Universidade de Évora - Instituto de Investigação e Formação Avançada

Programa de Doutoramento em Biologia

Tese de Doutoramento

Modelling roadkill spatiotemporal patterns, movement routes and mitigation solutions for Mediterranean amphibians

Tiago António Pinto Ferreira

Orientador(es) | António Mira
Sara M. Santos
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Évora 2025

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The research conducted in this Doctoral thesis was financially supported by the Portuguese Foundation for Science and Technology (FCT – Fundação para a Ciência e Tecnologia) through the grant 2020.04581.BD [DOI: 10.54499/2020.04581.BD] awarded to **Tiago António Pinto Ferreira** and through the projects “LIFE LINES – LIFE14 NAT/PT/001081” financed by the European Commission and “POPCONNECT PTDC/AAG-MAA/0372/2014” co-financed by Programa Operacional Competitividade e Internacionalização (POCI) and supported by FEDER (POCI-01-0145-FEDER-016811).

This thesis is presented as a compilation of scientific articles either published or submitted for publication in journals indexed in the Web of Science (WoS) that integrate some of the central chapters. These articles were developed in collaboration with other authors. However, the candidate clarifies that he led all phases of the work of each article, including its conception, analysis and writing. The candidate also actively contributed to the data collection.

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This thesis should be cited as:

Pinto, T. (2025). Modelling roadkill spatiotemporal patterns, movement routes and mitigation solutions for Mediterranean amphibians. Doctoral thesis. University of Évora, Évora, Portugal.

"All we have to decide is what to do with the time that is given to us"

- J. R. R. Tolkien

Acknowledgements

This section was opened with the intention of being written several times; and closed just as many without a single word being added. Whether due to lack of time or lack of inspiration. And although this page has been revisited multiple times, its completion undoubtedly marks the conclusion of a long journey. Despite having more writing freedom here, the words seemed harder to come by than in any other sections of this document (perhaps one of the many PhD side-effects).

A PhD thesis is often associated with solitary work, even more when it is done entirely remotely and with no fieldwork involved. However, this thesis is the conclusion of an enormous effort, both professional and personal, to which several people have contributed, directly or indirectly. Without their support, not even this section would exist to be read. That being said, I believe that the acknowledgements should be expressed daily, through actions and words, and that these written words are merely a formality for future memory.

First and foremost, I would like to thank my supervisors. To Sara Santos, thank you for your willingness to take on this task despite having other students and responsibilities to manage. You have always been tolerant, supportive and full of ideas to make the work less painful and more engaging. Your ability to motivate has certainly helped me many times, especially during the most challenging moments. Your organisational skills have been very valuable throughout the entire process, and I am also grateful for your speed in handling all the bureaucratic matters. To Neftalí Sillero, thank you for accepting me as your PhD student even before I had a clear plan in place. You have constantly been available to assist me in any matter, providing innovative ideas and an expertise that greatly enriched this thesis. I also appreciate how you taught me new ways of thinking and how to be more critical. It was a pleasure having you as my master's thesis examiner, but even more so, to have you as a PhD supervisor. To Professor António Mira, thank you for believing in me, for giving me the opportunity to work in the field of conservation biology all these years and for introducing me to road ecology (even when that meant doing MOVE on

Sunday mornings). I truly hope I was able to meet your expectations. Your strategic and "outside the box" thinking, as well as your refreshing ideas have been invaluable to this work. My sincere thanks to all of you for trusting in my potential from the very beginning, for sharing your expertise and creativity while giving me the flexibility to explore and develop my own ideas. Your guidance has inspired me to approach my work with more confidence and dedication. I am also deeply grateful for your dedication and patience throughout the long and (sometimes!) tedious emails, as well as our meetings. Above all, thank you for your friendship. It has been an honour working with all of you, and I hope we have more opportunities to continue to do so in the future.

To my closest friends, thank you for helping me to relax along the process (especially when this thesis consumed most of the hours of my days). Whether through sharing stupid videos on social networks, exchanging messages, or going out, you helped me out to maintain my mental sanity.

To Giovanni Manghi, for always being just a chat away and saving me countless times when there was an issue with my WS. To Eduardo Ferreira for the phone calls and sharing concerns about the PhD student life. To Nuno Pedroso for cutting through much of the bureaucratic processes. And of course, to the overall UBC group, for all the fieldwork that contributed to this thesis and all the discussions that, directly or indirectly, allowed me to obtain the important results of my thesis.

Institutionally, I would like to thank to Fundação para a Ciência e Tecnologia (FCT) for supporting this thesis through a PhD grant (2020.04581.BD). To MED – Mediterranean Institute for Agriculture, Environment and Development, as the host institution and for their financial support. To the "LIFE LINES – Linear Infrastructure Networks with Ecological Solutions (LIFE14 NAT/PT/001081)" and the "POPCONNECT (PTDC/AAG-MAA/0372/2014)" projects for providing much of the resources that allowed the completion of this thesis. I also would like to thank to CHANGE – Global Change and Sustainability Institute, to IIFA – Instituto de Investigação e Formação Avançada da Universidade de Évora and to CICGE – Centre for Research in Geo-Spatial Sciences.

Um especial obrigado aos meus pais, por sempre me terem motivado a prosseguir com o meu sonho de fazer um doutoramento e com a minha carreira científica. Por terem feito tudo o que estava ao seu alcance para eu poder completar esta etapa. Por serem compreensivos e nunca terem pedido nada em troca, mesmo sabendo que estou em (muita) falta para com eles. Obrigado por todos os momentos de carinho e toda a força que sempre me deram e me continuam a dar. Esta tese é para vocês. À minha irmã pelo apoio incondicional e por acreditar em mim. Mesmo longe fisicamente, obrigado por me desejares "coragem e força para a escrita" todos os dias destes últimos meses. Obrigado por seres a melhor irmã do mundo (também nunca tive outra!).

Por fim, um obrigado à Dinora. Por todo o apoio e (às vezes a falta de) paciência, pelas trocas de ideias e inúmeros monólogos onde explicava as minhas dúvidas, que tantas vezes me ajudaram a descontrair e até a encontrar resolução para muitas delas. Se hoje fecho este capítulo, muito o devo a ti. Obrigado por seres quem és, e por seres como és – muitas vezes uma verdadeira luz ao fundo do túnel. Esta tese é também tua.

Outputs included in this thesis

Peer-reviewed publications in international journals included in the thesis

Published publications

Pinto, T., Santos, S. M., Mira, A., & Sillero, N. (2023). Importance of water availability for amphibian roadkill in a Mediterranean landscape. *Biodiversity and Conservation*, 32(7), 2513-2537 https://doi.org/10.1007/s10531-023-02616-9

Pinto, T., Sillero, N., Mira, A., & Santos, S. M. (2024). Using the dead to infer about the living: Amphibian roadkill spatiotemporal dynamics suggest local populations' reduction. *Science of the Total Environment*, 927, 172356 https://doi.org/10.1016/j.scitotenv.2024.172356

Pinto, T., Sillero, N., Mira, A., Sousa, L. G., Oliveira, A., & Santos, S. M. (2024). Effectiveness of permanent drift fences in reducing roadkill risk of amphibians. *Journal of Environmental Management*, 368, 122049. https://doi.org/10.1016/j.jenvman.2024.122049

Submitted publications

Pinto, T., Santos, S. M., Mira, A., Sillero, N. Tell me where you go, and I'll tell you where you die: landscape connectivity as a tool to predict amphibian roadkill risk. Submitted to *Journal of Environmental Management*.

