found in comparisons between the weekly and the two other monitoring frequencies (Fig. 5; Supplementary Table S2d).

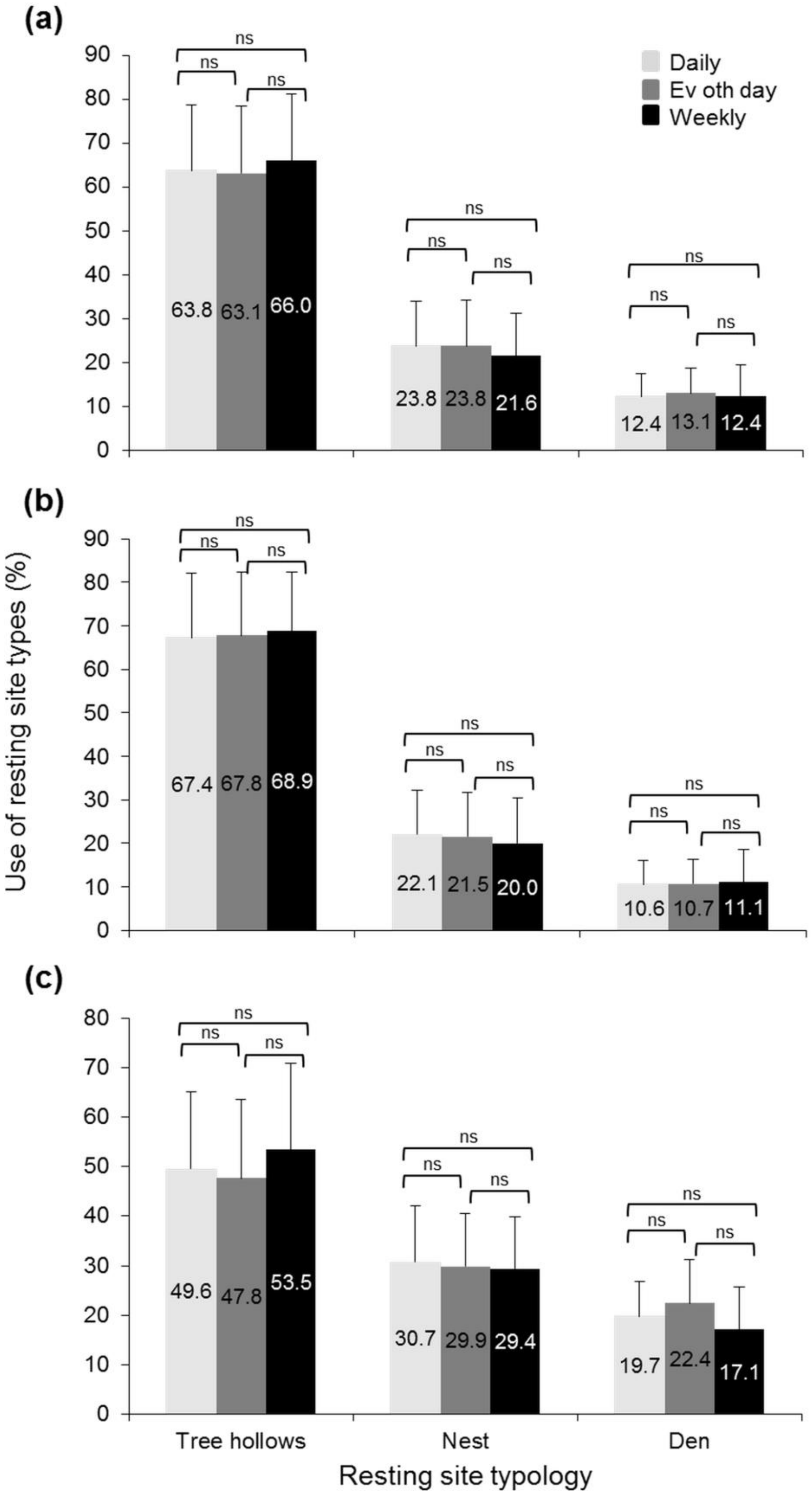

Figure 3 Average percentage use (%) of resting site typologies (±95%CI) per animal obtained for total (a), wet (b) and dry season (c), under each of the monitoring frequency schedules. Daily – (every day); Ev Oth Day – (every other day); Weekly – (once a week). No statistically significant differences among monitoring scenarios were detected using Kruskal-Wallis tests (P > 0.05).

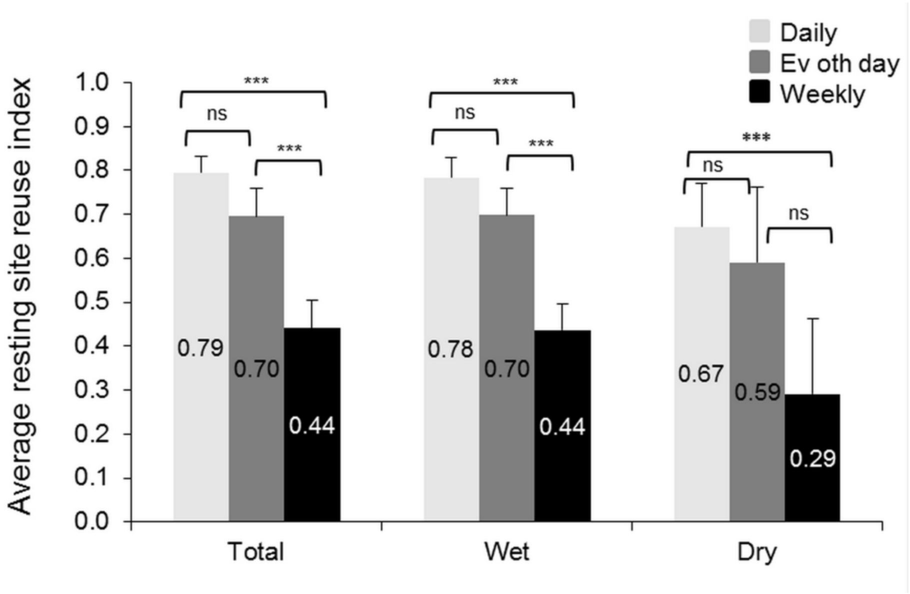

Figure 4 Average resting site reuse index (±95%CI) per animal for all the year, wet and dry season, under three monitoring frequency schedules. Daily (every day); Ev Oth Day (every other day); Weekly (once a week). In cases where statistical differences between monitoring schedules were detected using Kruskal-Wallis tests (P < 0.05), we represent the results of post-hoc pairwise tests: ns – not significant; * p < 0.05; ** p < 0.01; *** p < 0.001.

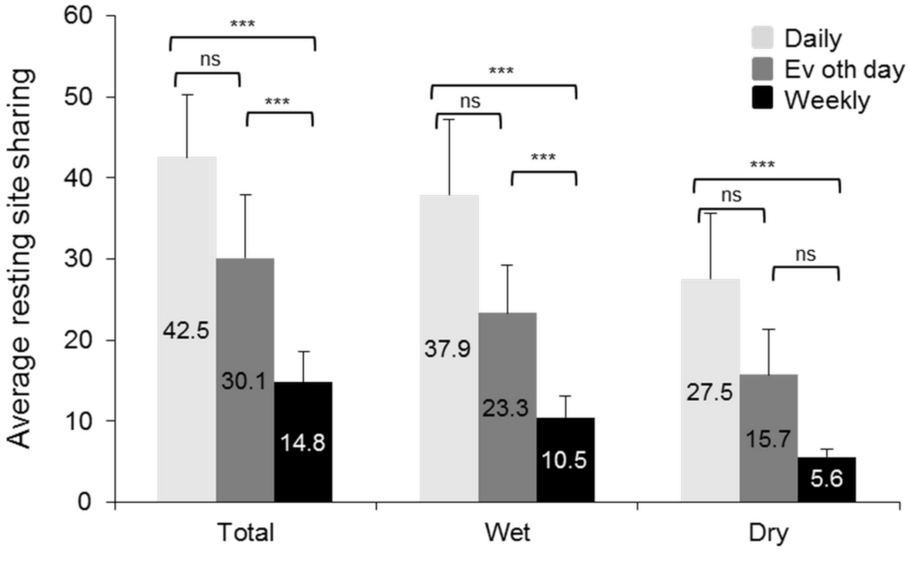

Figure 5 Average (%) resting site sharing (±95%CI) per animal for all the year, wet and dry season, under three monitoring frequency schedules. Daily (every day); Ev Oth Day (every other day); Weekly (once a week). In cases where statistical differences between monitoring schedules were detected using Kruskal-Wallis tests (P < 0.05), we represent the results of post-hoc pairwise tests: ns – not significant; * p < 0.05; ** p < 0.01; *** p < 0.001.

Decreasing monitoring frequency was associated with increasing uncertainty in average models relating the probability of tree hollow use to explanatory variables (Table 1), with decreasing capacity to identify significant effects, and with changes in effect sizes (Table 2). Uncertainty was underlined by the number of models included in the 95% Confidence Interval of supported models for both wet and dry seasons, which was low for analysis based on daily monitoring (6 and 12), and large to very large for the every other day (12 and 43) and weekly (45 and 33) monitoring schedules (Table 1).

Table 1 Summary results of model selection and averaging procedures for relationships between the use of tree hollows by genets and explanatory variables, in the wet and dry seasons, under three monitoring frequency schedules. In each case we provide the number of models included in the 95% confidence set of top models rank, the Akaike weights for the best fitting model (> w_i), and the variables included in the best model: 1 – human disturbance, 2 – riparian, 3 – forests edges, 4 – forest with shrubs, 5 – forests, 6 – precipitation, 7 – shrub cover, 8 – maximum temperature.

	Daily	Every other day	Weekly
Wet season			
Models in 95%CI	6	43	45
Best model w_i	0.56	0.09	0.12
Variables	12345678	23567	267
Dry season			
Models in 95%CI	12	12	33
Best model *wi*	0.40	0.25	0.15
Variables	123567	123578	2378

Likewise, the Akaike weights and number of supported effects were high for daily monitoring, and low for the two other monitoring frequencies (Table 2). In the wet season, the positive effects of distance to riparian galleries, and the negative effects of shrub cover, on the probability of tree hollow use were strongly supported ($w_+ > 0.80$ and 95% Confidence Intervals of coefficients not overlapping zero) irrespective of sampling schedule, and there was little variation in coefficient estimates, particularly in the case of precipitation (Table 2). In contrast, the models developed using daily monitoring data provided support for the positive effects of precipitation, forest cover, forest edges and human disturbance, and the negative effect of temperature, but none of these was strongly supported by models based on lower monitoring frequencies (Table 2). In the dry season, strong support irrespective of monitoring frequency was found for the positive effects of shrub cover, and the negative effects of distance to riparian galleries (Table 2). The positive effect of forest cover, and the negative effect of forest edges, were supported both using the daily and every other day schedules, and the negative effects of precipitation and human disturbance were only supported strongly using daily sampling data (Table 2). Overall, in both seasons the largest variation between sampling schedules was generally found for variables with the smallest effect sizes as judged by the corresponding standardised regression coefficients, which were identified as influential when using the daily sampling data, but lost importance when using the every other day and weekly monitoring data (Table 2).

Discussion

To the best of our knowledge, our case study using common genets showed for the first time the strong impact of different temporal intervals between locations on the analysis of resting site use by mammals. Results suggest that reducing monitoring frequency contributed for underestimating the number of sites used per animal, the resting site reuse rate, and the resting site sharing rate. However, differences in estimates were small between the daily and every other day sampling regimes, while they were large between these and the weekly sampling regime. Also, the study showed that decreasing monitoring frequency results in increasing uncertainty of models relating resting site use to environmental variables, and thus decreasing the capacity to detect important environmental effects. These results have important implications for the design and interpretation of resting ecology studies of mammals. For instance, it is possible to hypothesize that differences in sampling regime affected to a considerable extent the patterns of variation between and within species observed in recent (> 1995) radiotracking studies of mammalian resting ecology (Table 3; Purcell et al. 2009).

Table 2 Average models describing the estimated effects of explanatory variables on the probability of tree hollow use by genets in the wet and dry seasons, and for each monitoring frequency schedules. For each season, multi-model averaging was based on the 95% confidence set of models. For each variable, we show the standardised regression coefficient (*B*), the unconditional standard errors (*SE*), the 95% confidence interval of coefficient estimate (*CI*), and the selection probability (w_+). Variables selected in the best models are underlined and coefficient estimates whose 95%CI exclude zero are in bold. The number of observation by season and scenario are inside brackets.

	B	SE	CI	w_+	B	SE	CI	w_+	B	SE	CI	w_+
Wet season	Daily (n = 2291)				Every other day (n = 1171)				Weekly (n = 333)			
Intercept	**4.469**	**0.981**	**(2.546, 6.392)**	**–**	**4.650**	**0.947**	**(2.790, 6.500)**	**–**	**4.080**	**0.960**	**(2.200, 5.970)**	**–**
Maximum temperature	**− 0.562**	**0.130**	**(− 0.817, − 0.307)**	**1.000**	− 0.124	0.176	(− 0.469, 0.221)	0.500	− 0.067	0.202	(− 0.464, 0.329)	0.310
Precipitation	**0.298**	**0.114**	**(0.075, 0.522)**	**0.940**	0.330	0.191	(− 0.044, 0.705)	0.890	0.584	0.418	(− 0.235, 1.400)	0.820
Shrub cover	**− 3.209**	**0.271**	**(− 3.742, − 2.678)**	**1.000**	**− 3.290**	**0.397**	**(− 4.070, − 2.510)**	**1.000**	**− 3.510**	**0.716**	**(− 4.920, − 2.110)**	**1.000**
Riparian	**2.315**	**0.243**	**(1.839, 2.791)**	**1.000**	**2.420**	**0.341**	**(1.750, 3.090)**	**1.000**	**1.740**	**0.439**	**(0.875, 2.600)**	**1.000**
Human disturbance	**0.662**	**0.229**	**(0.215, 1.110)**	**1.000**	0.229	0.302	(− 0.362, 0.821)	0.530	− 0.021	0.187	(− 0.386, 0.345)	0.250
Forests	**0.633**	**0.285**	**(0.075, 1.191)**	**0.820**	0.389	0.419	(− 0.432, 1.210)	0.620	0.266	0.441	(− 0.598, 1.130)	0.430
Forests with shrubs	**− 0.649**	**0.331**	**(− 1.297, − 0.002)**	**0.730**	− 0.038	0.225	(− 0.479, 0.402)	0.260	0.062	0.321	(− 0.566, 0.691)	0.270
Forests edges	**0.988**	**0.304**	**(0.392, 1.583)**	**1.000**	0.500	0.398	(− 0.279, 1.280)	0.750	− 0.029	0.224	(− 0.468, 0.408)	0.250
Dry season	Daily (n = 1043)				Every other day (n = 599)				Weekly (n = 163)			
Intercept	0.286	0.993	(− 1.661, 2.232)	–	0.112	0.960	(− 1.770, 1.990)	–	0.349	0.862	(− 1.340, 2.040)	–
Maximum temperature	0.103	0.153	(− 0.198, 0.403)	0.320	0.219	0.228	(− 0.228, 0.665)	0.640	0.321	0.392	(− 0.448, 1.090)	0.570
Precipitation	**− 0.398**	**0.156**	**(− 0.704, − 0.092)**	**0.930**	–	–	–	–	0.584	0.269	(− 0.235, 1.400)	0.270
Shrub cover	**− 2.679**	**0.260**	**(− 3.188, − 2.169)**	**1.000**	**− 2.670**	**0.364**	**(–3.390, –1.960)**	**1.000**	**− 2.300**	**0.605**	**(− 3.490, − 1.120)**	**1.000**
Riparian	**0.788**	**0.179**	**(0.438, 1.138)**	**1.000**	**0.990**	**0.248**	**(0.504, 1.480)**	**1.000**	**0.981**	**0.455**	**(0.088, 1.870)**	**0.960**
Human disturbance	**− 0.671**	**0.250**	**(− 1.161, − 0.181)**	**1.000**	− 0.448	0.389	(− 1.216, 0.315)	0.730	–	–	–	–
Forests	**1.127**	**0.321**	**(0.498, 1.756)**	**1.000**	**0.874**	**0.421**	**(0.049, 1.700)**	**0.940**	0.346	0.544	(− 0.720, 1.410)	0.460
Forests with shrubs	− 0.151	0.254	(− 0.650, 0.348)	0.310	0.121	0.263	(− 0.394, 0.635)	0.370	0.037	0.303	(− 0.558, 0.631)	0.270
Forests edges	**−1.181**	**0.477**	**(− 2.215, – 0.247)**	**0.890**	**−1.890**	**0.712**	**(− 3.290, – 0.499)**	**0.990**	− 1.330	0.904	(− 3.100, 0.441)	0.830

Table 3 Summary results of studies on resting site use by mammalian carnivores. In each case we provide the species involved, geographic region, number of animals tracked (N), the study duration in months (Months), the monitoring frequency (Monitoring), the number of resting sites (Sites), the reuse index (RI), the method of tracking (homing vs. triangulation), and the reference of the study. The table compiles data retrieved from a comprehensive review of recent (>1995) radiotracking studies on the resting ecology of mammalian carnivores published in journals listed in the Science Citation Index, for which quantitative information on sampling regime was provided. [a] Average number of resting sites used per animal and total number of resting sites detected (in brackets)

Specie	Region	N	Months	Monitoring	Sites[a]	RI	Method	Reference
Genetta genetta	Southern Europe	21	28	Daily	28.2 (592)	0.79	Homing	Carvalho et al. 2014
Genetta genetta	Southern Europe	21	28	Every other day	22.9 (481)	0.70	Homing	This study
Genetta genetta	Southern Europe	21	28	Weekly	12.7 (267)	0.44	Homing	This study
Genetta genetta	Southern Europe	16	33	Weekly	4.7 (75)	0.88	Triangulation	Camps 2011
Genetta genetta	Southern Europe	3	5	Every other day	34.7 (104)	0.52	Homing	Santos-Reis et al. 2004
Martes foina	Central Europe	10	24	5 days week^{-1}	12.8 (128)	0.95	Homing	Herr et al. 2010
Martes foina	Southern Europe	3	5	Every other day	23.7 (71)	0.68	Homing	Santos-Reis et al. 2004
Martes foina	Southern Europe	8	59	Every other day	12.0 (97)	0.89	Homing	Genovesi and Boitani 1997
Martes martes	Central Europe	9	58	Every other day	91.0 (816)	0.38	Homing	Zalewski 1997
Martes martes	Northern Europe	22	48	Not applied	16.3 (358)	not applied	Homing	Brainerd et al. 1995
Martes americana	Northern America	13	7	Every other day	25.5 (52)	not applied	Homing	Slauson and Zielinski 2009
Martes pennanti	Northern America	15	10	Weekly	5.3 (79)	0.87	Homing	Gess et al. 2013
Martes pennanti	Northern America	11	15	2.5 days week^{-1}	7.1 (78)	0.91	Homing	Purcell et al. 2009
Martes pennanti	Northern America	21	52	Every other day	23.5 (493)	0.08	Homing	Zielinski et al. 2004
Felis sylvestris	Central Europe	3	6	5.8 days week^{-1}	15.0 (45)	0.89	Homing	Jerosch et al. 2010
Otocolobus manul	Central Asia	21	28	2.5 days week^{-1}	4.8 (101)	not applied	Homing	Ross et al. 2010

The reduction in the number of observable events (i.e., use of a daytime resting site by an animal) associated with lower monitoring frequencies is probably the main reason for the patterns observed. The number of new sites used by an animal and detected by a researcher accumulates over time, and so it should be expected that over a given time frame the number of sites detected per animal should decline when less days are sampled. However, the accumulation curves suggest that the number of sites detected increases quickly during the first 20-30 sampling days and then tends to level off slowly after about 50 days, which may explain why differences were relatively small between the daily and the every other day sampling regime, but large between these and the weekly sampling regime. These patterns may differ among species and ecological settings, because the error should be larger when the true number of resting sites used by an animal approaches (or surpasses) the number of days actually sampled using a given monitoring frequency. For instance, the effect of reducing the monitoring frequency should be particularly large in the extreme case of an animal using a new site every day, as the maximum number of sites detectable over a period of 100 days would be 100 with the daily schedule, 50 with the every other day schedule, only 14 with the weekly schedule. A similar reasoning applies for the site reuse index and the sharing rate, as the likelihood of detecting a given site used several times by one animal, or by different animals on distinct occasions, should be low when the true number of resting sites per animal is high compared to the number of monitoring days. This may explain why the estimates of both the reuse index and the sharing rate declined with declining monitoring frequencies, though once again the differences were relatively small between the daily and the every other day sampling regimes. The monitoring frequency did not affect estimates of the proportion of different resting site typologies used by genets. This was probably because the number of observations obtained was always relatively high compared to the number of typologies considered (only three), thereby providing reasonable estimates of proportional use irrespective of the monitoring frequency.