Table of Contents

Modelling roadkill spatiotemporal patterns, movement routes and mitigation solutions for Mediterranean amphibians

Abstract

Roads have detrimental impacts on ecosystems by fragmenting habitats, restricting wildlife movement, and increasing roadkill risk. The limited understanding of how different animal groups respond to these road-related threats hinders the development and implementation of effective road mitigation measures. Ultimately, this issue constrains the efforts to maintain and restore landscape connectivity. This thesis aims to analyse the influence of landscape drivers and its connectivity on the spatiotemporal patterns of amphibian roadkill, with remote sensing satellite data. To achieve this, I first identified key drivers of amphibian mortality spatial patterns. Secondly, I examined the spatiotemporal trends of amphibian roadkill. Thirdly, I tested the effectiveness of permanent mitigation measures for amphibians in reducing the roadkill risk. Lastly, I evaluated whether landscape connectivity could be used as a predictor of amphibian roadkill risk. My findings revealed that reduced water availability is likely to increase roadkill risk for most amphibian species, whose populations may already be struggling with the combined effect of road negative impacts and other anthropogenic threats, especially when considering their roadkill temporal trends. In addition, specific roadkill mitigation measures showed promising results in reducing the number of amphibians reaching the roads. Landscape connectivity also proved to be useful as an amphibian roadkill predictor, offering a cost-effective alternative to more demanding field-based methods. This study highlights the urgency of amphibian conservation actions at both the population and species levels, as many species' movement patterns remain unknown, impeding the development of more effective conservation measures. The integration of knowledge about amphibian movement, their roadkill patterns, and the response to roadkill mitigation structures is crucial for ensuring the survival and persistence of amphibians in the face of expanding road networks and environmental changes.

Keywords

Road mortality; remote sensing; spatial modelling; road mitigation; landscape functional connectivity; conservation.

Modelação dos padrões espácio-temporais de atropelamento, rotas de movimento e soluções de mitigação para anfíbios mediterrâneos

Resumo

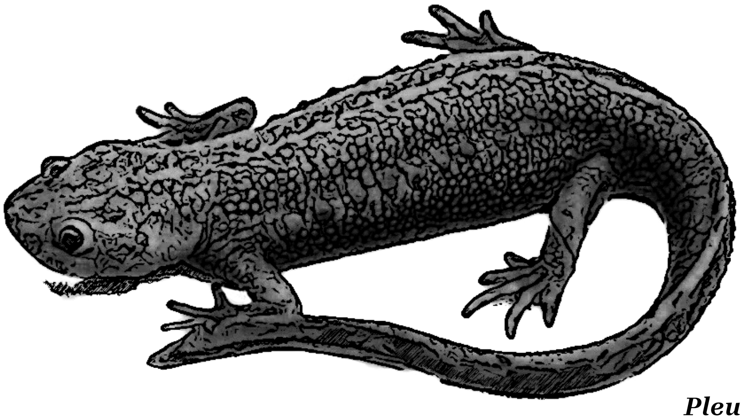
As estradas têm impactos negativos nos ecossistemas ao fragmentarem os habitats, restringirem o movimento dos animais e aumentarem o seu risco de atropelamento. O conhecimento reduzido de como diferentes grupos respondem a ameaças relacionadas com estradas limita o desenvolvimento e implementação de medidas de mitigação eficazes. Isto dificulta os esforços para manter e restaurar a conectividade da paisagem. Esta tese visa analisar a influência dos fatores de paisagem e da sua conectividade nos padrões espácio-temporais de atropelamento de anfíbios. Para tal, identificámos os principais fatores que influenciam os padrões espaciais de mortalidade de anfíbios, e examinámos as tendências espácio-temporais dos atropelamentos dos anfíbios. Em terceiro lugar, testámos a eficácia de medidas de mitigação na redução do risco de atropelamento para este grupo taxonómico. Por fim, avaliámos se a conectividade da paisagem pode ser utilizada como preditor do risco de atropelamento. Os resultados revelaram que a redução da disponibilidade de água provavelmente aumentará o risco de atropelamento para a maioria das espécies, cujas populações podem já estar afetadas com o impacto combinado das estradas e de outras ameaças antropogénicas, especialmente tendo em conta a tendência temporal dos atropelamentos. Adicionalmente, as medidas de mitigação reduziram significativamente o número de anfíbios que chegam às estradas. A conectividade da paisagem também se mostrou útil como preditor de atropelamentos, oferecendo uma alternativa economicamente mais vantajosa quando comparada com métodos mais tradicionais. Destacamos a urgência da conservação dos anfíbios, tanto ao nível das populações como das espécies, dado que os padrões de movimentos de muitas ainda são desconhecidos, impedindo o desenvolvimento de medidas de conservação mais eficazes. A integração do conhecimento sobre os movimentos dos anfíbios, os seus padrões de atropelamento e resposta a estruturas de mitigação é crucial para assegurar a sobrevivência e persistência dos mesmos face à expansão das redes viárias e mudanças ambientais.

Palavras-chave

Mortalidade nas estradas; deteção remota; modelação espacial; mitigação; conectividade funcional da paisagem; conservação.

Chapter 1

General introduction



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1.1. The global road network: current trends and future directions

Ecosystems are globally recognised for providing essential services for the economic and social well-being of human societies, ranging from food and water to climate regulation and natural pest control, as well as aesthetic and spiritual values (Tzoulas et al., 2007; Malhi et al., 2020). Human actions over the last centuries have triggered profound ecosystem changes, increasing extinction rates to magnitudes only equivalent to previous large mass extinctions (Dirzo et al., 2014). Although there is no exact information on the number of species lost, the estimations point to a current extinction rate greater than any known in the last 100,000 years (Eldridge, 1998). Human impacts on the planet have been one of the main drivers of this phenomenon, which Dirzo et al. (2014) described as *defaunation*. Despite overall efforts to contain this general biodiversity loss, evidence reveals that human societies are failing to accomplish this objective (Miller, 2005; Kleijn et al., 2011; Arenas-Castro & Sillero, 2021).

One of the contributing agents for this loss is linear infrastructures, which represent a pivotal role in human economic progress, facilitating the transportation of humans and goods, and providing access to new locations and resources necessary for society development (Laurance et al., 2014; van der

Ree et al, 2015; Meijer et al., 2018). Roads are one of the most widespread man-made infrastructures, covering approximately 64 million kilometres of the Earth's surface (Dulac, 2013). These infrastructures are conspicuous elements in almost all landscapes worldwide (**Figure 1.1**), with more than 80% of the continental U.S. and 50% of Europe within 1 km and 1.5 km of the nearest road of any type, respectively (Riitters & Wickham, 2003; Torres et al., 2016). These infrastructures are projected to expand even further in the future: by 2050, 25 million km of paved roads will be constructed (Laurance et al., 2014), especially in less developed countries (Laurance and Balmford, 2013; Meijer et al., 2018), doubling the number of vehicles (Gadonneix et al., 2011; Gross, 2016). The expansion of these infrastructures, associated with landscape fragmentation, has contributed to ecosystem changes and wildlife population declines, altering species distributions and ecological processes (Laurance & Balmford, 2013; Meijer et al., 2018). Habitat fragmentation is a consequence of habitat degradation and loss and occurs when natural areas are disrupted into smaller and less connected patches, with consequential repercussions on ecosystem functioning and wildlife populations (Forman et al., 2003; Fahrig, 2019). This phenomenon has been widely documented as a result of road network expansion (Coffin, 2007; Rytwinski and Fahrig, 2012).

The challenge for coexistence between humans, their infrastructures, and biodiversity may be addressed through strategies aimed at limiting environmental costs while maximising the benefits of human development (Laurance et al., 2014).

Figure 1.1. Global distribution of major roads (only primary, secondary and tertiary paved roads are presented). This map was generated with OpenStreetMap data (Haklay and Weber, 2008).