The reduction in sample size with decreasing monitoring frequency also affected the capacity to detect environmental correlates of rest site use, thereby compromising the capacity to understand the ecological processes affecting tree hollow use by genets, which were thoroughly discussed in a previous study (Carvalho et al. 2014). In fact, here we showed that reducing monitoring frequency resulted in higher uncertainty of models relating the probability of tree hollow use by genets to environmental variables, as well as the capacity to find support for the effects of such variables (Carroll et al. 1999; Zielinski et al. 2004). This should be expected, because lower sample sizes result in higher sampling variance, and thus wider confidence intervals of parameter estimates (Burnham and Anderson 2002). Typically, with relatively small sample sizes only the most important effects are unveiled, whereas increasing sample sizes results in the sequential identification of moderate effects, followed by yet smaller effects (Burnham and Anderson 2002). This is in line with the results of our study, where increasing the monitoring frequency from weekly to daily schedules resulted in sequential support to an increasing number of environmental effects on tree hollow use. For instance, during the dry season the weekly monitoring data supported the positive effects of distance to riparian galleries and the negative effects of shrub cover, the every other day data supported the same two effects plus the positive effect of forest cover and the negative effect of forest edges, and the daily data provided support for the former four effects plus the negative effects of distance to sources of human disturbance and precipitation. In general, therefore, low monitoring frequencies should only provide support for the few parameters with a strong effect on resting site, while more frequent monitoring will support, if necessary, more complex models describing a wider range, including more subtle effects (Burnham and Anderson 2002).

Failure to appreciate the impact of monitoring frequency may lead to misinterpretations and poor inferences in studies analysing mammalian resting behaviour, and so researchers need to interpret data from radiotracking studies cautiously by considering the decreased power unless sampling effort is enough. Accounting for this potential source of bias is important, because studies have used widely different monitoring frequencies, which may justify at least partly the differences in resting site use parameters observed across studies (Aubry et al. 2013). For instance, considering studies published on the resting ecology of forest carnivores in the last 20 years, results suggest that genets in our area used an exceptionally high number of different resting sites (Table 3). The difference is particularly marked (28.2 vs. 4.7) in relation to another study on genets by Camps (2011), who inferred that the very low number of resting sites used per animal was a consequence of high competition for resting sites due to high population density and low resting site availability. A simple alternative explanation, however, is that monitoring at weekly frequencies strongly underestimated the true number of resting sites used per animal, which would be much higher had a daily monitoring schedule been adopted. Selecting between these alternative explanations is not possible without further data, though it should be noted that even under our simulated weekly monitoring schedule the number of resting sites per animal detected by Camps (2011) was indeed lower (4.7 vs. 12.7) and the reuse rate was higher (0.88 vs. 0.44) than that observed in our study area. It is thus possible that the differences between the two studies derived both from errors introduced by a low monitoring frequency, different radiotracking methods, number of animals studied, time duration of the study and by true differences between study areas (Aubry et al. 2013).

Taken together, results from our study suggest that monitoring at daily intervals may be required for accurately estimating patterns of resting site use by mammals. If logistics and financial resources are restricted, however, it may be possible to adopt the every other day sampling regime, which appeared sufficient to estimate accurately the percentage use of different resting site types, while underestimating only slightly the number of resting sites per animal, and the reuse and sharing rates. This less demanding sampling regime may also be useful to estimate moderate to strong effects of environmental variables on resting site selection, though being unable to identify more subtle effects. In contrast to these two alternatives, we found that the weekly sampling regime performs very poorly and should be avoided, as it strongly underestimated all resting site parameters except the percentage use of different resting sites, and it failed to identify all but the strongest environmental drivers of resting site selection. Although some of the limitations of less frequently locating the target species might in principle be overcome by increasing the sampling period, this is often unfeasible in medium-sized carnivores and other medium to small mammals, for which the lifetime of batteries used in VHF transmitters, put a tight upper limit to the radiotracking period. As a consequence, it is strongly recommended that daily or at least every other day monitoring regimes should be adopted whenever possible, particularly when it is suspected that resting site use may strongly influence population parameters such as density, reproduction and survival (Boitani and Powel 2012; Baguette et al. 2013; Aubry et al. 2013), and where human activities are judged to be reducing resting site availability and thus compromise species persistence (Gibbons et al. 2008; Lindenmayer et al. 2012b; Brown et al. 2014; Carvalho et al. 2014).

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Supplementary Material

Monitoring frequency influences the analysis of resting behaviour in a forest carnivore

Table S1 Description and summary statistics of explanatory variables used to analyse rest site selection by the genet in Southern Portugal.

Variables	Abbreviation	Description	Transformation
Landscape composition and configuration (within 1 km of rest site)			
Forests	FOREST	Proportional cover by forests (> 30 % tree canopy density)	Asin[√x]
Forests with shrubs	FOREST_SH	Proportional cover by forests (> 30 % tree canopy density) with understory shrubs	Asin[√x]
Forest patch Size	FOREST_PS	Mean patch size (ha) of forest habitats	Log 10
Forest edges	FOREST_ED	Density of (m ha^{-1}) of edges between forests and other habitats	Log 10
Resource distribution			
Riparian	D_RIPARIAN	Distance (m) to the nearest riparian habitat	Log 10
Predation risk			
Shrub cover	SHRUB_CV	Proportional cover by shrubs within a 25 m of each rest site	Asin[√x]
Human disturbance			
Human disturbance	D_HUM_PER	Distance (m) of rest site to the nearest source of human disturbance (e.g., houses and roads)	Log 10
Weather			
Maximum temperature	T_MAX	Maximum daily temperature (ºC)	Log 10
Precipitation	PRECP	Daily precipitation (mm)	Log 10

Table S2 Summary results of Kruskal-Wallis and multiple comparison “two tail” posterior tests among monitoring frequency scenarios for (ns – not significant; * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001):

a) Rest-site numbers

Season	Total	Wet	Dry
Krukal-Wallis test			
Chi-squared (d.f. = 2)	24.6254	29.697	22.966
P-value	***	***	***
Post-hoc test (*P* < 0.05)			
Daily vs. Weekly	***	***	***
Daily vs. Every other Day	ns	ns	ns
Every other Day vs. Weekly	***	***	***

b) Rest-site selectivity

Season	Total	Wet	Dry
Trees			
Krukal-Wallis test			
Chi-squared (d.f. = 2)	0.038	0.019	0.188
P-value	ns	ns	ns
Nests			
Krukal-Wallis test			
Chi-squared (d.f. = 2)	0.116	0.143	0.009
P-value	ns	ns	ns
Dens			
Krukal-Wallis test			
Chi-squared (d.f. = 2)	0.684	0.487	0.992
P-value	ns	ns	ns

c) Rest-site reuse index

Season	Total	Wet	Dry
Krukal-Wallis test			
Chi-squared (d.f. = 2)	32.008	28.496	9.823
P-value	***	***	**
Post-hoc test (*P* < 0.05)			
Daily vs. Every other Day	ns	ns	ns
Daily vs. Weekly	***	***	***
Every other Day vs. Weekly	***	***	ns

d) Rest-site sharing

Season	Total	Wet	Dry
Krukal-Wallis test			
Chi-squared (d.f. = 2)	23.048	19.648	14.488
P-value	***	***	***
Post-hoc test (*P* < 0.05)			
Daily vs. Every other Day	ns	ns	ns
Daily vs. Weekly	***	***	***
Every other Day vs. Weekly	***	***	ns

4. JOINT EFFECTS OF ROADS AND SPATIAL HABITAT HETEROGENEITY ON LANDSCAPE FUNCTIONAL CONNECTIVITY IN A FOREST CARNIVORE.

Paper under review in Landscape Ecology

Carvalho F, Carvalho, R, Mira A, Beja P (*in prep*) Joint effects of roads and spatial habitat heterogeneity on landscape functional connectivity in a forest carnivore. *Under review in Landscape Ecology*

Joint effects of roads and spatial habitat heterogeneity on landscape functional connectivity in a forest carnivore

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Abstract

Landscape functional connectivity is likely shaped by a combination of factors, including the spatial heterogeneity of habitats and the distribution of infrastructures such as roads networks. These joint effects are poorly understood due to the shortage of empirical information on the environmental features affecting movement behaviour in human-dominated landscapes. We aimed to assess how spatial habitat heterogeneity and roads influence functional connectivity in a forest carnivore, the common genet. We used VHF radiotracking to collect movement data on 22 genets in a mixed forest-agricultural landscape of southern Portugal. We used information-theoretic model building and multimodel inference to develop path selection functions (PathSFs) discriminating between observed and random paths in relation to environmental variables. PathSFs were used together with land cover information to produce conductance surfaces. Genets showed preference for moving within forest patches and close to riparian habitats, and strongly avoided open agricultural land. The probability of movement declined with increasing road density, but increased with the proximity of culverts, viaducts and bridges. Landscape connectivity was favoured by large forest patches, and by the presence of riparian areas providing movement corridors within open agricultural land highly resistant to genet movement. Roads reduced connectivity by dissecting forest patches, but had less effect on riparian corridors due to the presence of crossing structures. Spatial habitat heterogeneity shaped the extent, location and configuration of areas most permeable to genet movement. The road network dissected the forest habitats, creating obstacles in areas otherwise permeable to animal movement.

Keywords Conditional logistic regression (CLR), Forest carnivores, *Genetta genetta*, Landscape conductance, Movement behaviour, Path selection function (PathSF), Road effects

Introduction

Human activities such as agriculture, forestry, urbanization and the construction of transport infrastructures are major drivers of landscape change, contributing to the loss and fragmentation of natural habitats (Foley et al. 2005; Lindenmayer and Fischer 2006). In these human-modified landscapes, many species are restricted to more or less isolated patches of suitable habitat surrounded by a hostile or poorly suitable matrix, and their landscape-level persistence is critically affected by the ability of individuals or propagules to move among habitat patches (Fahrig et al. 2011). Hence, the conservation management of human-modified landscapes require a detailed understanding of the factors influencing individual movements in relation to habitat structures, in order to be able to forecast the consequences for species persistence of increasing habitat fragmentation and changes in matrix permeability (Coulon et al. 2008; Cushman et al. 2013).

Connectivity is a fundamental component in human-modified landscapes, which translates the physical relationships (corridors) among habitat patches (structural connectivity) and the movement response of organisms toward them (functional connectivity) (Taylor et al. 1993). Despite its importance, there is still limited understanding of connectivity in real landscapes, because most studies have focused on structural rather than functional connectivity. This is probably because the former is much easier to estimate, as it only requires maps of land cover, which can easily be used within a geographic information system (GIS) to estimate metrics assumed to be related to connectivity (e.g. forest edge density, inter-patches distances, and mean patch size). However, this approach may be misleading, because the perception of landscapes is species-specific, and so patches that are isolated for one species may not be to others (Crooks and Sajayan 2006; Kadoya 2009). As a consequence, researchers have increasingly underlined the importance of looking at the functional aspect of the connectivity, where the reasons for movement behaviour need to be studied in order to assess the cost of movement through a landscape (Coulon et al. 2008; Walpole et al. 2012). This is a difficult task, however, and so many studies have estimated movement costs from expert opinion or from inferences drawn from published literature (e.g. Gurutxaga and Saura 2014; Dickson et al. 2013).

Telemetry devices such as VHF and more recently GPS collars provide a wealth of movement data that can be used to estimate movement costs and thus functional landscape connectivity (Crooks and Sajayan 2006). Until recently, however, the statistical approaches to estimate functional connectivity from such data were limited, thus providing a relatively weak basis for inference. In fact, most of the studies carried out so far have used point locations as the main basis of analysis, largely ignoring the movement patterns that could be inferred from the organization of the points along paths. To overcome this problem, recent statistical advances created the path-level (movement) randomization approach (Cushman et al. 2010; Reding et al. 2013), which provides a robust means to compare landscape factors influencing the path made by an animal (observed) with those that would be encountered in a large sample of available paths of identical length and topology (randomly generated), through conditional logistic regression (CLR). This approach avoids pseudoreplication and autocorrelation of observations, which otherwise became problematic in some analysis assessing point data (Cushman 2010; Zeller et al. 2012). This enables the development of species-specific landscape resistance models in which the resistance of any location, or pixel, in a landscape reflects the effects of all the main factors measured at one or several scales (Cushman et al. 2011).

Path-level analysis combined with CLR may provide a particularly useful approach to infer the effects of roads on functional connectivity. This is important because changes in connectivity caused by roads can have severe consequences on the viability of animal populations, by hindering the movements of individuals and genes across the landscape (Riley et al. 2006; Fahrig and Rytwinski 2009; Hartmann et al. 2013). Furthermore, the effects of roads can interact with those of other landscape characteristics such as the amount and spatial distribution of habitats, thereby requiring a detailed understanding of how animal movements are affected by specific combinations of landscape elements. For instance, the impact of roads may be particularly serious where they cross habitats potentially providing movement corridors in fragmented landscapes (Richard and Armstrong 2010; LaPoint et al. 2013). Although these effects have received some consideration in previous studies (Fu et al. 2010; Santos et al. 2013; Nogués and Cabarga-Varona 2014), none of which was based on empirical information on species-specific movement behaviour in relation to both habitat features and roads. However, integrating this type of information would be important to gain a mechanistic understanding of how spatial heterogeneity and human infrastructures jointly influence landscape connectivity patterns.

In this study, we analysed these issues based on path-level analysis of detailed radiotracking data of common genets in Mediterranean landscapes. Focusing on a mammalian carnivore is particularly relevant, because this group of species is highly vulnerable to losses of landscape connectivity (Crooks et al. 2011), including those caused by roads (Riley et al. 2006; Hartmann et al. 2013). The genet was selected because this is a relatively abundant, medium-sized carnivore, which is specialised in forest habitats and seems to be negatively affected by roads (e.g. Galantinho and Mira 2009; Carvalho et al. 2014). Specific objectives were 1) to describe the main landscape factors influencing path selection by genets; 2) to parameterize landscape conductance (inverse of resistance) across the landscape using empirical path-level models; and to 3) to quantify the joint effects of roads and spatial habitat heterogeneity on the overall landscape functional connectivity. The results obtained here provide new insights on road effects assessment and mitigation guidance on functional landscape connectivity in human-dominated landscapes.

Material and Methods

Study area

The study was carried out in a landscape of about 4572 km2 in southern Portugal (38°21'39'' to 39°01'36'' N, 08°24'22'' to 07°34'54'' W; Fig. 1). Climate is Mediterranean, with mean daily temperature ranging from 5.8 ºC to 12.8 ºC in winter, and from 16.3 ºC to 30.2 ºC in summer; annual rainfall averages 609.4 mm and is concentrated in October-March (Évora 1971–2000; IPMA, 2012). The relief is undulating (150m-430 m above sea level) and the landscape is dominated by agricultural land uses (≈45%), comprising mainly dry arable land and pastureland, with or without sparse oaks trees, and vineyards. There is also a large area (≈43.5%) of open to closed cork oak (*Quercus suber*) and holm oak (*Q. rotundifolia*) forests, where the understory may be herbaceous or shrubby depending primarily on grazing pressure and understory management to reduce fire risk and to provide easy access to livestock. Other land cover types include intensive olive orchards, and some scattered plantations of *Pinus* spp. and Eucalyptus spp. (≈8.8%). This landscape is dissected by numerous roads including the A6 highway constructed in 1996 with 2 lanes each side separated by a central splitter, and with a mean traffic flow of 977 vehicles night-1 range = 330-2494; Grilo et al. 2009). There are also national and regional roads, most of which have two lanes and paved verges, and a mean traffic flow of 873 vehicles night-1 (range = 439-1680; Silva et al. 2012) (Fig. 1).

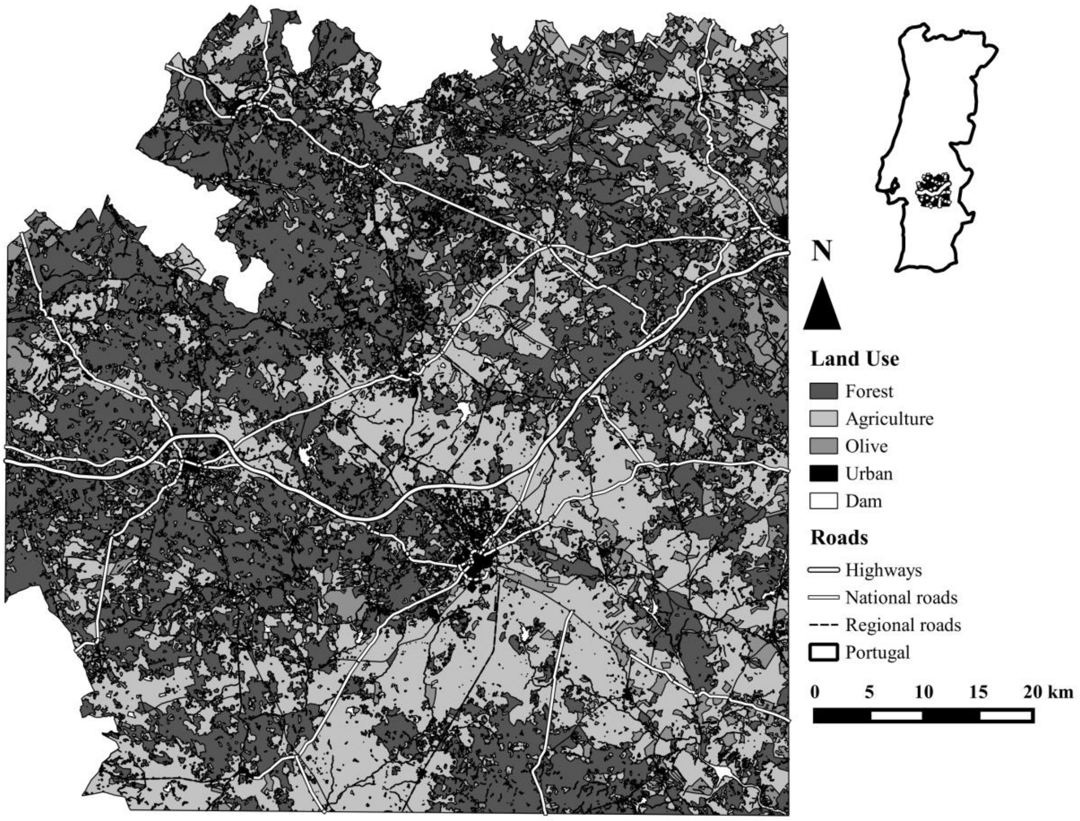

Figure 1 Map of the study area showing the dominant land cover types and the road network.

Observed and random paths

Genets were live-trapped in the study area following capture and handling procedures detailed elsewhere (Carvalho et al. 2014), and fitted with very high frequency (VHF) radio-collars. Movement data was then obtained for a total of 22 animals (13 females and 9 males), between May 2010 and July 2012, following an approach adapted from Reding et al. (2013). Each animal was tracked in average (± SD) during 9.0 ± 3.8 sessions (range: 4-16 sessions, n=198), with each session corresponding to an average (± SD) continuous period of 8.2 ± 3.5 hours (range: 4-14 hours), during which the position of the animal was estimated at average intervals (± SD) of 33.4 ± 9.4 min (range; 15-79 min, n= 2850 consecutive location records).

Animal positions were estimated through biangulations (2 bearings) performed by an observer in less than 5 minutes to reduce estimation errors. Based on a blind test of 50 known locations for each of the two observers involved in radiotracking (Millspaugh and Marzluff, 2001), we estimated an average (± SD) positional error of 92.6 ± 51.4 m (range: 14-179 m), which was considered satisfactory given the objectives of the study. The sequence of positions recorded was used to estimate the path taken by the animal during each tracking period. In a few cases (n=33) where position of the animal was lost for > 60 min, we dropped the corresponding time step and split the path in two, in order to minimize uncertainty regarding the true trajectory between successive locations (Zeller et al. 2012).

For each observed path we generated nine random paths within a buffer of 1.6 km, by first shifting the X and Y coordinates of the observed path by a random value in the interval [-1,600m; 1,600m], and then rotating its orientation by a random value between 0 and 360º (Cushman et al. 2011; Reding et al. 2013). The number of random paths per observed path was selected because it represented a compromise between adequately sampling the available landscape area and the need to limit overlap (< 20%) among paths (Reding et al. 2013; Roever et al. 2013), and because it was successfully used in a previous study on a comparable carnivore species (Cushman et al. 2011). In line with Reding et al. (2013), the buffer was selected to correspond approximately to the maximum annual home range of an adult animal estimated in the study area using the minimum convex polygon with all locations (800.6 ha). Random paths were generated using the Geospatial Modelling Environment (GME, version 0.7.2 RC2; Spatial Ecology, LLC).

Landscape descriptors

Digital land cover maps of the study area were prepared through photointerpretation of aerial photographs from 2011, which were refined with field surveys. Variables describing landscape composition and configuration were then estimated within 100-m and 500-m buffers around observed and random paths, thereby accounting for different scales at which animals may perceive the landscape (Reding et al. 2013; Borthagaray et al. 2014). The 100-m scale was selected because it was sufficiently small to reflect the immediate surroundings perceived by an animal when following a path, while it was larger than the estimated average positional error of genet radiotracking (e.g. Dickson et al. 2005; Pe'er and Kramer-Schadt 2008). The 500-m scale was used to reflect the wider area available to animals when making movement decision, and it was set as about twice the median step length (236.2m) between successive locations estimated during the radiotracking sessions, corresponding to about one hour of foraging activity (Reding et al. 2013).

Landscape composition was characterised using proportional cover by dominant land cover categories (Table 1): oak forests, corresponding to areas dominated by cork and holm oaks, and with >30% canopy cover; olive orchards; open agricultural land;

and urban areas. Because genets are mainly associated with forest habitats, landscape configuration was described from three variables reflecting forest fragmentation (Table 1): forest patch size; forest edge density; and tree canopy openness, measured as the average distance among trees within each buffer (Levin et al. 2007). Fragmentation by roads was described using two variables (Table 1): road density; and distance to the nearest road crossing structure potentially used by genets, including large culverts, bridges and viaducts (Ascensão et al. 2007; Serronha et al. 2013). Finally, we included three variables describing the average distance of each path to the nearest area with important resources for genets, including: forest edges; riparian galleries; and water bodies (Carvalho et al. 2014). Land cover maps were produced and landscapes variables were estimated using QGIS 2.2-Valmiera (Quantum GIS Development Team 2014).

Data analysis

Prior to statistical analyses, skewed variables were transformed to approach normality and to reduce the influence of extreme values, using either the angular transformation for proportional variables or the logarithmic transformations otherwise (Table 1). All variables were then standardised to zero mean and unit variance, to enhance comparability of effect sizes (e.g. Schielzeth 2010). Colinearity was investigated using pairwise correlations, and one variable from each pair correlated at >0.7 was eliminated from further analyses (Dorman et al. 2013). From each pair, we retained the variable with highest biological meaning based on previous studies on genet habitats (Palomar’s et al. 1994; Carvalho et al. 2014)

Modelling was based on the Path Selection Function (PathSF) approach, whereby the landscape characteristics around observed foraging movements (paths) were compared with random paths with similar topology using mixed effects conditional logistic regression (Cushman et al. 2010; Reding et al. 2013). Analysis followed a match-control design framework, using a binomial variable coding the observed movement path (1) vs. nine random movement paths (0), thereby creating a group “stratum” (e.g. Cushman et al. 2011). Animal identities were specified as a categorical random effect, to account for lack of independence resulting from eventual individual idiosyncrasies in habitat selection (Sour et al. 2009).

Table 1 Univariate description and summary statistics of explanatory variables used to analyse path selection functions for observed paths vs. random paths by the genet in Southern Portugal. AB – variable abbreviation, SD – standard deviation.

Variables	**AB**	**Observed paths**		**Random paths**		**B***	**P-value**
		Mean ± SD	Range	Mean ± SD	Range		
Landscape composition (100 m buffer)							
Forest areas (%)	FOR10	0.79 ± 0.19	0.01-1.00	0.63 ± 0.28	0.00-1.00	0.78	< 0.001
Agriculture areas (%)	AG100	0.17 ± 0.16	0.00-0.91	0.30 ± 0.25	0.00-100	-0.69	< 0.001
Olive orchards (%)	OLV10	0.02 ± 0.04	0.00-0.22	0.04 ± 0.11	0.00-1.00	-0.26	0.015
Urban areas (%)	UB100	0.00 ± 0.01	0.00-0.11	0.00 ± 0.02	0.00-0.25	-0.44	< 0.001
Landscape composition (500 m buffer)							
Forest areas (%)	FOR50	0.73 ± 0.17	0.02-0.99	0.63 ± 0.23	0.00-1.00	0.61	< 0.001
Agriculture areas (%)	AG500	0.22 ± 0.15	0.00-0.93	0.31 ± 0.21	0.00-0.97	-0.54	< 0.001
Olive orchards (%)	OLV50	0.02 ± 0.05	0.00-0.42	0.04 ± 0.08	0.00-1.00	-0.11	0.24
Urban areas (%)	UB500	0.01 ± 0.01	0.00-0.10	0.01 ± 0.01	0.00-0.12	-0.20	0.016
Landscape configuration (100 m buffer)							
Canopy Closure (m)	CCL10	13.66 ± 30.71	0.00-360.57	35.50 ± 56.43	0.67-773.20	-0.83	< 0.001
Forest Edge Density (m/ha)	FED10	33.27 ± 22.96	0.00-102.73	38.79 ± 22.86	0.00-129.91	-0.22	0.002
Forest Patch Size (ha)	FPS10	39.76 ± 30.45	0.29-162.87	25.96 ± 24.97	0.00-161.96	0.95	< 0.001
Landscape configuration (500 m buffer)							
Canopy Closure (m)	CCL50	19.45 ± 24.61	1.93-230.59	36.07 ± 47.37	1.24-621.19	-0.56	< 0.001
Forest Edge Density (m/ha)	FED50	36.93 ± 15.38	4.86-79.75	38.85 ± 14.25	0.00-84.51	-0.14	0.06
Forest Patch Size (ha)	FPS50	116.33 ± 100.54	1.27-466.26	84.92 ± 84.63	0.00-540.85	0.49	< 0.001
Foraging effects							
Distance to Forest areas (m)	DFOR	12.91 ± 32.47	0.00-391.69	42.83 ± 73.77	0.00-840.316	-0.69	< 0.001
Distance to a Riparian	DRIP	84.92 ± 139.43	0.00-863.74	137.85 ± 166.36	0.00-863.96	-0.47	< 0.001
Distance to Water (m)	DWT	592.93 ± 307.21	93.89-1554.81	613.62 ± 404.88	19.45-2292.89	0.05	0.521
Road effects (both 100 and 500m buffer)							
Road Density (m/ha)	RDS10	2.43 ± 7.72	0.00-67.24	6.41 ± 13.71	0.00-122.89	-0.62	< 0.001
Road Density (m/ha)	RDS50	4.80 ± 7.21	0.00-30.74	6.19 ± 9.25	0.00-63.76	-0.21	0.02
Distance to a Crossing	DCL	928.75 ± 697.96	9.65-3035.53	978.02 ± 824.35	5.97- 3379.14	-0.11	0.198

* The estimated coefficients (β) and associated probabilities (p) testing the null hypothesis (β = 0), which were obtained from univariate conditional logistic regression, are provided to illustrate the differences between observed and random paths for each of the variables tested.

Data analysis started with a preliminary univariate screening to estimate variables significantly differentiating observed from random paths. Multivariate Path Selection Functions were then built, considering either the entire set of variables or the same set excluding the two road related variables. Model building was based on information theoretical and multimodel inference approaches of Burnham and Anderson (2002) using the Akaike Information Criterion (AICc) and the corresponding Akaike weights (wi). Candidate models were built based on all possible subsets of non-collinear explanatory variables, including the null (i.e., without explanatory variables) and the full (i.e., with all explanatory variables) models. Models were ranked according to their Akaike weights (wi), and the average parameters and their unconditional standard errors (SE) were estimated based on the 95% confidence set of models (Burnham and Anderson 2002). The relative importance of each variable was judged based on the sum of Akaike weights of models where the variable was included (w+), and on the magnitude of the average model coefficient. Analyses were performed using the packages mclogit (Elff 2013) and MuMIn (Barton 2013) for R 2.15.3 software (R Development Core Team 2013).

Landscape conductance mapping

Landscape conductance in the study area was mapped in a GIS, based on a raster land cover map at a 100-m resolution, and by applying to each grid cell the average Path Selection Function derived from the model building procedure. In practice, for each grid cell the landscape variables were estimated in a 100 m buffer, and then the Path Selection Function was applied to estimate the probability of the grid cell to be in an observed versus a random movement path of genets (Harjur et al. 2013). Probabilities were assumed to be related to increasing flow and lower resistance to movement (McRae et al. 2008; Roever et al. 2013), and so the map of estimated probabilities for all grid cells was taken as a conductance map. The final conductance (probability) layer was then resampled to 100 m resolution to reflect the real fine-grain of habitat present in study area but at the same time not compromising the analysis due to computer processing constraints when working with finer-grained landscapes.

A conductance map was also prepared for a virtual roadless landscape similar to our study area, thereby providing a baseline for landscape connectivity in the absence of roads. The roadless landscape was created by replacing the road land cover class at each grid cell by the dominant class in contiguous cells. The conductance map was then estimated as for the real landscape, though using a Path Selection Function developed without considering the road-related variables.

Quantifying road effects

The effects of roads on fragmentation were quantified by computing the total area with high conductance, and the mean size of patches with high conductance, in the real and the roadless landscapes. In these analyses we used a probability threshold of 0.50 to identify the area of high conductance. Larger values were also tested (0.75 and 0.90), but they produced patches that were unrealistically small and isolated, tending to underestimate strongly the areas actually used by genets in the study area. In analysis, patches < 5ha were eliminated, because this was roughly the minimum size of isolated forest patches used by genets (Filipe Carvalho, Unpublished Data).

Changes in connectivity patterns induced by roads were also estimated using the probability connectivity (PC) index which is based on graph theory, on the habitat availability concept, and on inter-patch dispersal (Saura and Pascual-Hortal 2007). Specifically, we evaluated the importance of each individual patch for the preservation of overall landscape connectivity using dPC (e.g. Nogués and Cabarga-Verona 2014; Rubio et al. 2015), which quantifies the relative decrease in PC after each patch is removed individually from the landscape (Saura and Pascual-Hortal 2007). Estimates were made using three plausible dispersal distances (1, 5, and 10 km) for the common genet and using a 0.5 dispersal probability value (Fu et al. 2010). Connectivity metrics analyses were performed using the Conefor Sensinode 2.6 software (Saura and Torné 2009).

Results

Univariate analysis underlined major significant differences in paths crossed by genets in relation to random paths (Table 1). In average, genet paths had higher proportion of forest land cover and lower proportion of agricultural and urban areas, larger forest patch size and lower edge density, canopy openness and road density. Also, they were closer to forests and riparian galleries than random paths. From the original variables, only seven with no significant pairwise correlations were retained in subsequent analysis.

In multivariate modelling including the seven explanatory variables, four models were retained in the 95% confidence interval of top models. The average model confirmed the strong positive effects ($0.88 < w+ < 1.00$) of forest patch size, and the strongly negative effects of road density, urban areas, and distances to forests, riparian areas and road crossing structures (Table 2). The predicted performance of the model was considered reasonable, as judged by the value of AUC (0.74). The analysis performed without the road-related variables showed much the same results, and its predictive performance was also considered reasonable (AUC = 0.73).

Table 2 Average models describing the estimated effects of explanatory variables on the probability movement path use by genets for both full model and model without road effects variables. For each case, multi-model averaging was based on the 95% confidence set of models. For each variable, we show the standardised regression coefficient (β), the unconditional standard errors (SE), the 95% confidence interval of coefficient estimate (CI), and the selection probability (w+). Variables selected in the best models are underlined and coefficient estimates whose 95%CI exclude zero are in bold.

Full model

Parameters	**β**	**SE**	**CI**	**w_+**
FOR100_PS	**0.663**	**0.194**	**(0.283, 1.044)**	**1.000**
FED100	-0.040	0.095	(-0.227, 0.146)	0.220
DFOR	**-0.326**	**0.129**	**(-0.577, -0.073)**	**0.930**
DRIP	**-0.564**	**0.194**	**(-0.724, -0.404)**	**1.000**
UB100	**-0.274**	**0.125**	**(-0.519, -0.029)**	**0.880**
RDES100	**-0.607**	**0.156**	**(-0.912, -0.302)**	**1.000**
DCL	**-0.528**	**0.125**	**(-0.772, -0.283)**	**1.000**

Model without road effects

Parameters	**β**	**SE**	**CI**	**w_+**
FOR100_PS	**0.684**	**0.158**	**(0.374, 0.995)**	**1.000**
FED100	-0.055	0.094	(-0.239, 0.128)	0.290
DFOR	**-0.345**	**0.124**	**(-0.587, -0.102)**	**1.000**
DRIP	**-0.557**	**0.080**	**(-0.715, -0.399)**	**1.000**
UB100	**-0.255**	**0.117**	**(-0.484, -0.025)**	**0.850**

The landscape conductance map produced using land cover data shows that genet habitats in the study area are considerably fragmented, with patches of adequate habitat (probability > 0.50) surrounded by vast areas with poor conditions for the species (Fig 2 A). In this context, the road network strongly contributes to fragmentation by dissecting otherwise continuous areas of suitable genet habitat. In contrast, riparian galleries contribute to increase landscape conductance by providing corridors in otherwise unsuitable habitats. The negative effect of roads could be visualised by comparing the conductance map for the real and the virtual roadless landscapes (Figs. 2 and 3), although they differed little in terms of the total area with high (> 0.50) conductance (2389 vs. 2341 km2) and in terms of the global probability of connectivity (PC) index (0.26 vs. 0.25). However, the mean size of high conductance patches was significantly smaller (Welch two sample t-test on log-transformed data: t = 2.389, d.f. = 659.81, *P* = 0.017) in the real (558.05 ha ± 10078.43) than in the virtual roadless landscape (754.46 ha ± 12110.97). The mean importance of individual patches to overall landscape connectivity (dPC) was slightly smaller in the real than in the virtual landscape, but the differences were not statistically significant for plausible dispersal distances of 1km (0.288 ± 4.849 vs. 0.373 ± 5.703; Welch two sample t-test on angular-transformed data: t = 0.086, d.f. = 592.19, *P* = 0.931), 5km (0.292 ± 4.842 vs. 0.378 ± 5.700; t = 0.049, d.f. = 590.06, *P* = 0.961), and 10km (0.292 ± 4.833 vs. 0.379 ± 5.696; t = 0.043, d.f. = 592.19, *P* = 0.966).

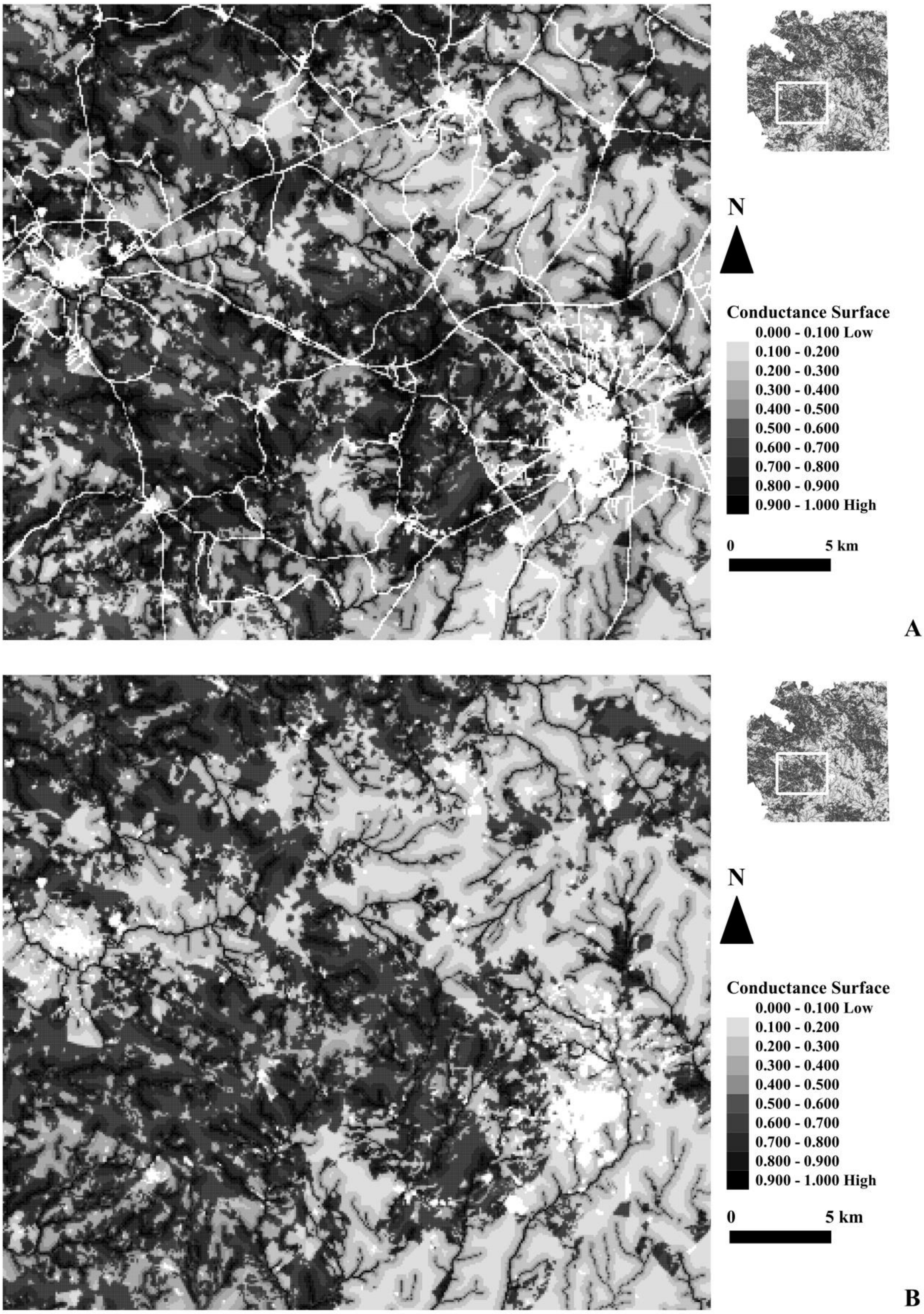

Figure 2 Conductance surface probability estimated for common genets in a section of the study area, using path selection functions and land cover maps from the real landscape (A), and from a virtual roadless (B) landscapes.

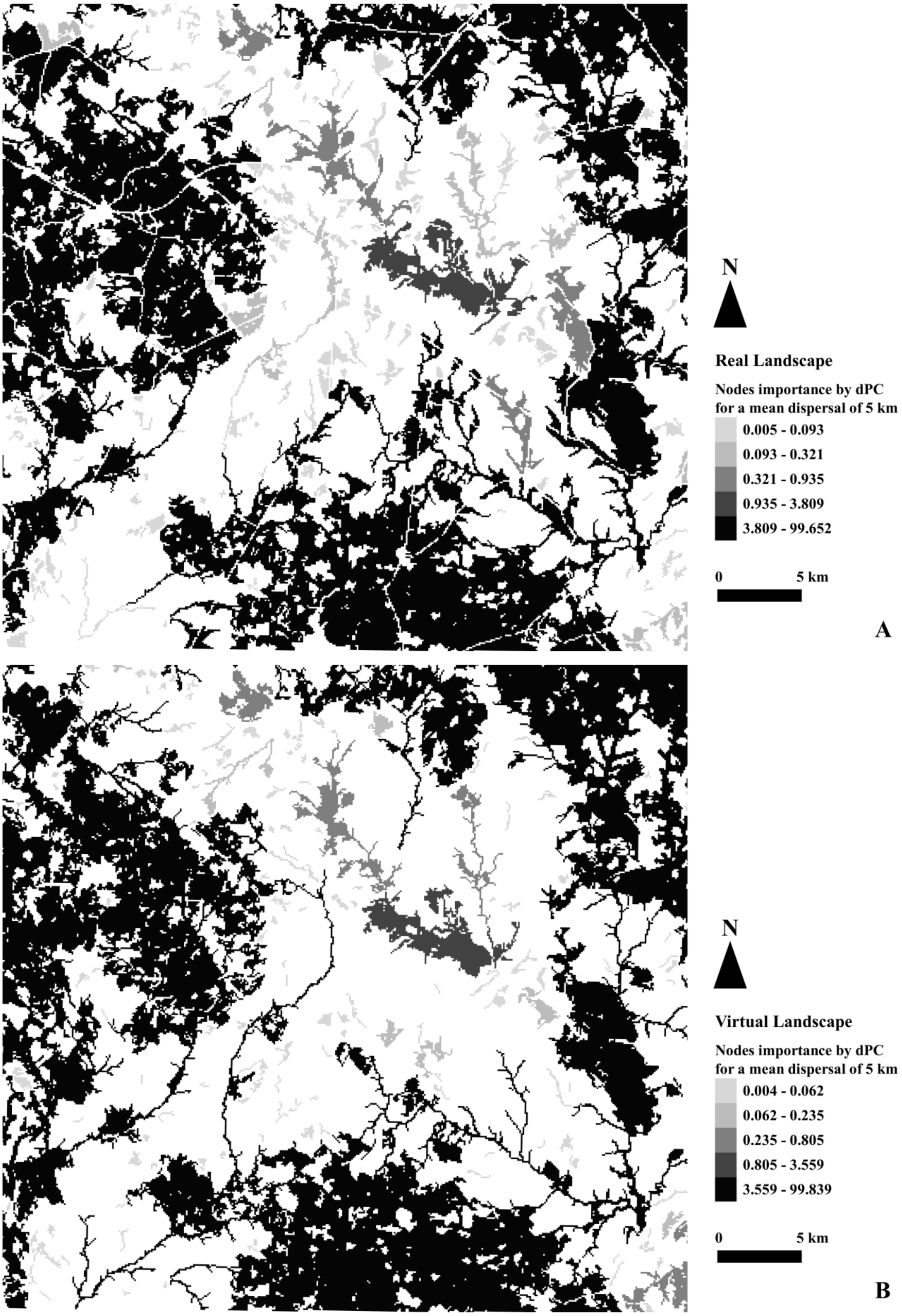

Figure 3 Map of a section of the study area depicting the relative importance of individual patches for overall landscape connectivity (node importance: dPC), for the real landscape (A) and for a virtual roadless (B) landscape. The importance of the patches is classified into five levels based on dPC as very high, high, medium, low, and very low (see e.g. Gurrutxaga and Saura, 2014), using the natural breaks data classification of QGIS.