1.2. Impacts of roads and their influence on ecosystems and wildlife

Inherent to social and economic development, roads also introduce environmental impacts to biodiversity (Laurance et al., 2014). These linear infrastructures provide human access to otherwise remote areas. The associated light and noise of vehicular traffic, as well as pollutants from both vehicles and roads (and even humans), are not limited to the road surface, extending its width many times into the adjacent landscape; this is known as the *road-effect zone* (Forman & Alexander, 1998). The road-effect zone is a useful framework to quantify the negative impacts of roads and traffic (van der Ree et al., 2015). In the Continental U.S., for example, 15-20% of the territory is estimated to be potentially impacted by these linear infrastructures (Forman & Alexander, 1998). The impacts start during road construction and can continue as long as the road remains active if no measures to mitigate their effects are installed (Coffin, 2007). Such transformations can alter abiotic components like hydrology and sediment transportation, resulting in contaminated water, high levels of chemical pollution, and erosion (Forman & Alexander, 1998; Coffin, 2007). These environmental road effects are frequently grouped into two categories: direct and indirect impacts (Bennet, 2017).

Direct impacts are often referred to as wildlife direct mortality by vehicle collisions (**Figure 1.2**), and the implications of these are well documented for a wide variety of taxonomic groups: mammals (Ceia-Hasse et al., 2017; Ascensão et al., 2019); birds (Santos et al., 2016; Pinto et al., 2020); reptiles (D'Amico et al., 2015; Hallisey et al., 2022); amphibians (Glista et al., 2008; Sillero et al., 2019) and even insects (Martin et al., 2018). In addition, millions of individuals are estimated to be killed every year on roads across all continents, except Antarctica (Grilo et al., 2021). For example, Loss et al., (2014) estimate that up to 340 million birds are killed on U.S. roads annually, while Grilo et al., (2020) report approximately 194 million birds and 29 million mammals road-killed yearly on European roads. In Australia, nearly 5.5 million reptiles and amphibians are estimated to be victims of roadkill every year (Forman et al., 2003).

Indirect impacts, however, cover a myriad of factors including habitat loss and/or degradation and the barrier effect (**Figure 1.2**; Laurance & Balmford, 2013; van der Ree et al., 2015). These impacts are often interrelated, as one typically leads to the other or is a consequence of another. This cumulative influence of road impacts creates a network of ecological disturbances that may even saturate landscapes with low road densities (Coffin, 2007). For instance, the loss of the habitat as a result of road construction can constrain the movement of a species, since fragmentation restricts access to other suitable habitat patches (Baguette et al., 2013); this, in turn, can result in a barrier effect (Shepard et al., 2008). The barrier effect is mainly influenced by road width and traffic volume, exacerbated by other agents such as light, noise, chemical pollution, and general disturbance from vehicles (Coffin, 2007). Ultimately, many species present a reduced tendency to cross wider roads or roads with high traffic volume, a phenomenon known as road avoidance behaviour (Forman & Alexander, 1998; Shepard et al., 2008; McClure et al., 2013). Jaeger and colleagues (2005) reported that species most sensitive to higher levels of noise and showing road surface avoidance were the most vulnerable ones. Road avoidance may also cause populations to become more isolated, resulting in additional habitat loss extending beyond the physical road itself. Other impacts such as noise also have significant effects, particularly on highly traffic-volume roads, such as highways, with detrimental consequences for both humans and wildlife (reviewed in Singh et al., 2018; Forman & Alexander, 1998; Silva et al., 2012; McClure et al., 2013). The barrier effect imposed by roads, coupled with the direct mortality associated with vehicle collision (or simply, roadkill), may also negatively affect numerous fundamental biological processes such as dispersal, migration, and gene flow, ultimately jeopardising the long-term viability of populations (Glista et al. 2008, Baguette et al. 2013; Ceia-Hasse et al., 2017). One of the most pronounced indirect impacts of roads on ecosystems begins with the preparation of the land preceding road construction or expansion (Forman and Alexander, 1998; Coffin, 2007). This disrupts continuous habitats into smaller patches (estimated to be around 600,000 patches worldwide, mostly with an area of less than 1 km^2, according to Ibisch et al., 2016), also reflecting a loss of continuous habitat in areas adjacent to roads, affecting the species occurring there. The disrupted effect of road activity reduces the suitability of the surrounding habitat, further contributing to habitat degradation (Rytwinski and Fahrig, 2012). These road-surrounding habitats are often smaller, which makes them subject to higher edge effects – known as the abrupt transition in structural and environmental conditions between two adjacent habitats (Forman and Alexander, 1998). Furthermore, they are usually simpler and of poor quality as they are frequently subject to high human intervention (e.g., road verge management). Road verges typically consist of a strip running parallel to the road surface that may be subject to varying degrees of management. Vegetation in these sites usually presents vigorous growth due to increased light and moisture from road drainage (Forman & Alexander, 1998). These conditions may attract predators or scavengers (that feed on species inhabiting those strips) increasing the risk of roadkill (Barrientos & Bolonio, 2009; Silva et al., 2019). Road verges usually also provide habitat for opportunistic groups, including exotic species, that frequently use roads as corridors to spread across the landscape (reviewed in Lázaro-Lobo & Ervin, 2019; Schwartz et al., 2018).

However, despite the aforementioned negative effects, roads can also bear positive effects on biodiversity, mainly at local scales: if properly managed, the presence of verges along roads can provide a suitable habitat for some taxonomic groups when surrounding landscape is highly modified by humans (e.g., intensive agricultural or grazing areas) (Valtonen et al., 2006; Coffin, 2007; Sabino-Marques & Mira, 2011; Galantinho et al., 2020; Galantinho et al., 2022). In these artificialised landscapes, verges may represent the only debris of native vegetation available (Forman and Alexander, 1998), even capable of supporting species of high conservation value (Pita et al., 2006). Nevertheless, roads bear more negative effects on biodiversity than positive effects.

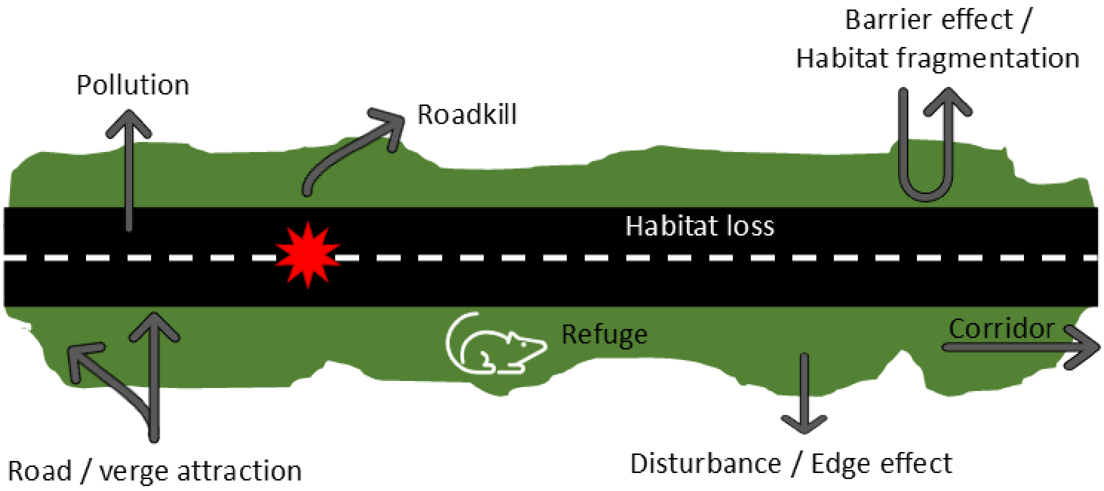

Figure 1.2. Major impacts of roads on wildlife. Roadkill may be the most notorious negative impact of roads, but other effects such as habitat loss and fragmentation, pollution and edge effects are evident as well. Road verges can also act as a refuge for some species and dispersal corridors for others. Adapted from Seiler (2001).