Discussion

Our findings support the view that connectivity in human-dominated landscapes is jointly influenced by spatial habitat heterogeneity and the spatial distribution of human infrastructures such as roads. Although this idea is often assumed in studies estimating resistance to movement, most of these are based on expert opinion or landscape genetics patterns (Zeller et al. 2012), and only a very few provided compelling supportive evidence through detailed empirical movement data and adequate statistical analyses (Reding et al. 2013). In our particular case study focusing on a forest carnivore, spatial habitat heterogeneity was important because it shaped the extent, location and configuration of areas most permeable to movement, as well as those areas rarely crossed by the animals. The road network was also influential because it dissected highly permeable habitats, thus creating obstacles in areas otherwise permeable to animal movement. These results have direct conservation implications, providing a basis for identifying road sectors particularly detrimental to landscape connectivity, where the application of mitigation measures should be a priority. Overall, the study point out the relevance of path-level analysis to provide a mechanistic understanding of how spatial heterogeneity and linear infrastructures jointly influence landscape connectivity patterns.

Despite some limitations and potential shortcomings of our study, we believe that the key results were not shaped by methodological artefacts in any significant extent. Connectivity modelling may be affected by the selection and definition of environmental variables, including for instance the variables used, their measurement scale and their spatial resolution (Zeller et al. 2012). In our case these problems were probably minimal, because variables were carefully chosen and defined based on previous studies on the spatial ecology and behaviour of the common genet (e.g. Palomares and Delibes 1994; Galantinho and Mira 2009; Pereira and Rodríguez 2010; Carvalho et al. 2014). Another pervasive issue in connectivity modelling is that movement decisions by animals may not be made based on the same preferences they use in selecting habitat, thus requiring that the two behaviours need to be separated when estimating resistance values (Zeller et al. 2012). This problem may have affected our data, because it was not possible to discern the use of local resources (e.g., food and cover) from the movements through the environment to find and obtain such resources. However, this was minimised by tracking animals during their nocturnal activity periods, when we found them moving frequently across their home ranges, and by defining paths using fixes taken at about half hour intervals, thus avoiding area-restricted movement behaviour during foraging bouts. Furthermore, by tracking a large number of animals during long periods we expected to incorporate in analysis a wide range of path-level types, which should have avoided problems associated with idiosyncratic behaviour by some individuals (Ascensão et al. 2014) and contributed to identify the factors affecting movement decisions. A potentially more serious problem is that most animals tracked were resident adults, with no information collected on dispersing individuals. This may be important because dispersers may be less attached to particular habitat types, and they may be more prone to cross obstacles such as roads (F. Carvalho, Unpublished Data). As a consequence, it is possible that a more connected landscape would have been inferred if we had tracked dispersing rather than resident animals (Squires et al. 2013), though this should be the subject of further research.

The habitat factors affecting movement behaviour by the common genet were much similar to those influencing its habitat selection patterns (e.g. Palomares and Delibes 1994; Galantinho and Mira 2009; Pereira and Rodríguez 2010; Carvalho et al. 2014). Common genets were found moving more frequently than expected by chance within forest patches and close to riparian habitats, while strongly avoiding open agricultural land, which is in line with the pattern found for other medium-sized forest carnivores, including stone martens (*Martes foina*), American martens (*Martes americana*), pine martens (*Martes martes*) and bobcats (*Lynx rufus*) (Rondinini and Boitani 2002; Cushman et al. 2011; Mergey et al. 2011; Reding et al. 2013). The restriction of movements to forest and riparian areas may be a consequence of genets preferring to travel within habitats where food resources are abundant. This is supported by studies carried out in other Mediterranean areas, where genets were found to prey heavily on the wood mouse (*Apodemus sylvaticus*) (Virgós et al. 1999; Rosalino and Santos-Reis 2002), which like the genet can be arboreal and very abundant within oak forests (Rosalino et al. 2011). Genets may also prefer to move within forested and riparian areas due to the availability of protective cover for reducing predation risk (Cushman et al. 2011; Mergey et al. 2011), as they often rest in tree hollows and arboreal nests (Carvalho et al. 2014), and in open areas may be highly vulnerable to predators such as eagle owls (*Bubo bubo*), foxes (*Vulpes vulpes*) and domestic dogs (*Canis familiaris*). Whatever the reason, the patterns observed highlight the importance of forest cover as a surrogate for landscape connectivity in the common genet, which is consistent with previous studies for forest carnivores (Walpole et al. 2012; Nogués and Cabarga-Verona 2014). Also, they confirm the importance of riparian areas as movement corridors in otherwise unsuitable, open agricultural habitats, which was already suggested for the common genet (Pereira and Rodríguez 2010) and other species (Klar et al. 2012; Balestrieri et al. 2014).

The negative effect of roads on genets was suggested by the lower probability of movement in areas with higher road density, which is consistent with previous studies on the species (Galantinho and Mira 2009; Carvalho et al. 2014), and is in line with recent observations for other forest carnivores such as stone and pine martens (Santos and Santos-Reis 2010; Mergey et al. 2011). Reasons for this avoidance are uncertain, but they may be related to a negative behavioural responses to noise and other types of human disturbance (Santos and Santos-Reis 2010; Carvalho et al. 2014), which individuals possibly take as indicators of risk to their survival. Despite this avoidance, however, we found that movements were located closer to road crossing structures such as culverts, bridges and viaducts, than what might be expected by chance. This result was likely to be driven at least partly by the association of these structures with riparian habitats along streams and rivers, which were shown to have a strongly positive effect on genet movements. However, it cannot be discarded the possibility of the structures themselves attracting genet movements, as they are known to be used by the species for road crossing (Serronha et al. 2013; Villalva et al. 2013).

As a consequence of its movement preferences described through the path selection function, the landscape connectivity surface developed for the genet was directly influenced by spatial habitat heterogeneity and the spatial distribution of roads. Connectivity across the landscape was strongly favoured by the presence of large forested areas, though these were surrounded by an open agricultural matrix highly resistant to genet movements. This largely hostile matrix, however, was crossed by riparian habitats which appear to act as movement corridors. The road network introduces important modifications in these connectivity patterns, mainly by separating forest patches that otherwise would be highly connected. As a consequence, patches were smaller and their relative importance for overall connectivity (dPC) was higher than what would be the case in a roadless but otherwise similar landscape, though the later effect was not significant probably due to the very large variation between patch sizes within each landscape type. Roads also crossed the riparian corridors, but their effect on connectivity was probably low due to the presence of viaducts, bridges and other crossing structures which provide permeability to movements (Grilo et al. 2012; Serronha et al. 2013; Villalva et al. 2013). As observed for tawny owls (*Strix aluco*) in the same landscape (Santos et al. 2013), connectivity was high despite the fragmentation by roads, because there was still a large proportion of forested habitat in the landscape. Therefore, further analysis across a gradient of habitat loss would be needed to fully appreciate how the interactions between spatial heterogeneity and human linear infrastructures affect landscape connectivity (Crouzeilles et al. 2014).

Our results have important implications for the conservation management of landscapes fragmented by roads, which may apply both to genets and to other forest carnivores such as marten species (Grilo et al. 2009; Ascensão et al. 2014), wildcat (*Felis silvestris*) (Klar et al. 2009; 2012) and Iberian Lynx (*Lynx pardinus*) (Ferreras et al. 2010). First, crossing of large forest patches by roads should be avoided as much as possible, because it strongly reduces the main movement pathways for forest carnivores in fragmented landscapes. Second, riparian areas should be given high conservation priority because they provide the main movement corridors for forest carnivores in otherwise highly hostile matrices (Balestrieri et al. 2014). These corridors may be crucial to link distant forest patches, thus playing a key role in metapopulation demographic processes and gene flow across the landscape. Third, crossing structures such as culverts, bridges and viaducts associated with riparian habitats should be regarded as key elements to assure connectivity across the landscape (Grilo et al. 2012; Serronha et al. 2013; Villalva et al. 2013). Thus efforts should be taken to assure the integrity of riparian habitats close to roads and under viaducts and bridges, thereby favouring the use of these structures as safe road crossing points by the animals. Finally, environmental planning of new roads and other linear infrastructures should be based on detailed information on landscape connectivity patterns for target species of conservation concern, which is required to identify routes with minimal impacts on wildlife, and to design cost-effective mitigation measures where impacts cannot be avoided (Rudnick et al. 2012; Cushman et al. 2013; Polak et al. 2014).

Our results join a very small subset of studies based on empirical movement data and path-level analysis to model landscape connectivity (e.g. Cushman and Lewis 2010; Cushman et al. 2011; Reding et al. 2013). Although these studies are laborious and time consuming, they seem to provide a stronger basis for inference than other alternatives such as expert opinion and landscape genetics approaches (Cushman 2010; Zeller et al. 2012). In our particular case study, the path-level approach was essential to determine the combined effects of spatial habitat heterogeneity and human linear infrastructures on landscape connectivity, which were unlikely to be detected with other methods. Given the paucity of studies carried out so far, it would be important to foster the application of the path-level modelling approach to a wide range of ecological settings, thereby providing a basis for generalization across species and landscape types. Ultimately this should prove useful to inform practical applications in the fields of landscape conservation management and impact assessment of human infrastructures, most of which will remain reliant on expert opinion due to resource limitations.

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5. GENE FLOW ACROSS A MOTORWAY IN A FOREST CARNIVORE: JOINT EVIDENCE FROM ROADKILLS, RADIOTRACKING AND GENETICS

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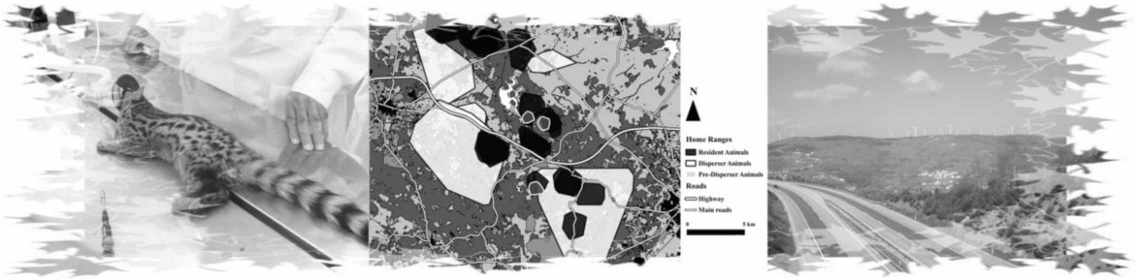

Gene flow across a motorway in a forest carnivore: joint evidence from roadkills, radiotracking and genetics

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Abstract

Roads can have negative effects by restricting connectivity across the landscape. Using a combination of roadkill, radiotracking and microsatellite data for common genets, we evaluated patterns of dispersal, gene flow and genetic differentiation across a motorway in a Mediterranean landscape from south-western Europe. Genets were often found roadkilled, particularly subadults (65%), with a peak during the dispersal period. Resident adults almost never crossed the motorway, which was often coincident with a range boundary; home range sizes were similar near (453.1 ha; n = 7) and far (456.1 ha, n = 12) from the motorway. In contrast, dispersing subadults and nomadic adults often crossed the motorway, and their home ranges were almost ten times larger than that of residents, encompassing both sides of the motorway. We found five father-offspring pairs and 19 half-sibling relationships across the motorway, and estimated high migration rates to the north and south directions as 0.398 and 0.098, respectively. STRUCTURE and GENELAND analyses did not show any evidence for spatial genetic structure associated with the motorway. Overall, our results suggest that the motorway allowed high levels of gene flow, despite the traffic casualties and the constraints to adult movements. These results contrast with most studies reporting highly negative genetic consequences of roads, but they may be common where there are low traffic volumes, numerous road crossing structures, and for species that have the physical ability to negotiate obstacles easily, with high population sizes on both sides of the road, and without significant social constraints to effective migration.

Keywords: carnivores; dispersal; home ranges; gene flow; motorway; territoriality

Introduction

Successful movements of animals across a heterogeneous landscape are important drivers of ecological and evolutionary processes (e.g. mating, foraging, movement), being particularly critical not only for long-term stability of populations dynamics but also for adaptation (e.g. distribution range shifts) on a changing environment (Nathan et al. 2008; Morales et al. 2010; Baguette et al. 2013). Hence, disruption of movement patterns through landscape changes such as habitat fragmentation may have a profound effect on populations' persistence (Fahrig 2003; Fischer and Lindenmayer 2007; Crooks et al. 2011). There is thus a need to understand how animals move across heterogeneous landscapes, and how these movements are affected by human infrastructures and land use changes (Hartmann et al. 2013; Poessel et al. 2014).

Linear infrastructures (e.g. roads, railways) are recognized as a major threat to biodiversity, largely contributing for the fragmentation of natural landscapes (Forman et al. 2003; Fahrig and Rytwinski 2009). Roads may interfere with animal movement and gene flow by either constituting a physical obstacle due to road mortality (Van Langevelde and Jaarsma 2004; Roe et al. 2006; Jackson and Fahrig 2011) or through behavioural avoidance (Riley et al. 2006; Shepard et al. 2008; Leblond et al. 2013). This decrease of connectivity between areas adjacent to roads may genetically isolate populations, compromising their long-term viability (Frankham 2005; Holderegger and Di Giulio 2010). Despite all studies conducted on this subject, proper assessment of barrier effects is not a simple task mainly due to two confounding factors: (1) barrier's characteristics such as traffic volume, lane width and age (Van Langevelde and Jaarsma 2004; Landguth et al. 2010); and (2) species life history traits and behaviour flexibility (Riley et al. 2006; Rytwinski and Fahrig 2012; Beyer et al. 2013). Therefore, understanding how these complex interactions govern movement patterns is crucial for the implementation of effective mitigation measures (Corlatti et al. 2009).

Mammalian carnivores have received special attention regarding negative impacts of roads. They exhibit intrinsic features (e.g. low population densities, high probability of encountering roads due to their high vagility and large area requirements) which make them especially vulnerable to genetic and ecological effects of habitat fragmentation (Benítez-López et al. 2010; Crooks et al. 2011; Rytwinski and Fahrig 2012). Additionally, they represent a clear example of the difficulty associated with road impact studies because carnivores exhibit complex behavioural traits such as territoriality or dispersal variation between sexes and life stage (Boitani and Powell 2012; Stoner et al. 2013), which influence their susceptibility towards roads at the individual level (Riley et al. 2006; Poessel et al. 2014). Thus, barrier effects may be context specific and in particular cases very hard to analyse. For example, the study conducted on stone martens by Ascensão et al. (2014) is an illustrative example of this complexity, where the authors found inconsistent behavioural responses patterns among individuals. Therefore, it is possible that linear infrastructures may be a barrier to some individuals but not for others, which may have far-reaching implications for population genetic connectivity across human dominated landscapes. Analysing this issue is difficult, however, because it requires detailed information on variation in movement patterns for a large number of individuals varying for instance in age, sex, and territorial status, and how they react to potential barriers created by linear infrastructures (Riley et al. 2006, 2014; Hartmann et al. 2013; Poessel et al. 2014).

Combining multiple complementary sources of information such as radiotracking and molecular data may help overcome these difficulties. Studies which aimed to assess the effects of linear infrastructures are generally limited to either direct registration of movement by radiotracking (Klar et al. 2009; Ascensão et al. 2014; Carvalho et al. submitted) or molecular data (Frantz et al. 2010, 2012; Sawaya et al. 2014). Therefore, incomplete data may be gathered and used to analyse how anthropogenic barriers affect connectivity, possibly having severe consequences for mitigation planning (Corlatti et al. 2009). While telemetry registers contemporary dispersal patterns at the individual level, providing data about fine-scale movement behaviour and other ecological processes (e.g. food, resting sites, intraspecific interactions), genetic data reflects effective dispersal during several generations that led to gene flow (i.e. reproductive events; Spear et al. 2010; Habel et al. 2015). Therefore, to obtain a clearer picture about genetic and ecological consequences caused by linear infrastructures, the combination of both types of data may provide a more robust framework than using each method separately. So far, only Riley et al. (2006) managed to collect both radiotracking and genetic data in order to address the genetic and behavioural effects of a freeway on genetic structuring and dispersal behaviour on coyotes (*Canis latrans*) and bobcats (*Lynx rufus*). Whereas microsatellites revealed that the motorway was indeed an effective genetic barrier, radiotracking provided additional data regarding home ranges distribution, behavioural interactions among adults and subadults and migration patterns of the latter. The authors ended up with fundamental information about connectivity dynamics on their study area for both species, that otherwise would not be possible to attain if only one approach was used instead.

In the present study we used a combination of roadkill, radiotracking and genetic data to examine the barrier effects of the A6 motorway on a Mediterranean forest carnivore, the common genet (*Genetta genetta*, Linnaeus 1758). Our objectives are: (1) to investigate differences in home range size and shape for resident animals living close and far from the motorway; (2) to evaluate genetic differentiation between both sides of the motorway; (3) to identify and quantify potential discrepancies of crossing events between resident vs. subadult individuals, using radiotracking and parentage data; and (4) to estimate gene flow between both sides of the motorway. Based on genets’ life history traits (Camps and Alldredge 2013; Carvalho et al. 2014) and other empirical studies concerning carnivores (e.g. Riley et al. 2006; Frantz et al. 2010;

Hartmann et al. 2013; Sawaya et al. 2014), it is predicted that at least, adult individuals will show a higher degree of avoidance to the presence of a motorway (i.e. their home ranges will be delimited by the motorway). Nevertheless, given the intrinsic features of A6 (recently constructed and low traffic volume) and the bolder behaviour towards the motorway shown by dispersing subadults, genetic differentiation should be minimal and gene flow high between both sides of the A6. The results will be used to discuss the value of combining different sources of information to understand the barrier effects of roads, and to evaluate measures to mitigate such effects.

Material and Methods

Study area

The study was conducted in an area of about 4600 km^2 in southern Portugal (38°21'39'' to 39°01'36'' N, 08°24'22'' to 07°34'54'' W; Fig. 1). The area is crossed centrally by the A6 motorway, which is a low traffic flow < 1000 vehicles night-1 (mean = 977; range = 330-2494; Grilo et al. 2009) motorway with two lanes each side separated by a central splitter. The motorway was established on 1997, and runs parallel to a national road along a ≈ 13 km stretch between the cities of Montemor-o-Novo and Évora. The motorway is not associated with any major natural barrier (e.g. mountain or river) and is rarely hidden (through noise barriers or embankments). Climate is Mediterranean, with mean daily temperature ranging from 5.8 ºC to 12.8 ºC in winter, and from 16.3 ºC to 30.2 ºC in summer; annual rainfall averages 609.4 mm and is concentrated in October-March (Évora 1971–2000; IPMA, 2012). The relief is undulating (150m-430m above sea level) and the landscape encompasses a large area (≈ 43.5%) of open to closed cork oak (*Quercus suber*) and holm oak (*Q. rotundifolia*) forests, where the understory may be herbaceous or shrubby depending primarily on grazing pressure. Agricultural areas are the main habitat (≈ 45%), comprising mainly dry arable land and pastureland, with or without sparse oaks trees, and olive orchards and vineyards. Other land cover types include intensive olive orchards, and some scattered plantations of *Pinus* spp. and *Eucalyptus* spp. (≈8.8%).

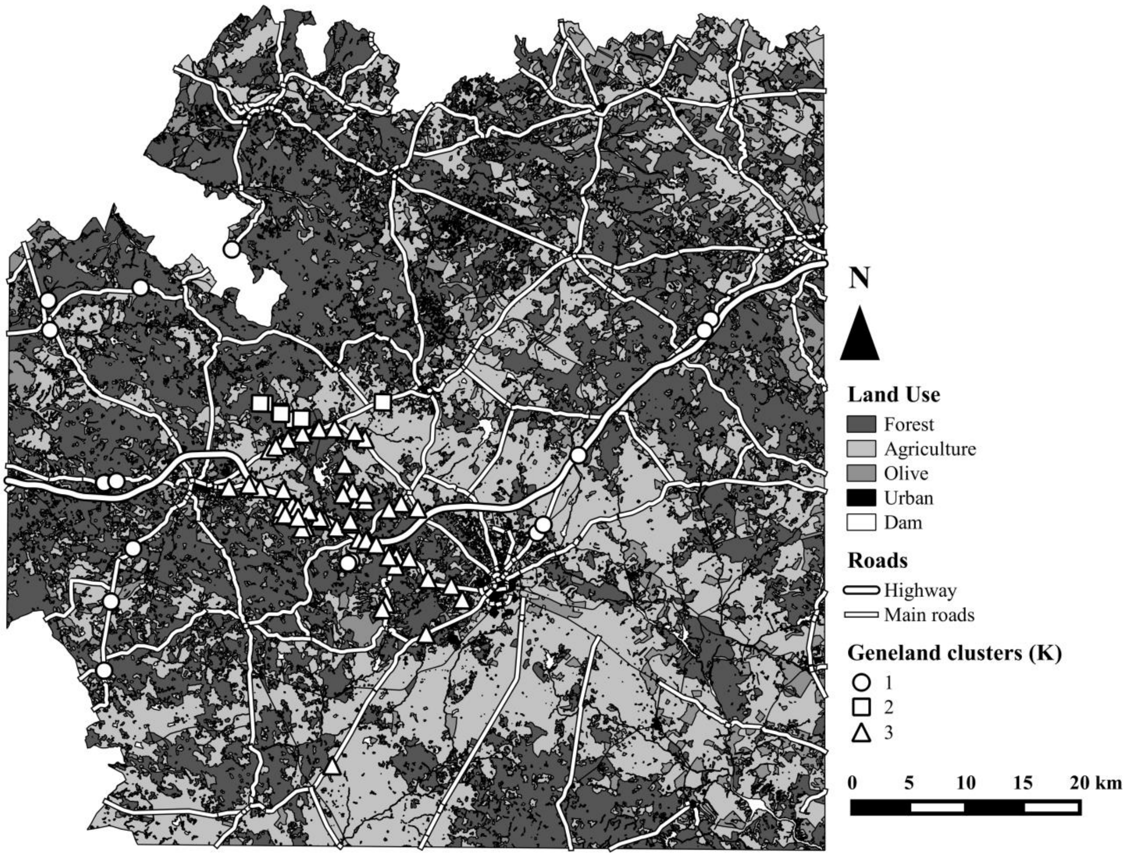

Figure 1 Map of the study area showing the main land use classes, road types and locations where common genets were sampled for genetic analyses. For each sample we indicate the cluster membership (K=3) inferred by GENELAND.

Study design

Inferences of genet responses to the A6 motorway were based on a combination of roadkill, radiotracking and genetic data. Data on genet roadkills were used to estimate the periods of peak road mortality, aiming primarily to assess whether peak mortality coincident with the main periods of juvenile and subadult dispersal, but also, to provide surrogate information regarding individual barrier behavioural responses towards the motorway during different life stages. Radiotracking was used to evaluate whether the motorway influenced the size and spatial organization of genet home ranges, particularly the extent to which home ranges of residents were crossed or not by the motorway. In addition, radiotracking was used to directly record road crossing events, and to estimate whether crossing rate was influenced by the social status of the animals. Genetic data was also used to estimate the occurrence of crossing events and movement rates across the motorway, based on parentage and sibship relatedness among individuals. Finally, we estimated genetic differentiation and spatial population structure to assess the degree of gene flow and infer critical biological periods that may be mainly responsible for gene flow.

Roadkill data and analysis

Roadkilled genets were recorded through systematic road surveys performed in a vehicle driven at slow speed (20 km h^{-1}) on a weekly (2007 and after March 2013) and daily basis (from January 2008 until March 2013), along approximately 51 km (and 150 km after March 2013) of national and municipal roads. Temporal mortality patterns in these roads were assumed to be similar to those in the A6 motorway, which could not be surveyed due to access restrictions. Based on carcass retention rates estimated in our study area (Santos et al. 2011), this sampling schedule was deemed adequate to detect virtually all genets roadkilled in the surveyed roads during the study period. The position of each roadkill was recorded using a GPS, and the carcass was taken to the laboratory for age determination based on teeth condition (milk teeth – cubs; definitive new teeth – juveniles; and different degree of waste – adults (most > 2 years)), and for biometric measurements such as body length and weight (Rodriguez-Refojos et al. 2011).

We used Kruskall-Wallis to test differences in genet roadkill rates (roadkills/100km/year) across four key periods in the genet life cycle: dispersal (October-November and March), breeding (December-February), birth (April-May) and rearing of young (June-September) (Grilo et al. 2009; F. Carvalho, Unpublished Data). If significant variation was detected, we used a posteriori multiple comparison “two tail” tests for pairwise differences between periods (Siegel and Castellan 1988; Sokal and Rohlf 1997).

Radiotracking data and analysis

Radiotracking data was obtained from 27 genets, which were live-trapped on both northern (n=11) and the southern (n=16) side of the A6 motorway, between May 2010 and December 2011 (Table S1). Live-trapping was carried out using home-made box traps. Captured animals were immediately carried to the veterinarian hospital at Évora University, where sexed, aged, measured for total length and weight, and instrumented with a VHF radio-collar, following the legal and ethical protocols. After release, radiotracking locations (fixes) of each animal were obtained through standard biangulation (2 bearings) techniques performed by an observer in a car, at less than 5-minute intervals to reduce location errors (e.g. Millspaugh and Marzluff 2001). For the two observers involved in radiotracking, we estimated the mean positional error as 92.6 m (SD = 51.4 m, range: 14-179m), based on 50 blind comparisons for each observer, between real positions evenly distributed across the main habitat types and the locations retrieved through telemetry. Mean number (±SD) of radiotracking locations per animal used in home range estimation was 165.5 ± 67.3, which corresponded to daily resting site locations (≈ 23.4 % of total) and at least two night radiotracking cycles per animal per month. Detailed description of trapping, handling and radiotracking procedures are provided in Carvalho et al. (2014) and Carvalho et al. (*submitted*).

The home range of each animal was estimated with the Minimum Polygon Convex (MCP) method, using 95% of the fixes (e.g. Millspaugh and Marzluff 2001). We used non-parametric Wilcoxon sign-rank tests for non-paired samples to compare average home range sizes between residents and dispersers living far (animals, which home range border was at least 2 km from the motorway) and near the motorway (Sokal and Rohlf 1997). Animal still depending on mothers (pre-dispersers) and that typically showed small home ranges were discarded from this analysis. The two sexes were combined, because genets show no sexual differences in resting site behaviour (Carvalho et al. 2014) and home range size (Santos-Reis et al. 2004; Carvalho et al. 2014). Home range areas were calculated using the plugin HRS under the QGIS environment (Home range estimation with R, version 2.1.8; Ghisla 2012).

Radiotracking data were also used to estimate the number of crossing events per 100 days (night) per animal, using crossings observed during night radiotracking cycles, and from pairs of consecutive daytime resting sites in opposite sides of the motorway. We used non-parametric Wilcoxon sign-rank tests for non-paired samples, to assess possible crossing patterns differences among residents and dispersers living in the vicinity of the motorways (Sokal and Rohlf 1997). We caution that results may be conservative and some crossings may have not been tracked. However, by analysing our night cycles during radiotracking, we noticed that returns after a first road crossing during the same night (foraging period) were rare and happened only in two resident animals which had secondary roads embedded in their home ranges. Likewise, we believe that our road crossing index truly represents crossing patterns of residents and dispersers genets in our population.

Genetic data and analysis

A total of 92 samples for genetic analyses were collected (live-trapped, n=32; roadkilled n=60; Table S2), corresponding to 33 adults, 49 subadults, and 10 individuals with undetermined age. A total of 45 and 47 samples were obtained to the north and to the south of the A6 motorway, respectively. Muscle (n=51) or blood (n=29) samples for genetic analysis were taken from each animal, except in a few roadkilled carcasses (n=12) where we took plucked hair samples due to an advanced state of decomposition. From this initial dataset, three individuals had to be discarded because the hair samples were too degraded, and so only 89 individuals (males, n = 42; females, n = 46; unidentified sex, n = 1) were genotyped. DNA was extracted using EasySpin Genomic DNA Tissue Kit (Citomed), in accordance with manufacturer's protocol. In the case of hairs, about ten hairs with visible root bulbs were used to increase quantity and quality of DNA extract (Goossens et al. 1998). Extraction quality and quantity was checked on a 0.8% agarose gel. From an initial set of 20 microsatellites developed specifically for the common genet (Gaubert et al. 2008; Fernandes et al. 2009), a total of 17 were selected and combined on multiplex reactions for Polymerase Chain Reaction (PCR) and used for genotyping. Details regarding primers, multiplexes, and PCR conditions are provided in the Supplementary Methods and Table S3. Amplification success was evaluated on a 2% agarose gel. PCR products were run on an ABI3130XL capillary sequencer (Applied Biosystems) and scored using GeneMapper version 4.0 (Applied Biosystems). To minimise genotyping errors, a second independent amplification was performed on a subset (≈34%) of randomly chosen high quality samples (blood and tissue) (Bonin et al. 2004; Guichoux et al. 2011). In the case of low quality samples (hairs), consensus genotypes were constructed according with the observation of the same genotypic locus profile two times for heterozygotes and three times for homozygotes (see Gagneux et al. 1997; Bonin et al. 2004). Loci that consistently failed to amplify were coded as missing data. Individual sexing was made using two new primers – genY7 and genX5 – designed using PRIMER3 (Untergasser et al. 2012), based on sequenced PCR products generated by primers that amplify conserved regions of X and Y chromosomes in mammals (DBY intron 7 and DBX intron 5, Hellborg and Ellegren 2003, 2004, repectively; see details in Supplementary Methods, Table S4 and Fig. S1).

We estimated allele dropout and false allele rates per genotype from the two PCR replicates, using Pedant 1.0 (Johnson and Haydon 2007) with 10000 steps. Due to different error proneness, high quality samples and hair samples were assessed separately. Unbiased null allele frequencies for each marker were calculated in INEst

2.0 (Chybicki and Burczyk 2009) using Population Inbreeding Model, with 200 000 cycles, burn-in 20 000 and each iteration was saved every 100 cycles. A standard one tailed z-test was performed to test if the estimated frequency for each locus was significantly higher than zero ($\alpha = 0.05$). Deviations from Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium (LD) were assessed in Genepop 4.2 (Rousset 2008). Parameters for exact tests were set to 10000 dememorizations, 5000 batches and 25000 of batch length. False Discovery Rate (FDR) method (Benjamini and Hochberg 1995) was employed to correct the level of significance of exact tests, once multiple comparisons are carried out among loci pairs. As HWE and LD analyses may be biased in the presence of related individuals, we repeated the analysis excluding all but one of each group of individuals sharing parent-offspring or full-sibling relationships (see Colony2 analysis). Number of alleles, observed and expected heterozygosities for each locus was calculated in GenAlEx 6.5 (Peakall and Smouse 2012).

We inferred parental and sibship relationships through the full-likelihood approach implemented in Colony2 (Jones and Wang 2010), assuming polygamy for both sexes, no inbreeding, high likelihood precision and medium run length. Values of allele dropout, false alleles and null alleles calculated in Pedant and INEst were included in Colony2. As high quality samples were more represented in the dataset and have considerably less genotyping errors than hair samples, we used a weighted mean based on the ratio of high to low quality samples (about 9:1) to infer the rate of genotyping errors. We carried out two independent analysis, assuming a probability of an offspring having a parent in our candidate parent sample of either 0.2 or 0.3 (these values are based on the mean adult genet home range size, which give us an idea of the breeding population size in our study area). Three independent runs (i.e., with different random seeds) were run for each probability scenario to assess results convergence. We accepted family relationships as “true” when a pair of individuals exhibited a posterior probability of ≥0.95 at least for two replicates of each considered probability scenario. A movement across the motorway was assumed when we found parent-offspring, siblings or half-siblings sampled on opposite sides of the motorway. In the case of parent-offspring, we generally assumed that the motorway crossing was made by the offspring during dispersal movements, which is supported by our roadkill and radiotracking data, and also scientific studies (Grilo et al. 2009; Quaglietta et al. 2013). In the cases of pre-dispersal offspring (i.e. small-sized juveniles that presumably were still dependent on the mother), we assumed that the crossing was made by the parent. In the case of sibship groups with n individuals sampled on one side of the road and m individuals sampled on the other side of the road, and if $n > m$, we assumed that there was a minimum of m crossing events and that the direction was from n to m. The spatial pattern of kinship inferred in Colony2 was spatially visualized using the option “Kinship Links” from Arctoolbox Landscape genetics 1.2.3 (Etherington 2011).

Migration rates across the motorway were estimated genetically using the likelihood method of Wang (2014) implemented in MigEst software. Unlike other widely used methods (e.g. Wilson and Rannala 2003), this approach does not require high differentiation among population (F_{ST}>0.02; Wang 2014), and so it could be used with our particular dataset. MigEst is based on parentage assignment data, estimating migration through the number of offspring individuals that are in population i and whose have assigned parents at population j. The method assumes that adult reproductive individuals do not migrate to other populations. This assumption was validated by our radiotracking data, which revealed that movements across the motorway were mainly made by subadult dispersers. However, care should be taken when interpreting the results, because resident males may perform extraterritorial movements for reproductive purposes (Soulsbury et al. 2011), which may have occurred in our study (see Results). We assumed that there were no unsampled populations. Parentage data was derived from the results of Colony2, using a proportion of either 0.2 or 0.3 candidate parents on each side of the motorway.

Genetic differentiation between populations separated by the motorway was estimated using pairwise F_{ST} estimates, computed using GenAlEx 6.5. Population genetic structure was estimated using the Bayesian clustering algorithms implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000) and GENELAND 4.0.4 (Guillot et al. 2005; see details in Supplementary Methods). These approaches were chosen because they have been used successfully to detect recent barriers (e.g. Riley et al. 2006; Frantz et al. 2012; Sawaya et al. 2014) and performed well in simulations in terms of both success rate and required generations to detect a barrier to gene flow (Blair et al. 2012). In STRUCTURE analysis, the best value of K was estimated according the method described by Evanno et al. (2005), as implemented on Structure Harvester v0.6.93 (Earl and vonHoldt 2012). For the best estimated K, the proportion of population ancestry (q) of each individual was calculated by averaging q from five replicate runs. Results were visualized using DISTRUCT 1.1 (Rosenberg 2004). GENELAND uses a spatially explicit model to infer population structuring, based on the coordinates for each individual sample (Table S3). The best K was determined from the modal value of the posterior distribution along the MCMC chain and individual cluster membership was averaged across the five replicate runs for the K inferred.

Results

Roadkills

During the study period we detected 89 roadkilled genets, corresponding to a mean roadkill rate of 12.8 individuals/100km/year. There were marginally significant differences in roadkill rates among biological periods (Chi-squared = 6.68; *P* = 0.083), with a tendency for a much higher mean roadkill rate (± 95%CI) in the dispersal period (10.7 ± 5.2) than in the breeding (7.0 ± 2.3), birth (7.00 ± 2.0) and rearing of young (4.8 ± 1.5) periods. About 65% of roadkilled genets were subadults.

Home range patterns

Overall, we radiotracked 27 common genets, including 11 at the southern side and 16 at the northern side of A6 (Fig. 2; Table S1). A total of 19 individuals were considered residents, because they were adults and they had relatively small and well-defined home ranges. Mean 95% MCP home range size for residents was 455.0 ± 124.5 ha. There was no significant difference (W = 38.01; *P* = 0.77) between the size of home ranges close (453.1 ± 95.4 ha, n = 7) or far (456.1 ± 142.8 ha, n = 12) from the motorway. Likewise, there were no differences (W = 52.03; *P* = 0.54) between the sizes of home ranges located to the north (464.1 ± 143.8 ha) and to the south (442.6 ± 100.2 ha) of the motorway. None of the 95% MCP home ranges of residents were crossed by the motorway. Instead, the motorway corresponded to a range border for genets radiotracked close to it. Overall, we detected only four crossings of the motorway by resident genets, which were made by just one animal using a large bridge and a viaduct.

Two radiotracked genets were subadult males and they had very large home range sizes (4908.0 ha and 3429.7 ha) comparatively to residents (Fig. 2; Table S1), and were thus considered dispersers. We also radiotracked one adult female with a very large home range (5748.9 ha) and nomadic movement behaviour, which was considered a disperser in the context of this study. These three dispersers were detected crossing the motorway 11 times, thereby showing a significantly higher (W = 1.02; *P* = 0.02) crossing rate (crossing index 4.10 ± 2.75) than the seven resident genets living close to the motorway (crossing index 0.21 ± 0.57). An additional animal (SG13) was captured as a juvenile at south of the motorway on 2008 on the scope of other study (Ramalho 2009), and was later recaptured as adult resident on the opposite side of the motorway in our study, thereby representing another motorway crossing event.

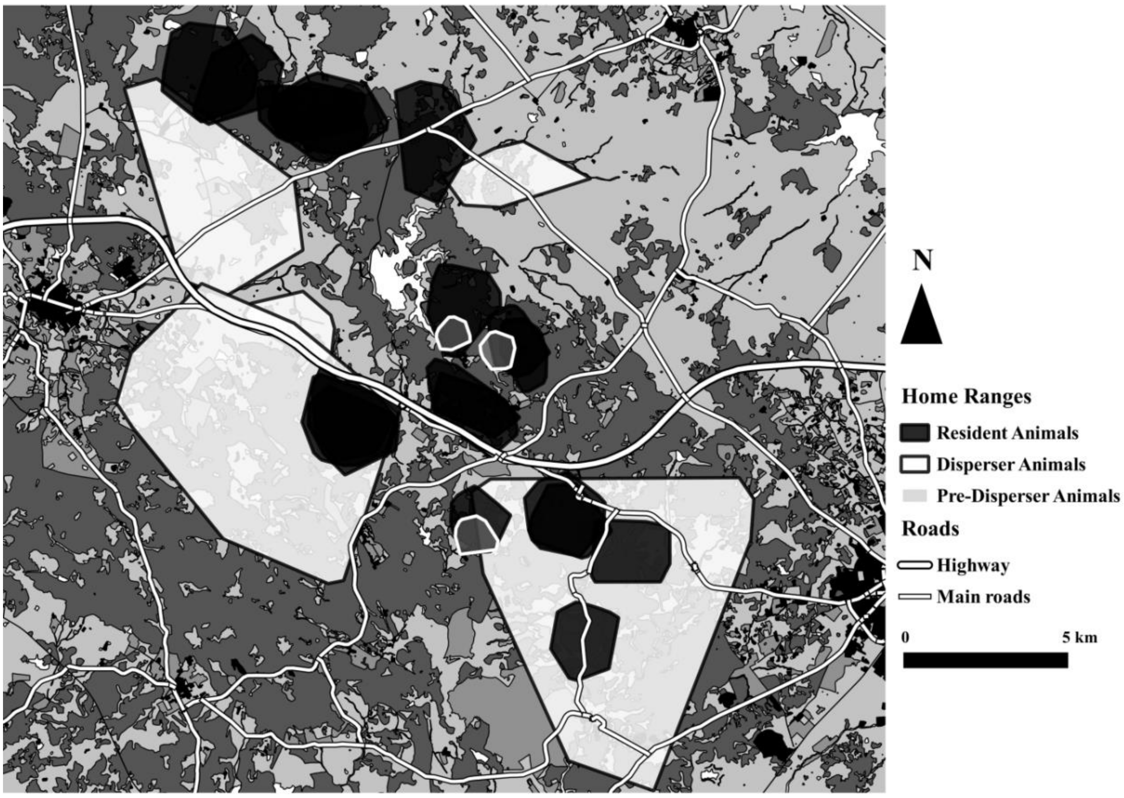

Figure 2 Home ranges of the 27 radiotracking common genets in both sides of the motorway.