The ability of a species to avoid vehicles or exploit highly variable ecological conditions plays a relevant role in determining its susceptibility to roads (Fahrig & Rytwinski, 2009). For example, species with greater home ranges may be at a disadvantage in road-dominated landscapes, as individuals will need to frequently cross different land uses and elements, increasing their roadkill risk when compared to species with lower dispersal abilities and smaller home ranges (Carr and Fahrig, 2001; Forman et al., 2003; Rytwinski and Fahrig, 2012). Likewise, species that exhibit low adult survival rates, diminished fecundity, and delayed sexual maturity, tend to display reduced population growth rates and thus, are more susceptible to experiencing population declines when confronted with increased road mortality (Rytwinski and Fahrig, 2011;

Rytwinski and Fahrig, 2012; Grilo et al., 2021). Indeed, road mortality accounts for nearly 35% of the annual deaths of the endangered Florida panthers (Taylor et al., 2002). Similarly, approximately 76% of anthropogenic deaths of Iberian lynx in the Iberian Peninsula reported in 2023 were attributed to vehicle collisions (Censo de lince-ibérico: Espanha e Portugal, 2023). Nevertheless, species with lower vagility may also be negatively affected by road mortality over longer periods (e.g., isolation leading to a higher chance of extinction risk because of lower demographic and genetic input; Holderegger and Di Giulio, 2010; Zeller et al., 2012; Baguette et al., 2013). Unlike natural predation, roadkill is neither compensatory nor necessarily influenced by population densities (Puky, 2005) and can affect individuals regardless of their health condition (Polak et al., 2014). Additionally, since it consistently removes a proportion of a population, its impact is most severe on rare species (Puky, 2005). Roadkill is also not randomly distributed across the landscape (Malo et al., 2004; Santos et al., 2017), nor high mortality aggregation sites (also known as roadkill hotspots) are static over space and time (Medinas et al., 2021): significant annual and inter-annual variations have been associated with environmental and anthropogenic factors (Seiler & Helldin, 2006; Shilling & Waetjen, 2015). For example, migratory behaviour may place certain species at higher roadkill risk, especially if movement occurs with a high number of individuals, and in a specific season or weather condition (Cushman, 2006; Glista et al., 2008).

1.3. Mitigation measures for road impacts

Since the publication of the first studies alerting to the negative effects of roads on wildlife, there has been a growing interest in trying to understand and manage those impacts (van der Grift et al., 2015). Mitigation measures such as crossing structures – which ensure both human and wildlife safety – facilitate movements from one side of the road to the other (Schmidt and Zumbach, 2008; Glista et al., 2009; van der Ree et al., 2015), while fences prevent wildlife from reaching the road and, consequently, being road-killed. When used together, these measures allow safe movement between both sides of the road, promote connectivity between habitat patches and populations, and help maintain natural patterns of distribution (McRae et al., 2012; Huijser et al., 2016).

There are over 40 types of road mitigation measures aiming to affect either driver behaviour or animal behaviour (van der Ree et al., 2015; Rytwinski et al., 2016). The first includes road warning signs, traffic speed and volume reduction, and temporary road closure (Huijser et al., 2015), while the second involves the creation of attractive areas for animals away from roads, measures to avert animals from the road (e.g., reflectors and deterrent sound devices; e.g., Sousa-Guedes et al., 2020), and structures to direct animal movement to safe passages underneath or over roads (Clevenger et al., 2001; Hamer et al., 2015; Sijtsma et al., 2020). Several of these mitigation measures have been implemented (**Figure 1.3**), over the last 30 years, particularly in Central Europe (e.g., Forman et al., 2003; Rytwinski et al., 2016; Petrovan & Schmidt, 2019), and some target specific animal groups.

Figure 1.3. Examples of road mitigation measures: a) a green bridge in Singapore and b) an underpass in Portugal with a dry ledge to provide a safe cross during floods. Photo credits: Carolyn Khew and LIFE LINES.

1.4. Amphibians as a model group

1.4.1. Amphibian life history and ecological pressures

Amphibians represent the most ancient terrestrial vertebrate group, with a history spanning more than 350 million years (IUCN SSC Amphibian Specialist Group, 2024). This taxonomic group is more diverse than mammals, with approximately 8,650 described species – of which nearly 90% belong to the order Anura, 8% to Caudata and 2% to Gymnophiona – distributed across all continents except Antarctica (IUCN SSC Amphibian Specialist Group, 2024), with new species discovered every year (Catenazzi, 2015). As the word *amphibian* implies, these organisms are closely related to terrestrial and freshwater ecosystems, with many species relying on a source of fresh water (typically water bodies) at least once in their lifespan. Some species are less water-dependent (e.g., using it only for reproduction), while others occur almost exclusively in these ecosystems.

Amphibians are currently the most threatened vertebrate group on Earth, facing a myriad of threats that have led to global population declines over the past decades (Houlahan et al., 2000; Stuart et al., 2004; Catenazzi, 2015; Luedtke et al., 2023). This was initially acknowledged in 1989 (Blaustein et al., 1994; Alford & Richards, 1999), with several populations of this taxonomic group reported as declining or classified as species of special conservation concern (Alford & Richards, 1999). According to the International Union for the Conservation of Nature (IUCN) Red List of Threatened and Endangered Species, one in every three amphibian species is threatened with extinction globally (Baillie et al. 2004; Luedtke et al., 2023), and in Europe, nearly a quarter of all occurring amphibian species are at risk (Temple & Cox, 2009).

There is no doubt that disturbances from anthropogenic origin are the basis of many amphibian species' depletion (**Figure 1.4**; Catenazzi, 2015; Luedtke et al., 2023; IUCN SSC Amphibian Specialist Group, 2024) and that many of these declines are not a consequence of single factors, but rather several stressors that may act synergistically through complex interactions, such as: habitat loss and fragmentation (Carr and Fahrig, 2001; Cushman, 2006; Gallant et al., 2007); climate change (Carey & Alexander, 2003); pollution and UV-radiation (Blaustein et al., 2003); introduction of exotic species (Kats & Ferrer, 2003); spread of emerging diseases (Pounds et al., 2006; Fisher et al., 2009); and linear infrastructures (Matos et al., 2012; Beebee, 2013; Dornas et al., 2019; Sillero et al., 2019). Roads represent an increased risk to amphibians during their terrestrial phase (both as adults and juveniles), particularly during dispersal and migration movements (Eigenbrod et al., 2008; Sillero, 2008; Beebee, 2013).

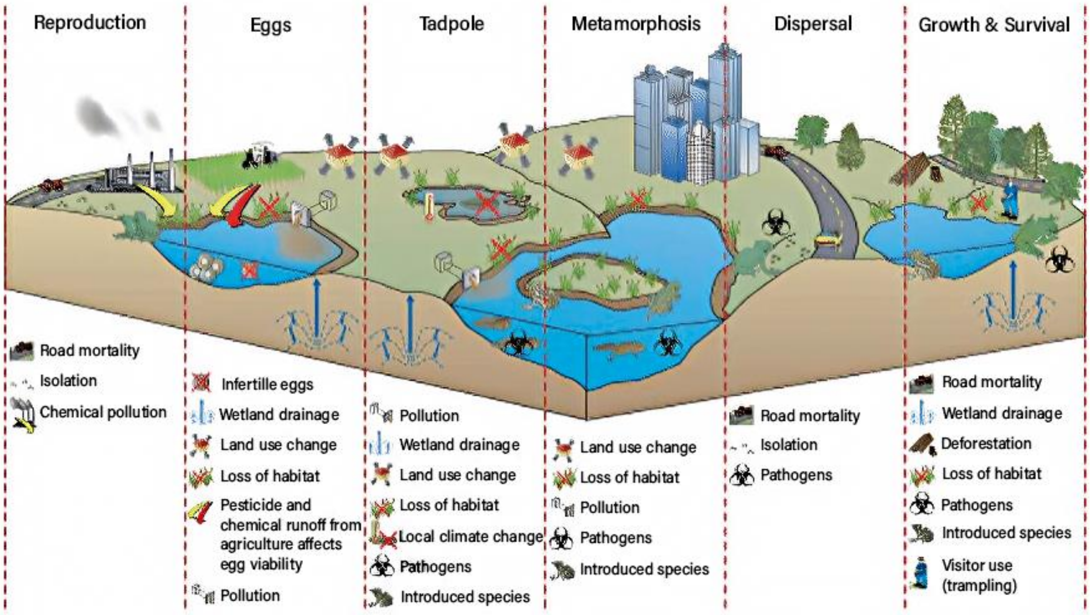

Figure 1.4. Anthropogenic ecological pressures for amphibians across different life stages (adapted from Lookingbill et al., 2007).