Parentage, Migration rates and genetic differentiation

The 17 genotyped microsatellites were polymorphic, with a number of alleles ranging from 3 to 11 (Table 1). The mean (± SD) observed heterozygosity per locus was 0.662 ± 0.122, while mean expected heterozygosity was 0.669 ± 0.124 (Table 1). Allele dropout and false allele rates were low, although their mean values were higher for hair (AD = 0.045; FA = 0.018) than for high quality samples (AD = 0.004; FA = 0.002). Ggen2.1 was the only microsatellite showing a null allele frequency significantly different from zero (nf = 0.074; P = 0.017), and was thus removed from subsequent analysis. No marker showed HWE deviations, while LD calculations revealed that pair Ggen4.10/Ggen3.3 was on linkage disequilibrium (P = 0.0003, adjusted FDR α = 0.0004). After removing ten related individuals inferred in Colony2, this pair was no longer on LD (P = 0.004, adjusted FDR α = 0.0004) and thus both markers were retained.

Table 1 Diversity measures (Na – number of alleles; Ho – observed heterozygosity; He – expected heterozygosity) and genotyping errors statistics (AD – allele dropout; FA – false alleles; nf – null alleles frequency) calculated for high quality (hq) and hair (ha) samples calculated for each locus. Overall calculations for diversity measures do not include Ggen2.1 once it as removed.

Locus	Na	Ho	He	AD (hq)	FA (hq)	AD (ha)	FA (ha)	nf
A104	4	0.674	0.629	0	0	0	0	0
A108	4	0.337	0.382	0.051	0	0.131	0	0.061
C101	5	0.663	0.678	0	0	0.233	0	0
D111	7	0.607	0.713	0	0	0.083	0.062	0.074
D4	5	0.708	0.737	0	0	0.088	0	0.005
Ggen2.1	11	0.742	0.863	0.024	0.026	0.130	0	0.074
Ggen2A16	8	0.798	0.774	0	0	0	0	0
Ggen4.10	11	0.802	0.859	0	0	0	0	0.045
Ggen4.12	5	0.596	0.700	0	0	0	0	0.070
A110	7	0.541	0.510	0	0	0	0	0
A5	3	0.674	0.652	0	0	0	0.056	0
B105	6	0.562	0.616	0	0	0	0.057	0.021
Ggen2A13	4	0.632	0.647	0	0	0.105	0	0.014
Ggen3.3	8	0.759	0.737	0	0	0	0.085	0.026
A112	3	0.640	0.595	0	0	0	0	0
B104	7	0.862	0.827	0	0	0	0.056	0
Ggen2A15	8	0.730	0.779	0	0	0	0	0.030
Overall	5.99	0.662	0.669	0.004	0.002	0.045	0.019	0.025

A total of 96 pairs of individuals sharing kinship affinities were identified using Colony2, from which nine were father-offspring, four mother-offspring, four full-sibling and 79 half-sibling relationships (Table 2; Table S5). The five father-offspring pairs provided evidence for at least four movements across the motorway (Table 2; Fig. 3), including the likely dispersal from north to south of two subadults (SG3 and MG27). The two other cases probably reflect the movements of adults rather than dispersal by subadults, because the offspring (SG4, SG26, SG27) were young and presumed to be still dependent on their mothers. One of these cases refer to a possible extraterritorial movement of a radiotracked male (SG12) with a well-defined home range on the northern side of the motorway, which apparently sired an offspring (SG4) on the opposite side of the motorway. The other case was a male (SG1) captured as an adult and radiotracked for a short period due to transmitter failure, that two years later appeared to have sired two offspring (SG26 and SG27) on the opposite side of the motorway. From these parentage data, MigEst inferred migration rates of 0.398

(95%CI: 0.060-0.824) in the South-North direction, and 0.098 (95%CI: 0.004-0.810) in the North-South direction. Estimates were equal either assuming a proportion of 0.2 or 0.3 of candidate parents sampled. From the half-sibling dataset, we counted a minimum of 11 crossing events (Fig. 3; Table S5) from which we were able to identify the migration into south of at least one subadult (SG16).

Table 2 Parent-offspring (OF–offspring) and full-sibling relationships estimated with Colony2, and inference of movements across the motorway. Asterisks indicate individuals from kin pairs sampled on opposite sides of the motorway, which were used in MigEst.

Parent	OF 1	OF 2	OF3	Motorway crossing	Direction
Father-offspring					
SG7*	SG3*	MG27*	-	SG3, MG27	North → South
SG12*	SG4*	SG8	SG14	SG12	North → South
SG13	SG10	SG15	-	-	-
SG1*	SG26*	SG27*	-	SG1	South → North
Mother-offspring					
SG11	SG9	-	-	-	-
SG22	SG24	PG5	-	-	-
SG14	PG10	-	-	-	-
Full siblings					
-	MG14	MG24	-	-	-
-	MG21	MG32	-	-	-
-	MG67	SG19	-	-	-
-	MG22	MG38	-	-	-

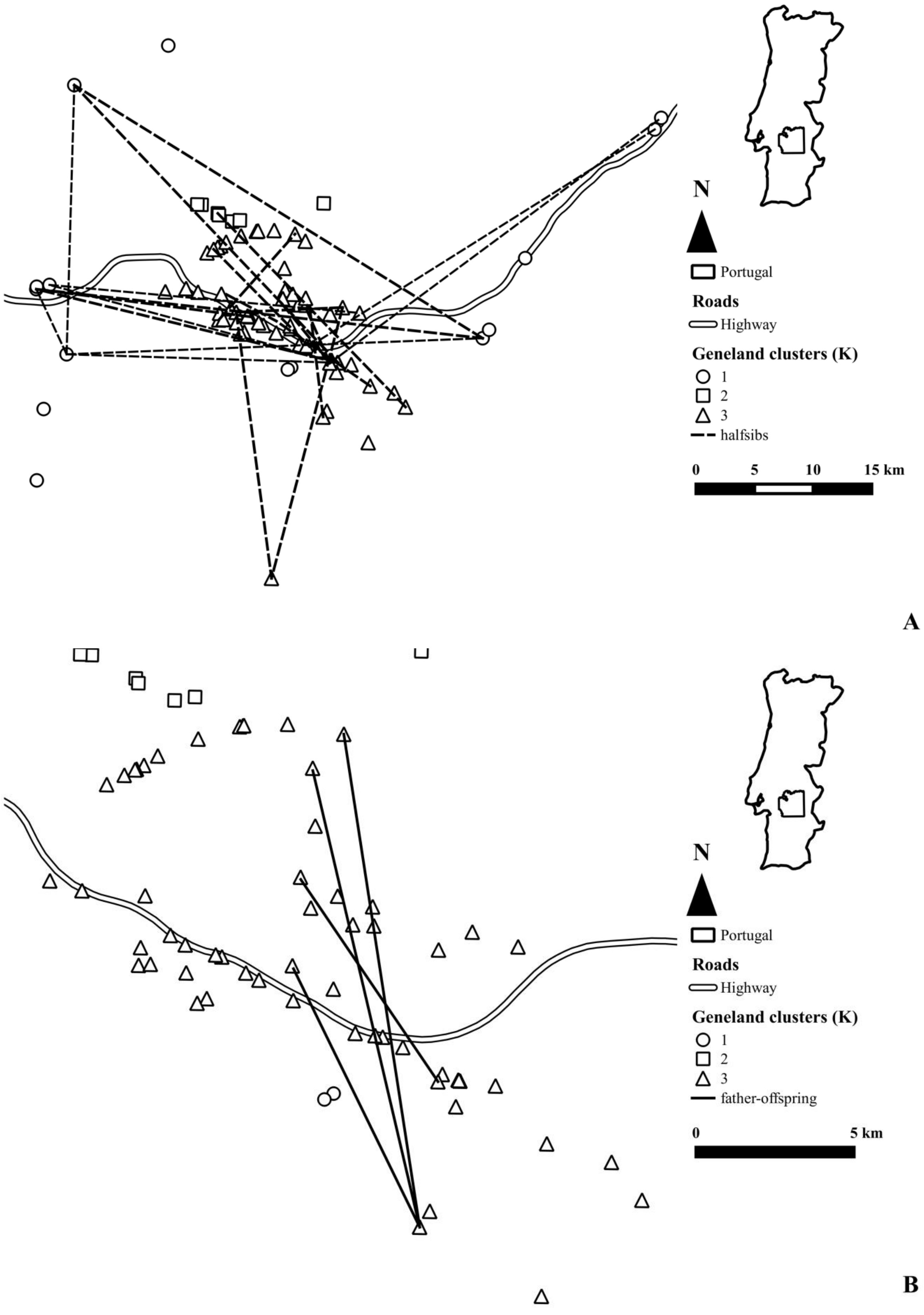

Figure 3 Genet sampling locations and movements across the A6 motorway inferred through half-sibling relationships (A) or parent-offspring (B).

STRUCTURE identified K=2 as optimal according to Evanno's Method, but there was no geographic meaning in relation to the presence of the A6 motorway (Fig. 4). From 45 individuals sampled at north, 24 individuals (≈53%; mean (± SD), q = 0.852 ± 0.130) were assigned to south of A6. This trend was also observed on the 44 sampled individuals at south, where 25 individuals (≈57%; mean (± SD), q = 0.836 ± 0.103) from 44 sampled at south were assigned to the north. GENELAND inferred K=3 as the most likely number of clusters, but there was also no evidence for spatial population structuring caused by the motorway (Fig. 1). Sixty four individuals (≈72%) were assigned to a cluster in the centre of the study area where sampling was more aggregated, while 16 genets (≈18%) mainly at east and west of the study area comprised other genetic group. Nine individuals (≈10%) located at north of the study area formed the remaining cluster.

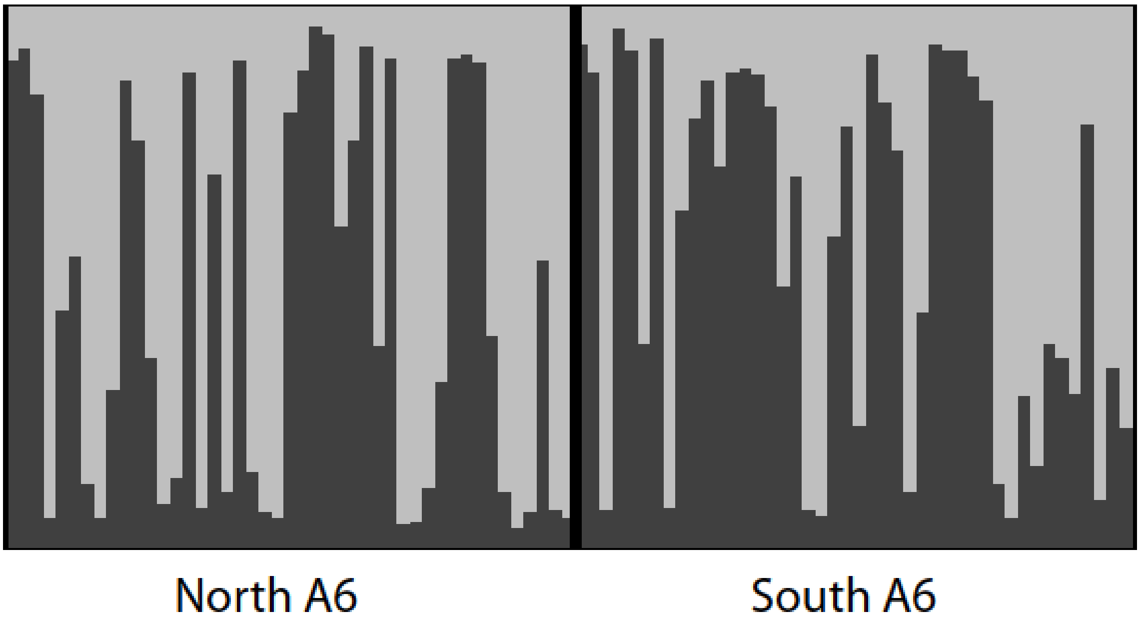

Figure 4 STRUCTURE bar plot showing admixture values averaged across ten runs for the best supported cluster number (K=2) of north and south sides of the motorway.

Discussion

The combination of roadkill, radiotracking and genetic data provided interesting evidences that a motorway was a barrier to animal movement, although concurrently allowing considerable levels of gene flow in both lateral directions. We confirmed previous studies suggesting that genets and other medium-sized carnivores are highly vulnerable to traffic casualties, particularly during dispersal period, which hinders free movement of individuals across the landscape and thus reduce connectivity (Grilo et al.

2009; Quaglietta et al. 2013). Additionally, the higher crossing rates registered by radiotracking also supported the premise of subadults exhibiting a bolder behaviour towards roads comparatively to adults. Kinship analysis further confirmed these results, with familiar relationships detected across the motorway. Combining all these evidences, we suggest that gene flow may be mediated by subadults dispersers, the nomadic movement behaviour of a few adults, and by extraterritorial excursions of resident males. As a consequence, gene flow rates across A6 were very high, contributing for the absence of population genetic structure associated with the presence of the motorway. Overall, our results point out the value of combining different information sources to gain a mechanistic understanding of the barrier effects of roads (e.g. Riley et al. 2006; Finnegan et al. 2012), and suggest that in some circumstances there may be considerable gene flow across roads despite major constraints to animal movement.

Gene flow across the motorway might be probably driven to a large extent by the dispersal movements of genet subadults, as suggested by a much higher crossing rate observed for dispersers than for resident adults, by some familiar relationships observed across the motorway and the disparate high number of subadults road fatalities. Like other carnivores (Riley et al. 2006; Mergey et al. 2011; Bonte et al. 2012), dispersing genets probably travel long distances, and likely adopt a bolder behaviour and travel through less suitable habitats than adults. This bolder behaviour may also be illustrated by our roadkill dataset, where about 65 % of fatalities were subadults, supporting the fact that subadults are more prone to mortality during dispersal (Grilo et al. 2009; Quaglietta et al. 2013). Nevertheless, other factors may explain this roadkill pattern, such as the greater knowledge/experience of adults when crossing roads (Ascensão et al. 2014), but given our radiotracking data, the former explanation may be most likely. By contrast to dispersing juveniles, resident adults appeared to rarely cross the motorway. Their home ranges were often coincident with the motorway, and thus it appeared to function as an artificial boundary, further supporting the idea that it constrained the movement of resident individuals. This may reflect an avoidance behaviour of roaded areas which are perceived as risky, as suggested in a recent study using path-level analysis (Carvalho et al. *Submitted*). However, we also found evidence that in at least some circumstances genet adults may move across the motorway, with potential consequences for gene flow. One possibility seems to be the extraterritorial excursions sometimes made by resident males (Aubry et al. 2004; Soulsbury et al. 2011), eventually leading to an animal siring offspring on both sides of the motorway. This idea was compatible with one father-offspring relationship identified through parentage analysis, in which the offspring was too young and thus still dependent on the mother, suggesting that motorway crossing was made by an adult male and not by a dispersing subadult. There is also the possibility of gene flow associated with the nomadic behaviour of some adult individuals, which may eventually settle in a vacant breeding territory or mate extraterritorially with a locally resident individual. Some evidence for these possibilities was provided by the nomadic behaviour of a radiotracked adult male (SG1) that ranged widely on both sides of the road, and by a father-offspring relation involving one adult male and two pre-dispersal offspring. Therefore, adults may also contribute to gene flow even when their movements are strongly constrained by a road barrier, though the extent of this contribution is difficult to quantify.

Genetic clustering and migration analyses further add evidence for the existence of high gene flow rates. First, although STRUCTURE and GENELAND identified two and three population clusters, respectively, these were not geographically associated with the motorway or any other obvious barrier to gene flow, and may not represent true population units. Given that, most likely the motorway is not an effective barrier to gene flow, patterns of spatial autocorrelation will arise across the study area, and Bayesian clustering algorithms tend to spuriously overestimate the number of clusters on those situations (Frantz et al. 2009; Meirmans 2012). These results indicate that gene flow maintained by the effective migration of subadults and some adult genets was probably large enough to counteract the eventual effects of drift and high rates of direct road mortality (Wang 2004; Lowe and Allendorf 2010; Jackson and Fahrig 2011). This is also supported by the high migration rates estimated through MigEst, though these should be taken as rough approximations and thus interpreted carefully, as they were based on just a few parent-offspring pairs, yielding very wide confidence limits. Furthermore, MigEst assumes that movements are made only by dispersing offspring, though adults could also perform migration events and contribute to gene flow.

It is thus apparent that the studied motorway was highly permeable barrier to gene flow for genets, in contrast to what has been reported for a number of other mammalian species (Riley et al. 2006; Frantz et al. 2012; Sawaya et al. 2014). The absence of a strong barrier effect was likely a consequence of multiple factors, most related with intrinsic features of the motorway and species biology and ecology. A6 motorway has a very low daily traffic volume (about 15 times lower than the motorway investigated in Riley et al. 2006) which implies lower disturbance levels (Van Langevelde and Jaarsma 2004). The low traffic volume and hypothetical high effective population size (see Gauffre et al. 2008; Prunier et al. 2014) in this area (Carvalho et al. 2015) may also contribute to the fact that despite the negative role of road mortality in the successful crossing rates (Grilo et al. 2009; Jackson and Fahrig 2011; also the large number of subadults fatalities in our study), a significant proportion of successful crossings most likely occur, being enough to maintain gene flow in the study area for this species. Additionally, radiotracking revealed that dispersing genets (and one adult) used occasionally a range of structures such as bridges, viaducts, culverts and other underpasses (Ascensão et al. 2007; Grilo et al. 2008; Serronha et al. 2013), further contributing to reduce the barrier effect of the motorway, though this is not always associated with the occurrence of high gene flow (Riley et al. 2006). Furthermore, there seemed to be no major social obstacles for dispersing individuals to move across the motorway and breed successfully, in contrast to results of Riley et al. (2006). In Riley's et al (2006) study, it was reported that dispersing bobcats and coyotes could easily cross a motorway, but effective dispersal was low due to tight packing of territories along the road, and the low turnover rate of breeding adults. As a consequence, gene flow was hindered because dispersing individuals could rarely find a vacant territory across the road. In genets, the size of home ranges was similar near and far from the motorway, suggesting that territory packing was not particularly high close to the road. It is possible that the turnover of breeding adults was relatively high due for instance to illegal predator control, which may be an important cause of genet mortality in the region (Beja et al. 2009). Therefore, it is likely that dispersing subadults could eventually find a vacant territory on the other side of the road, as supported by the parentage analysis, thereby assuring effective dispersal.

It cannot be ruled out, however, that the lack of differentiation was due at least in part to time-lag effects between barrier construction and population genetic responses (Landguth et al. 2010). Since its construction in 1997, only about 7-8 genet generations have experienced the motorway effects, which may be not enough time for the population to genetically respond. Despite existing theoretical (Blair et al. 2012) and empirical (Kuehn et al. 2007) evidence showing that a genetic signal can be indeed detected within a number of generations comparable to the number of genets' generations experiencing barrier effects in our study area, we acknowledge that the factors mentioned on the previous paragraph may increase time-lag. Although this possibility cannot be assessed at present, it should be noted that simulation (Blair et al. 2012) and empirical (Frantz et al. 2010; Sawaya et al. 2014) studies have shown that a genetic barrier signal will be evident within less generations for species with high dispersal capabilities such as genets and other carnivores, because the patterns of isolation by distance IBD are more rapidly disrupted.

Like Riley et al. (2006), we demonstrated the value of combining multiple sources of data to address specific questions concerning conservation issues in road ecology. At first sight, our field (roadkills and radiotracking) and molecular results seem contradictory, once the former revealed that the motorway imposes movement constraints (road mortality for subadults and behavioural avoidance for adults), while the latter showed high levels of genetic connectivity between opposite sides of the motorway. Instead, these approaches complemented in our study, offering a clearer picture regarding the complexity of ecological and genetic responses towards a linear barrier. Specifically, our integrative framework was especially useful regarding three aspects. First, the isolated use of each approach could have led us to spurious conclusions, i.e., the use of molecular data alone would point to an absence of any barrier effects, disregarding completely the social consequences caused by the motorway to resident adults. However, the isolated use of roadkill and radiotracking data could have led us to acknowledge A6 as a major barrier to movement, fact disproved by gene flow and population structuring analyses. Secondly, gave us some hints of possible important crossing paths that are leading to effective migration (such as viaducts). Despite we detected few crossing events at these structures, with more advanced techniques such as GPS satellite telemetry (lighter ones for small carnivores) more information could be retrieved concerning this aspect (Cushman et al. 2013; Riley et al. 2014). Third, we took advantage of radiotracking to increase parentage analysis confidence. By having access to precise age data and dispersal condition (disperser, nomad or territorial), we were able to solve ambiguous relationships. For example, this enabled us to detect extraterritorial movements across A6 for reproductive purposes, something that otherwise would not be possible.

Overall, our results are in contrast with most other studies examining the genetic effects of roads, which suggested that they represent very important barriers to gene flow, potentially promoting serious inbreeding and loss of genetic diversity (Riley et al. 2006, 2014; Jackson and Fahrig 2011). It is uncertain, however, whether the pattern reported here for the genet is exceptional, or whether it is relatively common but under reported due to publication bias against negative results. We believe that the latter may be true for roads and species exhibiting similar characteristics to the ones of our study, illustrating the importance of using a model system such as common genets to acquire important insights in the field of Road Ecology. Additionally, we demonstrated the invaluable importance of combining different sources of data to obtain more robust information, which could have important conservation implications at the level of planning and implementation of mitigation measures. For example, this information would help identifying the circumstances under which investments are most needed to mitigate negative road effects, thereby optimizing the allocation of limited human and financial resources. Therefore, when feasible, on behaviourally complex species, we recommend that the only viable option in conservation and mitigation plans is to incorporate different sources of data, to better understand the complex and dynamic relationships between a species and major anthropogenic barrier.

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Supplementary Material

Gene flow across a motorway in a forest carnivore: joint evidence from roadkills, radiotracking and genetics

Supplementary Methods

Microsatellite amplification

From the initial set of 20 microsatellites, we discarded Ggen2A25, Ggen2A2 and Ggen 1.30 due to consistent amplification failure during multiplex optimization. Therefore 17 microsatellites were multiplexed in three reaction sets (Table S3). To perform multiplex reactions, forward primers were modified at 5' end to enable tagging with fluorescently labelled oligonucleotides (6-FAM, VIC, NED or PET). A "PIG-tail" (GTTT) was added to 5' end of reverse primers to ensure full adenylation during PCR. Each PCR reaction contained 5 µl of Multiplex PCR Kit Master Mix (QIAGEN, Valencia, CA, USA), 3 µl of distilled H2O, 1 µl of primer multiplex mix and 1-3 µl of DNA extract, yielding a total volume of 10-12 µl per reaction. A negative control was used to identify any eventual contamination. Touchdown PCR cycling conditions were equal across all multiplexes: initial denaturation at 95ºC for 15 min, followed by 9 cycles of denaturation at 95ºC for 30 s, annealing at 58ºC with a temperature decrease of 0.5ºC each cycle and elongation at 72 ºC. Following this, 28 cycles of denaturation for 30 s at 95ºC, 60 s at 54ºC, 30 s at 72 ºC and finally, 8 additional cycles for 30 s at 95ºC, 45 s at 53ºC and 30 s for 72ºC, finishing with a final extension at 60ºC during 30 min

Primer development for sexing

Published primer sets which amplify conserved regions of X and Y chromosomes in mammals – DBY intron 7 (Hellborg and Ellegren 2003) and DBX intron 5 (Hellborg and Ellegren 2004) - were tested on a set of eight high quality samples (five known males and three known females) to check if these universal primers worked on this species. PCR reactions contained a total volume of 10-12 µl and were performed as follows: 5 µl Multiplex PCR MasterMix (QIAGEN, Valencia, CA, USA), 3.2 µl of distilled H_2O, 0.4 µl of 10 µM forward primer, 0.4 µl of 10 µM reverse primer and 1-3 µl of DNA sample. Optimized PCR program consisted of an initial activation step at 95ºC for 15 min, followed by 12 cycles of denaturation at 95ºC for 30 s, annealing at 61ºC with a temperature decrease of 0.5ºC each cycle and extension at 72ºC for 45 s, followed by 23 cycles of denaturation at 95ºC for 30 s, annealing at 56ºC during 30 s, extension at 72ºC for 45 s and a final extension step at 60ºC during 5 min. Sex determination was performed visually in a gel electrophoresis (one fragment – female; two fragments – male). Although individuals were correctly sexed (product size superior to 300 bp and 400 bp, for DBY intron 7 and DBX intron 5 respectively), primers failed to amplify on samples of lower quality, such as some hair samples or very degraded muscle tissue. To circumvent this problem, these PCR products were sequenced in order to redesign new primers – genX5 and genY7 (Table S2). It is expected that primers that generate shorter amplified fragments will increase PCR efficiency. Successful amplifications were purified using ExoSAP (constituted by a mix of exonuclease I and Shrimp Alkaline Phosphatase enzymes; Applied Biosystems, Foster City, CA, USA). Sanger sequencing reactions were carried out on 10 µl reaction volumes with the following composition: 0.4 µl of termination reaction reagent BigDye (Applied Biosystems, Foster City, CA, USA), 1 µl of BigDye buffer (Applied Biosystems, Foster City, CA, USA), 0.5 µl of 10 µM forward primer and 7.1 µl of distilled H_2O. Sequence reaction profile was as follow: initial denaturation at 94ºC for 3 min, followed by 24 cycles at 96ºC for 10 s, annealing at 50ºC for 5 s, finishing with a elongation step at 60ºC for 4 min. Reaction sequence products were sequenced on an ABI3130XL capillary sequencer (Applied Biosystems, Foster City, CA, USA). Sequenced fragments were screened on BIOEDIT (Hall 1999). From these fragments, primers genX5 and genY7 were designed using PRIMER3 (Untergasser *et al.* 2012). Primers were tested using 32 individuals (29 blood and 3 hair samples) where the sex was known. PCR reaction consisted on 5 µl of Multiplex PCR Master Mix (QIAGEN, Valencia, CA, USA), 1.5 µl of 10 µM genY7 forward and reverse primers and 0.1 µl of 10 µM genX5 forward and reverse primers. The cycling conditions were: 95ºC for 15 min, followed by 12 cycles at 95ºC for 30 s, 58ºC with a temperature decrease of 0.5ºC each cycle and 72ºC for 30 s, followed by another set of 28 cycles of 95ºC for 30 s, 53ºC for 30 s, 72ºC for 30 s and a final extension step at 60ºC for 5 min. Sex determination was performed via visual inspection in a gel electrophoresis (one fragment – female; two fragments – male ; Fig. S1)

STRUCTURE and GENELAND analyses

STRUCTURE was run using a recently developed model which allows the inclusion of sampling information (Hubisz et al. 2009). A higher prior weight is put on clustering results when they are correlated with the provided sampling information, providing more power to this analysis to detect fine genetic structure. It may be argued that STRUCTURE is forcing the outcome of false positives (i.e. detection of genetic structure when on reality is absent), but simulated data in Hubisz et al. (2009) showed that this model is not biased towards false positives. Admixture and correlated allele frequencies models were set for ten independent runs (to assess convergence) analysing K = 1 to 5, where each run had a burn-in period of 100 000 iterations and 1 000 000 Markov Chain Monte Carlo (MCMC) iterations.

In GENELAND, five independent runs with K varying between 1 and 5 were performed using the correlated model of allele frequencies, no spatial uncertainty, maximum rate of Poisson process fixed to 90 and maximum number of nuclei in the Poisson-Voronoi tessellation was 270. Total number of MCMC iterations was 1 000 000 with a thinning of 200.

Supplementary Tables

Table S1 Summary data of 27 radiotracked genets. Information regarding sex, radiotracking period, life stage (TER – Territorial; NOM – nomadic; DISP – disperser; PRED – pre-disperser) and home range area are represented. For each animal we provide the number of crossing events of A6, the crossing index (in brackets), and the crossing structures used (CUL - culverts; BRI - bridge; VIA - viaduct; ROAD - road surface; NA – not applicable). *Animals studied during 2008 but not in a daily basis.

ID	Sex	Period	Life stage	Home range (ha)	Motorway	Crossing structure
SG2	F	153	TER	374.5	0	NA
SG3	F	259	NOM	5748.9	0	NA
SG5	M	150	DISP	372.9	2 (1.3%)	CUL(2)
SG6	F	259	TER	480.8	4 (1.5%)	BRI(2);VIA (2)
SG7	M	80	TER	294.5	NA	NA
SG8	M	164	PRED	78.0^B	NA	NA
SG9	M	112	PRED	115.3^B	NA	NA
SG10	F	174	PRED	94.7^B	NA	NA
SG11	F	37	TER	268.6	NA	NA
SG12	M	193	TER	448.2	NA	NA
SG14	F	204	TER	240.6	NA	NA
SG15	M	200	TER	276	NA	NA
SG19	M	169	TER	602.4	NA	NA
SG20	F	211	TER	495.8	NA	NA
SG22	F	389	TER	620.7	NA	NA
SG23	M	46	TER	497.7	NA	NA
SG24	F	240	TER	441.5	NA	NA
SG25	F	326	TER	652.5	NA	NA
SG26	F	148	DISP	122.8^B	0	NA
SG28	F	134	TER	627.9	0	NA
PG2	M	133	TER	474.9	0	NA
PG5	F	325	TER	524.5	NA	NA
F1*	F	216	TER	393.7	0	NA
M1*	M	131	TER	429.8	NA	NA
M2*	M	144	DISP	4908	6 (4.2%)	CUL(6);BRI(10)
M3*	M	199	TER	501	NA	NA
M4*	M	44	DISP	3429.7	3 (6.8%)	BRI(4);CUL(8)

Table S2 Samples used on genetic analyses. Information concerning sample source (R-roadkilled; T-trapping), sample type (MU-muscle; BL-blood; HA-hairs), sex, age and if the sample was obtained at north (N) or south (S) of A6 are displayed. Additionally, we provide information regarding UTM coordinates and the criteria used to select coordinates as input in Geneland (RS-roadkill site; TS-trapping site; HRC-home range centroid). Asterisks denote samples not included on genetic analyses.

ID	Source	Type	Sex	Age	Side A6	Geneland	UTM X	UTM Y
MG2	R	MU	male	adult	N	RS	575787	4281626
MG3	R	MU	female	adult	S	RS	585927	4271838
MG4	R	MU	female	subadult	S	RS	583250	4273209
MG5	R	MU	male	subadult	S	RS	588657	4265063
MG6	R	MU	male	adult	S	RS	585842	4271007
MG8	R	MU	female	adult	N	RS	585210	4275953
MG9	R	MU	male	subadult	N	RS	575172	4281302
MG10	R	MU	male	adult	N	RS	578896	4282932
MG11	R	MU	male	subadult	S	RS	582620	4273270
MG12	R	MU	female	-	S	RS	576720	4276266
MG13	R	MU	female	-	S	RS	585902	4271851
MG14	R	MU	male	subadult	N	RS	578797	4282902
MG15	R	MU	male	adult	S	RS	578359	4275613
MG16	R	MU	female	subadult	S	RS	579546	4274900
MG17	R	MU	female	adult	S	RS	585399	4272022
MG18	R	MU	male	adult	S	RS	588741	4269885
MG19	R	MU	male	subadult	S	RS	585962	4271821
MG20	R	MU	female	subadult	S	RS	583489	4273166
MG21	R	MU	female	subadult	N	RS	575555	4281501
MG22	R	MU	female	subadult	N	RS	560103	4277637
MG23	R	MU	male	subadult	N	RS	560132	4277851
MG24	R	MU	female	subadult	N	RS	577489	4282490
MG25	R	MU	male	subadult	S	RS	591783	4268153
MG26	R	MU	male	subadult	S	RS	579127	4275118
MG27	R	MU	male	subadult	S	RS	573899	4277628
MG28	R	MU	female	adult	S	RS	590799	4269336
MG29	R	MU	female	subadult	N	RS	586268	4276537
MG30	R	MU	female	subadult	S	RS	572875	4277923
MG32	R	MU	male	subadult	N	RS	575509	4281480
MG33	R	MU	female	-	S	RS	601777	4281015
MG34	R	MU	male	subadult	S	RS	598265	4274124
MG35	R	MU	male	subadult	N	RS	577195	4278937
MG36	R	MU	female	adult	S	RS	556650	4234964
MG38	R	MU	male	adult	N	RS	563039	4295087
MG40	R	MU	female	subadult	S	RS	580605	4253356
MG57	R	MU	male	adult	S	RS	584135	4272853
MG58	R	MU	female	subadult	N	RS	576221	4281931
MG59	R	MU	female	-	S	RS	614561	4245007
MG60	R	MU	male	subadult	S	RS	587086	4271686
MG61	R	MU	female	subadult	S	RS	580642	4274274
MG62	R	MU	male	subadult	N	RS	578928	4282940
MG63	R	MU	male	subadult	N	RS	584503	4285381
MG64	R	MU	male	subadult	S	RS	598804	4274855
MG65	R	MU	female	-	N	RS	613178	4293171
MG66	R	MU	female	subadult	N	RS	555146	4291209
MG67	R	MU	male	subadult	S	RS	578161	4275673
MG68	R	MU	male	subadult	N	RS	549931	4279508
MG69	R	MU	male	-	N	RS	561219	4278024
MG70	R	MU	male	subadult	N	RS	574621	4280997

Table S2 Continue.

ID	Source	Type	Sex	Age	Side A6	Geneland	UTM X	UTM Y
MG71	R	MU	female	subadult	N	RS	612637	4292173
MG72	R	MU	female	subadult	S	RS	571111	4277620
SG1	T	BL	male	adult	S	TS	584766	4267180
SG2	T	BL	female	adult	S	HRC	585076	4267688
SG3	T	BL	female	adult	S	TS	584766	4267180
SG4	T	BL	female	subadult	S	TS	585265	4271789
SG5	T	BL	male	subadult	N	HRC	575898	4277504
SG6	T	BL	female	adult	N	HRC	581904	4274659
SG7	T	BL	male	adult	N	HRC	581822	4274885
SG8	T	BL	male	subadult	N	HRC	581145	4277207
SG9	T	BL	male	subadult	S	HRC	581682	4271162
SG10	T	BL	female	subadult	N	HRC	582484	4276702
SG11	T	BL	female	adult	S	HRC	581971	4271354
SG12	T	BL	male	adult	N	HRC	581336	4277925
SG13	T	BL	male	adult	N	TS	581987	4277597
SG14	T	BL	female	adult	N	HRC	583152	4276679
SG15	T	BL	male	subadult	N	HRC	583101	4277281
SG16	T	BL	male	subadult	S	TS	575728	4275303
SG17	T	BL	male	subadult	S	TS	575782	4275863
SG18	T	BL	female	adult	S	TS	576102	4275343
SG19	T	BL	male	adult	S	HRC	577903	4274289
SG20	T	BL	female	adult	S	HRC	577603	4274137
SG21	T	BL	female	subadult	S	TS	577238	4275090
SG22	T	BL	female	adult	N	HRC	577367	4283813
SG23	T	BL	male	adult	N	HRC	574078	4285081
SG24	T	BL	female	subadult	N	HRC	575567	4284219
SG25	T	BL	female	adult	N	HRC	573715	4285095
SG26	T	BL	female	subadult	N	HRC	582991	4282132
SG27	T	BL	male	subadult	N	TS	582094	4282721
SG28	T	BL	female	adult	N	HRC	580312	4283003
SG29	T	BL	female	adult	N	TS	581240	4279801
PG1*	R	HA	female	adult	S	RS	575441	4265795
PG2*	T	HA	male	adult	S	HRC	584335	4271526
PG3	R	HA	female	-	S	RS	560425	4261346
PG4	R	HA	female	subadult	N	RS	554934	4293825
PG5	T	HA	female	subadult	N	HRC	576723	4283693
PG6	T	HA	female	adult	N	TS	575480	4284369
PG7	R	HA	female	subadult	N	RS	587724	4276095
PG10	R	HA	male	adult	N	RS	571002	4298599
PG11*	R	HA	-	-	S	RS	604531	4284471
PG13	R	HA	-	-	S	RS	560906	4267437
PG19	R	HA	male	-	S	RS	562783	4272135
PG20	R	HA	female	subadult	N	RS	616477	4332779

Table S3 Characterization of the 20 microsatellites used in this study. Information regarding microsatellites distribution across multiplexes, nucleotide sequence added to primer forward (PF) 5' end to allow biding with fluorescent labels and concentration of primer forward and reverse (PR) used to construct multiplex mixes and on PCR 10 µl reactions are represented. Asterisks denote primers not included on genetic analyses. [a] and [b] - Microsatelites from Gaubert et al. 2008 and Fernandes et al. 2009, respectively.

Locus	**Multiplex**	**Label**	**PF 5' end modifications**	**PF concentration multiplex/pcr (µM)**	**PR concentration multiplex/pcr (µM)**
A104[a]	Gen1	VIC	TAA TAC GAC TCA CTA TAG GG	0.08 / 0.008	2.0 / 0.2
A108[a]	Gen1	6-FAM	TGT AAA ACG ACG GCC AGT	0.08 / 0.008	1.2 / 0.12
C101[a]	Gen1	VIC	TAA TAC GAC TCA CTA TAG GG	0.16 / 0.016	0.8 / 0.08
D111[a]	Gen1	6-FAM	TGT AAA ACG ACG GCC AGT	0.08 / 0.008	8.0 / 0.8
D4[a]	Gen1	NED	TTT CCC AGT CAC GAC GTT G	0.08/ 0.008	2.0 / 0.2
Ggen2.1[b]	Gen1	PET	GAT AAC AAT TTC ACA CAG G	0.12 / 0.012	0.8 / 0.08
Ggen2A16[b]	Gen1	NED	TTT CCC AGT CAC GAC GTT G	0.08 / 0.008	9.2 / 0.92
Ggen4.10[b]	Gen1	VIC	TAA TAC GAC TCA CTA TAG GG	0.16 / 0.016	1.4 / 0.14
Ggen4.12[b]	Gen1	PET	GAT AAC AAT TTC ACA CAG G	0.12 / 0.012	0.8 / 0.08
A110[a]	Gen2	6-FAM	TGT AAA ACG ACG GCC AGT	0.11 / 0.011	1.1 / 0.011
A5[a]	Gen2	PET	GAT AAC AAT TTC ACA CAG G	0.12 / 0.012	1.2 / 0.12
B105[a]	Gen2	VIC	TAA TAC GAC TCA CTA TAG GG	0.08 / 0.008	0.8 / 0.08
Ggen2A13[b]	Gen2	6-FAM	TGT AAA ACG ACG GCC AGT	0.08 / 0.008	0.8 / 0.08
Ggen3.3[b]	Gen3	NED	TTT CCC AGT CAC GAC GTT G	0.5 / 0.05	5.0 / 0.5
A112[a]	Gen3	VIC	TAA TAC GAC TCA CTA TAG GG	0.16 / 0.016	1.6 / 0.16
B104[a]	Gen3	NED	TTT CCC AGT CAC GAC GTT G	0.32 / 0.032	3.2 / 0.32
Ggen2A15[b]	Gen3	VIC	TAA TAC GAC TCA CTA TAG GG	0.16 / 0.016	1.6 / 0.16
Ggen2A25*[b]	-	6-FAM	TGT AAA ACG ACG GCC AGT	-	-
Ggen2A2*[b]	-	6-FAM	TGT AAA ACG ACG GCC AGT	-	-
Ggen1.30*[b]	-	6-FAM	TGT AAA ACG ACG GCC AGT	-	-

Table S4 Characterization of the primers redesigned for sexing common genets. Primer sequence, melting temperature (*Tm*), percentage of guanine and cytosine (G/C) and PCR product size are displayed.

Primer	**Primer sequence (5' – 3')**	***Tm*** **(ºC)**	**G/C (%)**	**Product size**
genX5	F: AGCCTGGGGATTGGTTTTCT	59.21	50.00	189
	R: TCCCATCTCAACATCGCTGA	58.81	50.00	
genX7	F:AGTTGTTGGCATAAAATGTTTGA	55.83	30.43	250
	R: GGCGTCCGTATCTTCCATTT	58.05	50.00	

Table S5 Common genets genotyped in the study area and their inferred half-siblings (HS). Dark gray and light gray shading represent individuals sampled on the southern and northern sides of the A6 motorway, respectively.