1.4.2. Effects of roads on amphibians and the importance of long-term monitoring

Amphibians are the vertebrate group with the highest mortality rates on roads, with estimates between 60% and 95% of road-killed vertebrates (Ascensão and Mira, 2005; Glista et al., 2008; Beebee, 2013; D'Amico et al., 2015; Garriga et al., 2017). The pronounced susceptibility of amphibians to road mortality can be attributable to various biological, physiological and behavioural traits such as: lower vagility; seasonal migrations to and from reproduction areas, often in great numbers (Carr and Fahrig, 2001; Sillero, 2008); immobility in the presence of approaching vehicles (Mazerolle et al. 2005; Lima et al. 2015); a greater amount of time required to cross the road surface (Hels and Buchwald, 2001); narrow habitat tolerances, exacerbating the effects of habitat loss and edge effects (Houlahan and Findlay, 2003; Pittman and Semlitsch, 2013); permeable skin with osmoregulatory and respiratory functions, increasing the sensitivity to road pollutants (Colino-Rabanal & Lizana, 2012); and behavioural attraction from some species (Forman et al. 2003).

Vocalisation is critical for some species' (anurans) communication, especially during the breeding season (e.g., to attract a mate) and high-traffic volume roads may have detrimental effects on breeding choruses of surrounding populations, particularly for the species that lack plasticity on their vocalisations to compensate for road noise (Nelson et al., 2017). Road contamination may also disrupt chemical trails (e.g. pheromones), negatively interfering with the communication and navigation of certain species (Andrews et al., 2006). Additionally, the dependency on a water source for reproduction, as well as winter and summer shelters that can be located further from the reproduction areas, increase movement frequency and consequently the mortality risk. Indeed, higher roadkill occurs at migration routes crossing roads between terrestrial and aquatic territories (coincident with hibernation/aestivation and reproduction habitats) (Orlowski, 2007; Eigenbrod et al., 2008; Sillero, 2008; Beebee, 2013). The maintenance of the connectivity between reproduction and shelter habitats is of special significance (Cayuela et al., 2020), particularly as amphibians inhabit both aquatic and terrestrial habitats and most species are subject to stressors from these different ecosystems (Blaustein et al., 1994; Becker et al., 2010).

Some effects of road mortality on amphibians are well documented. For instance, species exhibiting larger dispersal distances tend to experience a higher risk of roadkill (Carr and Fahrig, 2001; Cushman, 2006). Additionally, the proximity of water bodies or the presence of high-quality habitats near roads is associated with increased road mortality for many species (e.g., Ascensão and Mira, 2005; Sillero, 2008; D'Amico et al., 2015). Similarly, higher amphibian activity during specific times of the year (e.g. migration to and from reproduction and shelter sites) or under certain weather conditions (e.g., rainy nights with mild temperatures) often leads to identical outcomes (Glista et al., 2008; Orlowski et al., 2008). In contrast, the influence of landscape factors on amphibian roadkill patterns, and the extent to which they can be predicted, remains relatively unexplored. Similarly, there is a knowledge gap in understanding the impact of roads on amphibian mortality over time. Some particular reasons for this can be: 1) the difficulty in collecting data for some species, limiting the ability to predict specific roadkill patterns at different scales (Clevenger & Sawaya, 2010); and 2) temporal variation and lack of long-term monitoring, as most studies are conducted on short-term periods, or in specific seasons or weather conditions, restricting the understanding of how roadkill patterns change over time (Beebee, 2013). For instance, very few studies have assessed the negative impacts of roads on amphibian population trends, however, Carr and Fahrig (2001) stated that road mortality has more negative effects on populations of vagile frogs than on sedentary ones. Also, Hels and Buchwald (2001) estimated a loss of 10% of amphibian populations near roads in Denmark. The limited number of studies that address this issue are usually conducted in short-term periods, with occasional records, and report mostly seasonal variations in roadkill, which can lead to misleading results (Sillero, 2008).

To overcome the challenges of collecting amphibian data through field-based methods, roadkill data can constitute a valuable alternative, for example, in allowing researchers to make inferences about population trends (Schwartz et al., 2020). If roadkill data are collected over long periods, variations within and between years are included, supporting more robust inferences on the possible impact on population persistence. In some cases, roadkill might be the primary source of data for many species, therefore, collecting data over longer periods, increases the likelihood of gathering information on elusive species across all terrestrial amphibian life stages. Thus, long-term studies are particularly important as the full effect of roads on amphibian populations may take decades to become apparent if the initial populations are reasonably high (Findlay and Bourdages, 2000).

1.5. Landscape connectivity and wildlife movement

Wildlife movements are determined by the ability of an animal to move through the landscape. These movements (e.g., daily activities, migration, dispersal) are important for population persistence but, in highly fragmented habitats such as road-dominated landscapes, they can be markedly disrupted as suitable habitat patches may be lost or become isolated (Diniz et al., 2020).

Taylor and colleagues (1993) described the term *Landscape connectivity* as the degree to which the landscape facilitates or hinders the movement of an organism among resource patches. As landscape connectivity is influenced by both landscape structure and species-specific responses, it is commonly considered through two fundamental concepts: 1) structural connectivity – determined by the composition and spatial configuration of the landscape and influenced by factors such as the continuity of suitable habitats and presence of alternative pathways (Collinge and Forman, 1998); and 2) functional connectivity as the connectivity experienced by a species within the landscape, shaped by its distribution, dispersal capacities and behavioural tendencies, as well as habitat preferences, level of specialisation, or even response to predators (Tischendorf and Fahrig, 2000; Baguette and Van Dyck, 2007). The probability of an individual or species moving between patches will also be closely related to the landscape permeability or resistance between those patches (Baguette et al., 2013). Therefore, understanding animal movement is essential to develop effective connectivity-driven conservation strategies (Vasudev et al., 2015). However, traditional field-based methods (e.g. telemetry and capture-mark-recapture studies) require an impressive amount of sampling effort, which is expensive, logistically challenging, or even unachievable for many species (Kindlman and Burel, 2008; Kays et al., 2015), as is the case for amphibians (Cayuela et al., 2020). Yet, landscape connectivity can be assessed through methods such as species spatial distribution and habitat suitability models (e.g. through resistance surfaces), presenting a cost-effective method, when others are inexistent (Valerio et al., 2019).