Sample	HS1	HS2	HS3	HS4	HS5	HS6	Opposite side A6
MG11	MG20	-	-	-	-	-	-
MG12	MG35	-	-	-	-	-	-
MG14	MG21	MG32	SG10	SG7	-	-	-
MG15	MG22	-	-	-	-	-	Yes
MG16	MG30	SG19	-	-	-	-	-
MG18	SG5	MG59	SG7	-	-	-	Yes
MG19	MG22	MG38	PG19	MG34	MG69	-	Yes
MG20	MG71	-	-	-	-	-	Yes
MG21	MG23	MG24	MG58	MG70	SG10	SG7	-
MG22	PG19	MG34	-	-	-	-	Yes
MG23	MG32	MG58	MG70	-	-	-	-
MG24	MG32	SG10	SG7	-	-	-	-
MG25	SG25	SG18	-	-	-	-	Yes
MG26	SG21	-	-	-	-	-	-
MG27	SG3	-	-	-	-	-	-
MG29	MG35	MG40	-	-	-	-	Yes
MG32	MG58	MG70	SG10	SG7	-	-	-
MG33	PG19	SG11	-	-	-	-	-
MG34	PG3	MG38	PG19	-	-	-	Yes
MG35	SG16	MG40	-	-	-	-	-
MG38	PG19	-	-	-	-	-	Yes
MG40	SG2	-	-	-	-	-	-
MG58	MG70	-	-	-	-	-	-
MG64	PG7	-	-	-	-	-	Yes
MG65	SG4	-	-	-	-	-	Yes
MG67	MG72	-	-	-	-	-	-
MG69	SG3	-	-	-	-	-	Yes
MG72	SG19	SG21	-	-	-	-	-
MG9	MG20	-	-	-	-	-	Yes
PG3	PG13	-	-	-	-	-	-
PG4	PG10	PG6	-	-	-	-	-
SG1	SG2	SG15	-	-	-	-	Yes
SG10	SG15	-	-	-	-	-	-
SG12	PG10	-	-	-	-	-	-
SG16	SG27	-	-	-	-	-	Yes
SG23	PG5	-	-	-	-	-	-
SG24	PG5	-	-	-	-	-	-
SG26	SG27	-	-	-	-	-	-
SG4	SG8	PG13	SG14	PG3	-	-	Yes
SG5	SG7	-	-	-	-	-	-
SG6	SG12	-	-	-	-	-	-
SG7	SG10	-	-	-	-	-	-
SG8	SG14	MG13	-	-	-	-	Yes

Supplementary Figures

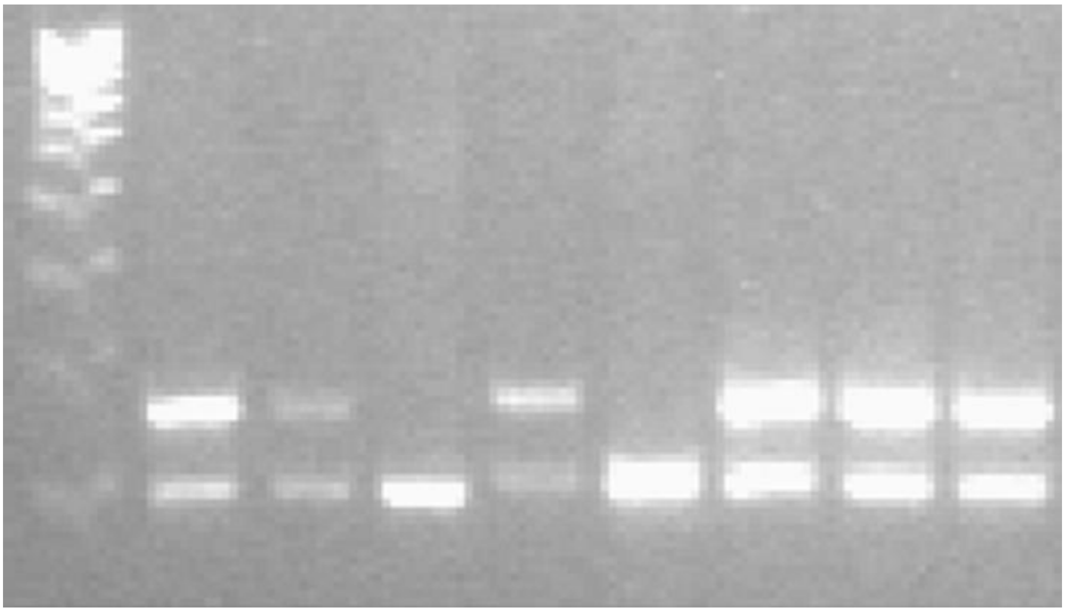

Figure S1 Sex identification of six males (PCR products with two fragments) and two females (PCR products with one fragment).

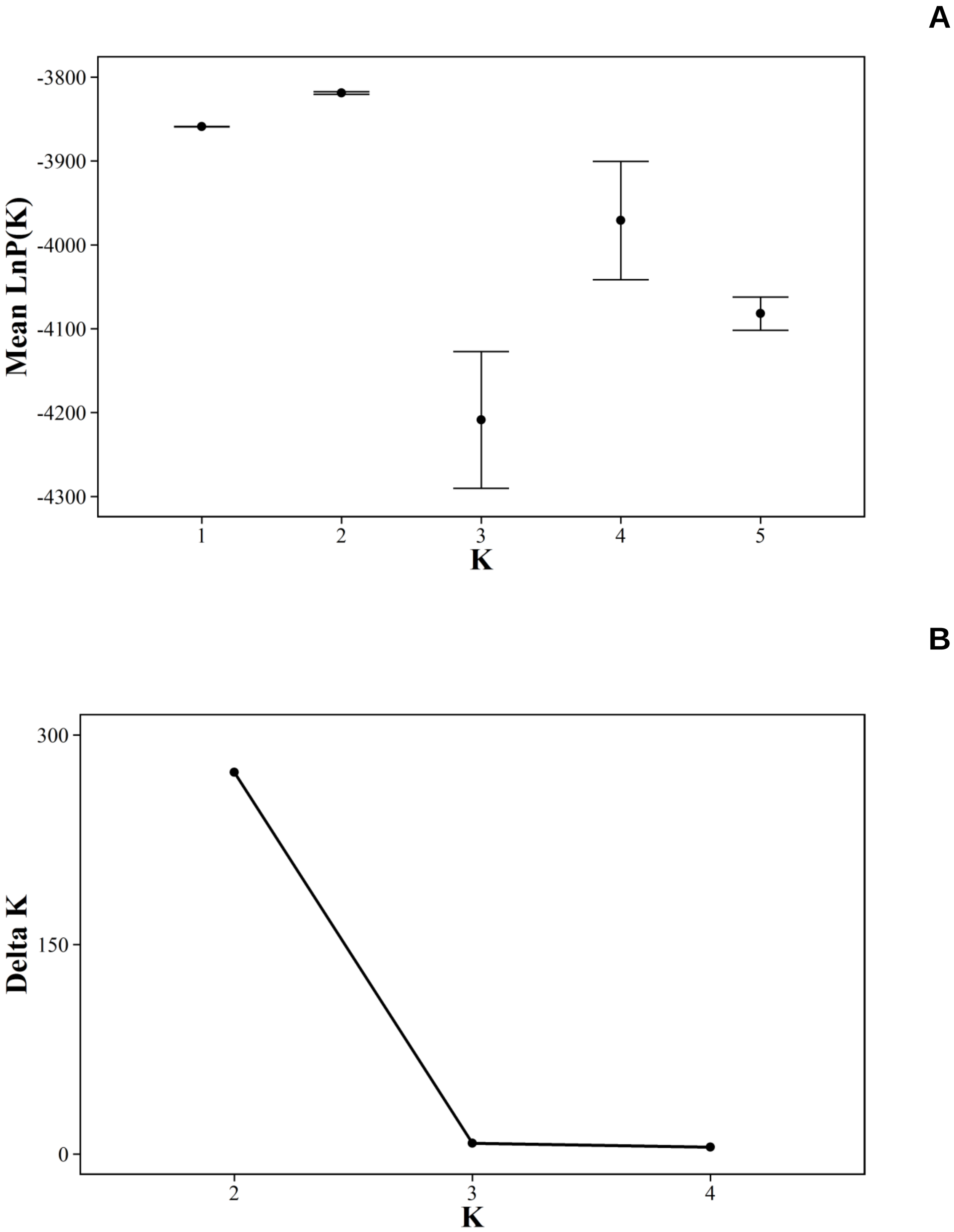

Figure S2 STRUCTURE HARVESTER plots exhibiting the best K (K=2) from using the criteria: (A) likelihood of data; (B) highest ΔK values.

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6. GENERAL CONCLUSIONS

Summary of the main findings

The main goal of this thesis was to identify anthropogenic (roads) and ecological factors shaping functional connectivity for a forest carnivore in a human-modified Mediterranean landscape. To address this issue, we analysed how roads interact with other environmental factors to influence common genet resting behaviour, foraging patterns and dispersal. Moreover, we also assessed possible population genetic structuring in a region dissected by a motorway.

In Chapter 2 (first paper), we described the main factors affecting resting behaviour at two spatial scales – micro and home range – in the wet and dry seasons using mixed model analysis. Common genets selected mainly tree hollows all year round, but this choice was stronger during the wet season, followed by nests and dens, while in the dry season genets used nests and dens more frequently than during the wet season (Carvalho et al. 2014). Tree hollows may be the safest choice against predators, harsh weather conditions and human disturbance, in sites with lower shrub cover (Carvalho et al. 2014). However, genets used alternative sites (nests and dens) more frequently in areas with high shrub cover at the micro-scale, and close to riparian habitats, probably because these areas were less prone to predation risk and starvation. In general, genets used mainly tree hollows for resting in more heavily roaded areas.

In Chapter 3 (second paper), we investigated the consequences of different sampling efforts (daily vs. every other day vs. weekly monitoring) for identifying patterns in resting site selection and use. Based on mixed effect modelling we showed that larger sampling intervals may underestimate the importance of several local resources for genets, thus lowering the power to identify significant explanatory variables (Carvalho et al. 2015). For instance, in the wet season, roads and urban areas (human disturbances) had the greatest influence on genets resting behaviour under the daily sampling regime, but the importance of these variables decreased greatly under less intensive sampling regimes. In the dry season, the differences between sampling regimes were even more evident, most likely because under the weekly scenario, anthropogenic effects became insignificant (Carvalho et al. 2015). These results highlighted the negative consequences of larger sampling intervals for connectivity assessments. Daily sampling regimes were the most efficient capturing the critical variables that should be considered for building complex functional connectivity movement models. Missing to detect the importance of such variables on landscape connectivity for a species may have seriously compromise the assessment of its long-term persistence.

Chapter 4 (paper 3) addressed effects of roads on landscape functional connectivity. Results showed clearly that genets preferred to move within forest patches close to riparian habitats and strongly avoided open agricultural land. The preference of genets for forest areas may be driven by three main factors: higher prey availability (Rosalino and Santos-Reis 2002); canopy cover to reduce predation risk (Mergey et al. 2011); and availability of tree hollows, which were proven to be the safest choice for resting (Carvalho et al. 2014). Roads decreased the probability of genet movement except near culverts, bridges and viaducts, especially when in continuity with riparian corridors. These negative effects of roads may be related to noise and other disturbances such as the risk of being hit by a car (Santos and Santos-Reis 2010). Overall, roads reduced landscape connectivity by dissecting forest patches to significantly lower mean sizes, but this effect diminished in riparian corridors due to the associated crossing structures. Riparian corridors were fundamental in promoting connectivity in open agricultural areas, as shown by our conductance surfaces (Chapter 4, Fig. 3A and B) (Pereira and Rodríguez 2010). We showed the relevance of path-level analysis as a simple way to infer how spatial heterogeneity and roads influence landscape functional connectivity, taking advantage of a robust empirical movement data set.

Finally, in Chapter 5 (paper 4), we studied the complex behaviour of common genets toward main roads, by combining data on: i) road-related mortality, ii) road crossing events, assessed by radiotracking, iii) and the genetic relationships among animals. First, 65% of all roadkills were subadults, occurring mainly on the dispersal period; this may have unknown consequences on the dynamics and social system of the population (Quaglietta et al. 2013; Chapter 5). Second, we found a significantly different crossing behaviour, where adults rarely crossed the motorway (Carvalho et al. submitted) (their home ranges were limited by it), while dispersers crossed it several times. However, parentage analysis revealed that both subadults and adults cross the motorway, which was not supported by using radiotracking data alone. Thus, although a road barrier effect may exist, this effect was not strong enough to block gene flow across the motorway. Genetic clustering and migration analyses further confirmed the existence of high gene flow rates across the A6 motorway. While the reasons for that are uncertain, but may be related to the recent age of the road (constructed in 1996), possibly comprehending only 7/8 generations, insufficient for producing any consistent pattern of genetic drift, or for visible deleterious effects of road-related mortality (Jackson and Fahrig 2011). Moreover, a low traffic flow (< 10000 vehicles per day) means lower levels of disturbance, and several viaducts and bridges surrounded by forest and riparian habitats allow safe passages. Both factors have probably contributed to successful crossings by willing subadults. Although the motorway is not yet a major barrier for gene flow in our study area, it seems to act as a filter for individual movement: subadults crossed roads regularly, but only some male adults crossed occasionally to mate (see also Soulsbury et al. 2011). This conclusion would be impossible if we had only relied on one of the three approaches followed in this thesis. While, parentage analysis suggested the absence of a barrier, radiotracking and roadkills showed that only subadults cross it regularly and frequently die. Combining multiple sampling techniques may be particularly useful to provide context-specific knowledge likely to increase the efficiency of mitigation actions, thereby optimising the limited human and financial resources (Barrueto et al. 2014, Torrubia et al. 2014).

Implications for connectivity management in road-dominated landscapes

From a conservation standpoint, our work provides management guidelines to maintain or even increase functional landscape connectivity for small forest carnivore species. We demonstrated road effects on genet´s behaviour at microhabitat, home range and landscape scales, and highlighted the consequences of these approaches on parameterizing and depicting landscape functional connectivity. From our results, the following management guidelines may be proposed to improve landscape functional connectivity when building new linear infrastructures:

- Retain tree hollows within 400m of the road. Roads and other human disturbances influence daily resting patterns and force animals to rest away from them. Genets, however, may remain near roads if the habitat is suitable and tree hollows – the safest resting sites – are available. Moreover, large trees along riparian galleries should also be maintained, as they are crucial additional resources, particularly during the dry season and during dispersal events, by providing secure resting stepping stones;
- Keep roads at least 3 km away from the core of dense forest areas. Forest areas, especially the denser ones, are part of the core areas of adult females and their cubs, and they are highly permeable and allow safe movements;
- Keep, whenever possible, available mean forest patch areas of around 800 ha, which is twice the area of a mean adult genet home range, to assure functional connectivity at the landscape scale;
- Maintain road underpasses open, naturally vegetated and free of waste. A natural (ideally) or artificial dry path should be kept throughout the year in underpasses

prone to flooding (genets and stone martens were found roadkilled just above flooded culverts during the study). Some animals used these structures frequently and the current number of culverts, bridges and viaducts (at least one each 500 m) seemed enough to maintain landscape functional connectivity in our study area if they are clean and operational;

- Prioritize road sectors based on path-level analysis. Path-level analysis revealed the road sectors that truly hinder movement for common genet, but also for other forest carnivores such as marten species, polecats, wildcats and Iberian lynx. At these sectors, it will be prudent to implement fences impermeable for carnivore species with climbing skills, and corridors guiding the animals to safe passages;
- Limit the access of small forest carnivores on road sections intersect by riparian corridors, particularly if they cross a hostile matrix such as open agricultural areas, because they were more prone to road-related mortality. These spots may be the only safe passages for dispersing within these areas, and animals should be channelled into those natural corridors;
- Use different sources of information to identify main routes of animal movement, and to design and locate cost-effective mitigation measures where impacts cannot be avoided, thereby optimizing the allocation of limited human and financial resources.

Future research

The value of landscape connectivity for conservation biology is unquestionable, especially under the current trends of habitat fragmentation worldwide. This awareness has led to different concepts of connectivity (e.g. functional and structural), which are akin to relatively new disciplines (e.g. Road Ecology and Landscape Genetics), which in turn have allowed the development of new analytical methods and concepts (e.g. least-cost theory, circuit theory, path-level analysis) and metrics (e.g. IIC, PC) associated with connectivity (Kadoya 2009). Although, current understanding of landscape connectivity emphasizes the importance of functional connectivity, in this thesis, we opted to account for both aspects of connectivity (i.e. structural and functional) for a forest carnivore in a Mediterranean human-modified landscape. However, some caveats should be highlighted regarding for instance long dispersal movement.

Although, we were unable to discern long-distance dispersal differences between females and males in genets, we stress that linking empirical radiotracking movement estimations with genetic data should provide a useful approach to achieve this goal. Intensive studies using collars with longer battery life should help to clarify whether male biased long-distance dispersal also prevails for common genets as described for felids, canids and some mustelid species (Schmidt et al. 2011). Moreover, radiotracking dispersers should be crucial for improving connectivity measures, because such individuals may be less attached to particular habitat types, and they may be more prone to cross obstacles such as roads (Chapter 4). Further information about dispersal events should thus shed light on habitat and path movement choices made during such a vital life stage.

High landscape functional connectivity, despite the fragmentation by roads, was also observed for tawny owls (*Strix aluco*) in the same study area (Santos et al. 2013). It is thus likely that the surveyed landscape still provides forest patches large enough to assure landscape connectivity for many forest species occurring therein. Accordingly, by simulating different levels of habitat fragmentation, it would possible to fully appreciate how the interactions between spatial heterogeneity and human linear infrastructures affect landscape connectivity (Crouzeilles et al. 2014).

New technology (e.g. intensive GPS data) will probably demand for new analytical methods to cope with such large and complex data. Although a wide modelling framework was beyond the scope of this thesis, these issues (e.g. locations errors, spatial and temporal autocorrelations) should be more deeply addressed in future studies. More complex modelling procedures, such as state-space models (SSM), show promising results for individual animal movement, allowing the simultaneous modelling of steps and angles (Patterson et al. 2008; Martini et al. 2013). SSM relates the speed and turning angles among successive locations to a possible behaviour state. For instance, shorter and more tortuous movements may mean searching for food in optimal forest habitat, while faster and unidirectional longer movements (travel) suggest speeding-up through a hostile matrix between two safe patches. These models account for errors associated to location positions, but will demand more accurate data (e.g. GPS collars) in the future (Patterson et al. 2008).

Apart these potential shortcomings and the new avenues recently available for improving modelling procedures, this thesis was successful in showing the complexity of mechanisms underlying space use by the common genet. This complexity, particularly toward main roads, should depend not only on the animal status (adult residents vs. subadult dispersers) and physiology, but also on habitat features and climate (Martini et al. 2013). Thus, we emphasize the need for more complex and integrated analyses, where studies we will be able to test and incorporate new covariates including, for instance, fine-scale thermal maps, prey availability maps and direct predation risk. Thermal maps with 100-meter resolution are already being developed. The scanning of possible thermal signatures in the landscape at fine scales will add explanations for the different choices of resting sites and movement paths. Assessing prey availability and predation risk at the fine scale will provide extra insights on the association between several physiological states (heart rate, hormonal stress), movement state (speed, turning angles) and the habitats where they occur (Schmidt et al. 2011; Vasudev et al. 2015).

Roadkills locations provide accurate data on crossing attempt locations that can be related to surrounding habitats. Cataloguing roadkilled animals by sex and age will show how roads affect the movement at the population level allowing for better assessments of mid- to long term road impacts (Ward et al. 2015). This last issue is the subject of a new on-going paper from our team based on a 10-year roadkill database. The main goal is to assess the utility of our robust movement model, based on path-level analysis, to accurately predict road crossing locations (and roadkills). The roadkill data will be used to (cross) validate the prediction of the path level model.

Summarising, although our results showed that roads influence both resting and foraging behaviour of common genets, with clear consequences for landscape functional connectivity for this species, those influences may differ for other similar species according to their own perceptual ranges. For instance, an interesting result was the obtained by Ascensão et al. (2014) for stone martens in a similar area, where they found adult stone martens as crossing more often the motorway and integrating them inside their home ranges, which contrasts with our study. Future research should focus on whether such different interactions with roads and spatial habitat heterogeneity at the local scale influence the overall landscape connectivity dynamics. This would provide further understanding of movement ecology in fragmented landscapes, with positive insights for new conservation strategies to effectively mitigate road effects on landscape functional connectivity.

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