Pond-breeding amphibians – most of the species occurring in Europe – are generally considered to be organised in metapopulations (Marsh & Trenham, 2001) as they are typically associated with water bodies scattered through the landscape and present lower dispersal capacities (but see Smith & Green, 2005). The metapopulation concept describes an assemblage of distinct populations belonging to the same species, that occur in the same region and exchange individuals through dispersal or migration (Hanski, 1998; Marsh & Trenham, 2001). For this reason, landscape connectivity ensures the long-term persistence of these populations, as it enables individuals to disperse between suitable habitat patches. In locations with rapidly changing landscape structures (e.g., regions where water bodies are highly susceptible to stochastic events (Cushman, 2006; Ribeiro et al., 2011)), this may be even more challenging, as it can result in increased distances between suitable patches and consequentially, higher rates of dispersal. If the distance between these patches exceeds the species' dispersal capacity, population survival may be jeopardised (Kindlmann & Burel, 2008). Reproduction sites are common aggregation sites for many amphibian species and the structural connectivity of water body networks represents an essential feature for their presence (Ribeiro et al., 2011). This is notably relevant for amphibian population persistence since they frequently experience local extinctions, even in non-fragmented landscapes (Trenham et al., 2003). The degree of functional connectivity across the landscape is species-specific and varies with the species' dispersal capacity and the number of dispersing individuals (Kindlmann & Burel, 2008; Baguette et al., 2013). Understanding landscape resistance to different species' movement and quantifying its connectivity, may ultimately help prioritise high-movement probability sites for implementing measures aimed at maintaining or increasing landscape connectivity, ultimately supporting populations' viability. This is particularly urgent in landscapes severely impacted by anthropogenic and environmental changes (Nowakowski et al., 2017), and where these measures may benefit the greatest number of species. Additionally, the increasing availability of free high-resolution (spatial and temporal) remote sensing satellite imagery (e.g. Landsat imagery catalogue) can provide significant opportunities to extend the knowledge of the spatial distribution and functional connectivity, with particular emphasis on tracking landscape dynamics over space and time – a topic that remains relatively underexplored for this taxonomic group. Remote sensing techniques, combined with GIS and landscape analysis offer effective methods for habitat mapping and ecological corridor design (Neumann et al., 2015).

Besides habitat corridor delineation and planning, landscape connectivity quantification can also be applied to road ecology (Koen et al., 2014). Identifying critical movement corridors through landscape connectivity analysis may help to determine optimal sites for implementing mitigation structures on existing roads, or even support a more sustainable road design during the planning of new infrastructures. Previous research has shown that road intersections with high connectivity corridors often show increased roadkill for multiple taxa (Clevenger and Huijser, 2011; van der Ree et al., 2015). Therefore, identifying these locations could also maximise conservation efforts, through the implementation of mitigation structures that may benefit a wide range of species with different traits (Valerio et al., 2023).

Finally, integrating landscape connectivity with road planning and mitigation may go beyond specifically reducing roadkill, as it may contribute to broader landscape-scale conservation by fostering gene flow and mitigating the impact of habitat fragmentation (Haddad et al., 2015) ensuring population persistence and viability.

1.6. Amphibian-oriented mitigation measures and their effectiveness

The first known roadkill mitigation measures for amphibians in Europe were implemented in Hungary in 1986 (Puky, 2003). Since then, a plenitude of structures with different designs and constructions have been developed and used, including: 1) road signs (**Figure 1.5a**), used to alert drivers to a particular section of the road that is frequently crossed by amphibians; 2) temporary or permanent drift fences (**Figure 1.5b**), built of canvas or plastic (temporary) or made from concrete, metal or UV-resistant polymers (permanent) that prevent animals from reaching the road surface; 3) temporary road closure, usually during amphibian movement peaks; and 4) underpasses or road tunnels (**Figure 1.5b**), with one entry on each side of the road, to allow animals to move safely between both sides of the road (reviewed in Jochimsen et al., 2004; Schmidt and Zumbach, 2008). Likewise, compensation measures can also benefit amphibian populations affected by roads. For instance, constructing new ponds and water bodies, typically away from roads, can help offset road mortality that cannot be prevented (Lesbarrères et al., 2010). Based on available information, drift fences and underneath tunnels are the most commonly used measures, as they provide the most favourable solution for reducing amphibian road mortality and barrier effects (Puky, 2003; Beebee, 2013; Hamer et al., 2015; Jarvis et al., 2019). Still, there are some amphibian species – such as those with climbing abilities – for which no structures yet exist to significantly prevent them from being road-killed (Dodd et al., 2004).

Several studies have attempted to reveal the success of many of these measures in reducing amphibian mortality, with some of them reporting promising results (e.g., Beebee, 2013; Helldin & Petrovan, 2019; Conan et al., 2023). Yet, impeding animals from being killed on roads might not always be a good solution. In reality, if an animal is not allowed to cross a road, the mitigation structure might be causing more harm than good because, by restricting animal movement, the likelihood of reproduction and dispersal might be at risk (Jaeger & Fahrig, 2004; Schmidt & Zumbach, 2008; Matos et al., 2019; Ottburg and van der Grift, 2019; Brehme et al., 2021). This is particularly difficult to examine in amphibians as there is scarce information about population status in sites where mitigation measures have already been implemented, and if these measures enhance population viability in the long term (Schmidt & Zumbach, 2008). For instance, despite a tremendous effort to save common toads (*Bufo bufo*) from roadkill over the last decades (volunteers collecting individuals during migration on roads and along fences to assist road crossing), this species is still declining in some European countries (Petrovan & Schmidt, 2016). Matos and colleagues (2019) studied the movements of the great crested newt (*Triturus cristatus*) along several mitigation measures in the UK. The authors detected few animals entering the tunnels, suggesting that individuals do not cross the road as part of their annual breeding migrations. Despite the majority of the newts being found alongside fences, these infrastructures generally acted as barriers to movement, diverting the newts away from the road and rarely directing them towards the tunnels. A reason for the lack of success in finding effective measures to reduce amphibian road mortality and barrier effects may be due to insufficient monitoring or lack of temporal scale (van der Ree et al., 2007), as it is often logistically impossible for many studies to monitor these structures several years before and after their implementation.

While knowledge of the mortality spatial patterns before the implementation of a particular measure is undoubtedly useful for determining the optimal location where that measure might be installed, its effectiveness can only be assessed through long-term monitoring, performed after the mitigation measure construction phase (Boyle, 2021). Failing to adopt this approach may lead to uncertain or misleading conclusions (Lesbarrères and Fahrig, 2012). When long-term studies are not feasible, complementary techniques such as ecological modelling or landscape connectivity analyses (aided by high-resolution remote sensing satellite imagery) may assist in determining the most suitable sites for implementing mitigation measures.

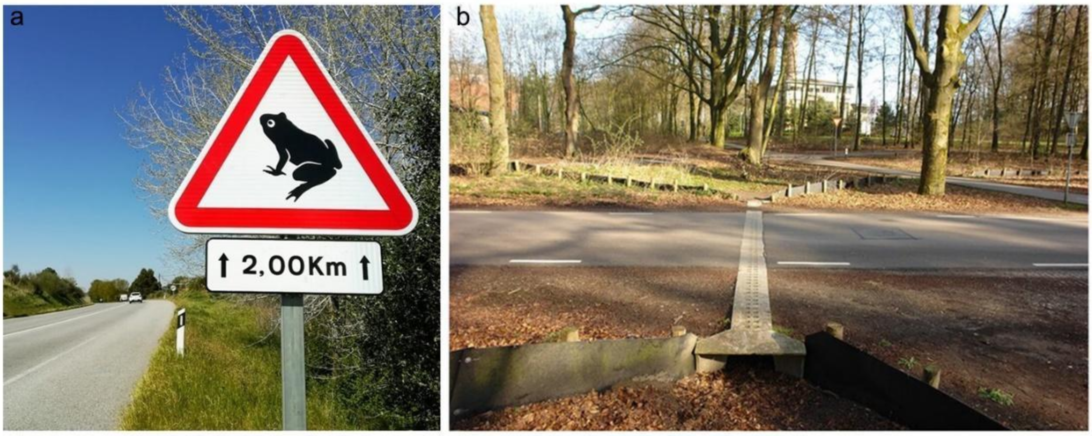

Figure 1.5. Examples of amphibian-oriented roadkill mitigation measures: a) a road sign and b) an underneath tunnel and drift fences. Photo credits: LIFE LINES and F. Ottburg.

1.7. Research goals

Amphibians are the most impacted vertebrate group by the negative effects of roads. However, it is also one of the least studied animal classes and there are still some knowledge gaps in the implementation of effective roadkill mitigation measures. As the resources to invest in species protection are typically limited, practitioners need to reduce uncertainties and find cost-effective solutions that fit multiple species, while restoring connectivity in less permeable areas (Lesbarrères & Fahrig, 2012; Sijtsma et al., 2020). To accomplish this, a combination of knowledge of populations' status, together with the effects caused by roads and their consequences on landscape functional connectivity is critical, although very few studies have managed to accomplish this, especially for amphibians (Carr and Fahrig, 2001). The solution to draw better conservation measures and practices for this taxonomic group may rely on long-term studies that integrate inter-annual variations of landscape derived from natural or human-induced changes. The large availability of remote sensing satellite data provides detailed landscape composition, allowing a myriad of possibilities for tracking environmental dynamics over long periods (Wang et al., 2010). This integrated approach may provide advantages in strategic mitigation planning by identifying locations with resilience to landscape changes over the long term (Arenas-Castro & Sillero, 2021), allowing the implementation of structures with proven efficiency to reduce roadkill risk for this target group. This is especially important in a highly biodiverse and water-scarce region such as the Mediterranean Basin.

Therefore, the main purpose of this work is to understand the landscape drivers and connectivity on the spatiotemporal patterns of amphibian roadkill, using remote sensing satellite data. The necessity of understanding amphibian roadkill patterns, accounting for spatiotemporal environmental variations at a fine scale, may be crucial for selecting the most impacted locations for the implementation of mitigation structures. Likewise, the urgency of finding structures that can effectively reduce amphibian roadkill while providing connectivity through different habitats is a matter of extreme importance in conservation biology (Jarvis et al., 2019; Boyle et al., 2021). Ultimately, assessing movement corridors for amphibians and where they are crossed by roads may help to delineate conservation actions for reducing roads' negative effects on this highly threatened vertebrate group.

Accordingly, this thesis has four specific research goals, each linked to respective hypotheses, as follows:

1) Identify the landscape and environmental drivers of amphibian roadkill spatial patterns. I hypothesise that low water availability in the landscape will be correlated with lower probabilities of amphibian roadkill, since

these conditions reduce suitable habitats for amphibians (Araújo et al., 2006). Moreover, water bodies closer to roads will be correlated with higher levels of amphibian roadkill because these are common aggregation sites for most amphibian species (D'Amico et al., 2015).

2) Assess the temporal trends of amphibian mortality and the landscape drivers of spatial-temporal patterns. I expect these patterns to fluctuate over space and time, mainly due to changes in climatic factors (e.g., seasonal variability) and landscape features (Glista et al., 2008).
3) Evaluate the effectiveness of mitigation measures in reducing amphibian mortality. I hypothesise that the mitigation measures significantly prevent amphibians from reaching the roads and that the presence of amphibians on roads does not increase in road sections adjacent to the mitigation measures (Helldin & Petrovan, 2019).
4) Evaluate the relationship between landscape connectivity, movement probability, and roadkill probability. I expect road sections with roadkill to be positively correlated with high landscape connectivity (Clevenger and Huijser, 2011; van der Ree et al., 2015).

1.8. Thesis structure

Considering the above, the thesis is structured in six main chapters: the introduction (chapter 1); four chapters that include the research developed for the specific research goals of the thesis (1 to 4), through scientific manuscripts either published (chapters 2, 3 and 4) or submitted to publication (chapter 5) in peer-reviewed international journals; and a final chapter (chapter 6) with the overall conclusions drawn from the research and new insights acquired throughout the study. The chapters have the following structure:

- Chapter 1 corresponds to the general introduction, where I present a brief background developing the theoretical framework and basal topics related to the research, as well as the thesis' main objectives and structure. I also present a description of the study area where the research was developed.

- Chapter 2 describes the key drivers of amphibian roadkill spatial patterns accounting for one of the most critical agents for amphibian survival in Mediterranean landscapes – water. For different species with diverse water dependencies, I investigate the roadkill spatial patterns, using a combination of landscape and other remote sensing-derived predictors. This chapter corresponds to the following publication:
    - Pinto, T., Santos, S. M., Mira, A., & Sillero, N. (2023). Importance of water availability for amphibian roadkill in a Mediterranean landscape. *Biodiversity and Conservation*, 32(7), 2513-2537 https://doi.org/10.1007/s10531-023-02616-9

- Chapter 3 focuses on unveiling the spatiotemporal patterns of amphibian roadkill through a timespan of over a decade, with the implementation of a methodology rarely used in road ecology – occupancy modelling. I model four amphibian species with different ecological and habitat requirements, and I make inferences on possible population trends through time. The content of this chapter is published in the following article:
    - Pinto, T., Sillero, N., Mira, A., & Santos, S. M. (2024). Using the dead to infer about the living: Amphibian roadkill spatiotemporal dynamics suggest local populations' reduction. *Science of the Total Environment*, 927, 172356 https://doi.org/10.1016/j.scitotenv.2024.172356

- In Chapter 4, I test the effectiveness of mitigation measures in reducing amphibian roadkill risk. By adopting a before-after-control-impact (BACI) sampling design that started before the construction of the mitigation structures, until several years after their implementation, I demonstrate the capacity of these measures to reduce the roadkill risk and enhance amphibian conservation. This chapter corresponds to the following publication:

  - Pinto, T., Sillero, N., Mira, A., Sousa, L. G., Oliveira, A., & Santos, S. M. (2024). Effectiveness of permanent drift fences in reducing roadkill risk of amphibians. *Journal of Environmental Management*, 368, 122049. https://doi.org/10.1016/j.jenvman.2024.122049

- In Chapter 5, I determine whether areas with higher amphibian movement probability have also increased roadkill risk when crossed by an existing road. I use ecological niche models (ENMs) and circuit theory to build habitat suitability and connectivity models, respectively, for two amphibian species inhabiting the study area. Lastly, I evaluate whether these modelling tools can be used to effectively predict the best locations for implementing roadkill mitigation measures by relating road sections with high movement probability to the presence or absence of roadkill. The content of this chapter is currently under review as the following:
  - Pinto, T., Santos, S. M., Mira, A., Sillero, N. (under review). Tell me where you go, and I'll tell you where you die: landscape connectivity as a tool to predict amphibian roadkill risk. *Journal of Environmental Management*.

- Chapter 6 summarises the main results of chapters 2, 3, 4, and 5 while pointing out additional research. Based on these thesis outcomes, I discuss the relevance of these findings to the current knowledge and its potential limitations, and describe further questions this study may have raised.

1.9. Study area

The study area lies within the Mediterranean Basin. Globally, this region is considered the second-largest biodiversity hotspot in the world (Médail and Quézel, 1999), and is unique because of its long history of coexistence between wildlife and humans. Nevertheless, since the middle of the twentieth century, human pressures have been strongly intensified through the construction of large road networks crossing or close to important natural areas, and agriculture expansion and intensification (Blondel et al., 2010). Moreover, the Mediterranean is also considered one of the most vulnerable regions in the world to the impacts of global warming (Tuel and Eltahir, 2020) and water scarcity (EEA, 2023). During the last century, the air temperature has risen in all regions of the Mediterranean, particularly in the Iberian Peninsula and southern France, with an increase of almost 2°C (UNEP/MAP, 2017). The predictions for this region also include a substantial reduction in rainfall, contributing to desertification (UNEP/MAP, 2017; Tuel and Eltahir, 2020). This is even more alarming since the Iberian Peninsula comprises almost 50% of European fauna and flora, with more than 30% of endemic species (Araújo et al., 2007) and within these, twenty-eight amphibian species including twelve endemisms (Sillero et al., 2014).

Given the special relevance in terms of climate vulnerability and amphibian richness, the selected area to conduct this study (with approx. 210,000 ha) is located in the Central Alentejo region in Southern Portugal, Iberian Peninsula (**Figure 1.6**).

The study area lies within the Southwest Iberian Mediterranean sclerophyllous and mixed forests ecoregion (Scarascia-Mugnozza et at., 2000), dominated by a typically Mediterranean climate, with hot and dry summers (mean temperatures between 16.5°C and 30.2°C) and mild and wet winters (mean temperatures between 5.8°C and 12.8°C). The annual precipitation ranges between 500 mm and 650 mm, occurring predominantly from October to March (IPMA, 2021), and the orography is mainly flat, characterised by gentle slopes, with altitudes ranging between 100 m to 400 m a.s.l. The landscape is essentially composed of dispersed trees of Cork oak (*Quercus suber*) and Holm oak (*Quercus rotundifolia*) with varying cover, mixed with pastures and agriculture crops, composing the complex agrosilvopastoral system known as *montado* (**Figure 1.7**; Pinto-Correia, 1993; Pinto-Correia et al., 2011). This landscape mosaic covers approximately 3.5-4.0 million hectares in the Southwest Iberian Peninsula (Olea & San Miguel-Ayanz, 2006) and is considered one of the ecosystems with the highest biodiversity in the western Mediterranean Basin (Pinto-Correia et al., 2011).

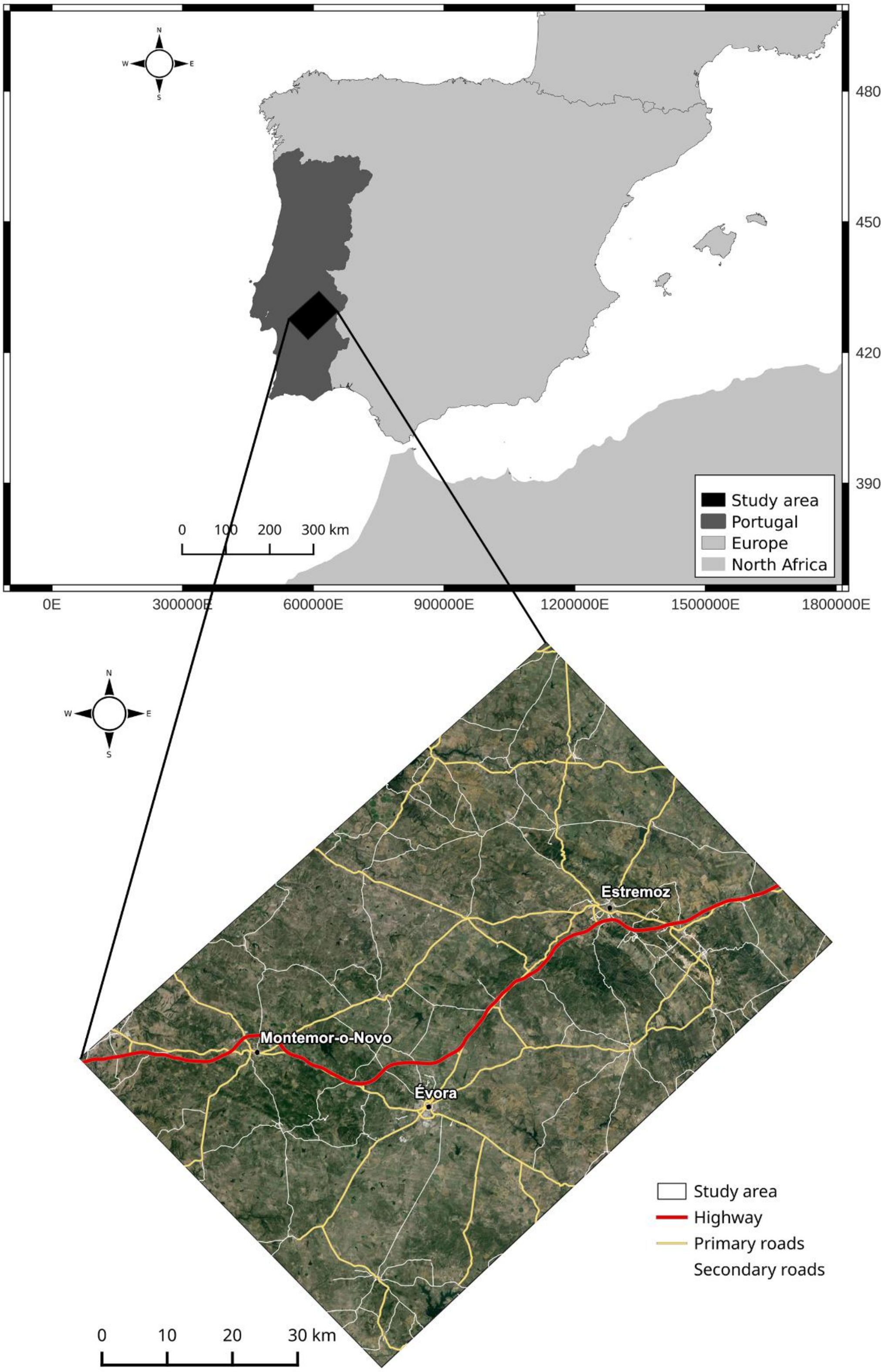

Figure 1.6. Study area where this thesis was conducted. It is located in the Iberian Peninsula – a well-known glacial refugia for herpetofauna with high levels of genetic diversity (Gómez and Lunt, 2007; Sillero et al, 2009) – within the major cities of Montemor-o-Novo, Évora and Estremoz, and is characterised by a high concentration of roads.

The *montado* is the product of centuries of human changes through practices such as the production of cork, acorns and firewood, as well as agricultural exploitation (Olea & San Miguel-Ayanz, 2006; Blondel et al., 2010). In the study area, production activities like cereal crops, combined with extensive livestock grazing are also present (Pinto-Correia and Mascarenhas, 1999), as well as orchards and permanently irrigated agricultural areas (powered by constructed dams) with increasing cultures of olive groves, vineyards, and water-dependent crops. Much of the existing water is concentrated in artificial, man-made water bodies (mean area = 2 ha) primarily used for agricultural practices. Consequently, these bodies are often subject to poor management and may harbour exotic species (e.g., predatory fish); some may even act as ecological traps (Wei et al., 2023). Temporary water bodies (usually referred to as ponds) are also present; however, they have been destroyed in the last decades, mainly due to agricultural intensification (Ferreira & Beja, 2013).

The study area is also characterised by a well-established road network (with a density of 1 km/km^2) and is crossed by the main transportation corridor linking Lisbon to Madrid. All these characteristics contribute to amphibian threats (including increased roadkill rates) and were the main reason for the choice of conducting the study at this location.

Figure 1.7. The *montado* ecosystem is representative of the study area: a) Holm oak sparse forest and open agricultural area and b) water body mainly used for livestock watering. Photo credits: Tiago Pinto.

Chapter 2

Importance of water availability for amphibian roadkill in a Mediterranean landscape

Bufo spinosus

Published in *Biodiversity and Conservation* as:

Pinto, T., Santos, S. M., Mira, A., & Sillero, N. (2023). Importance of water availability for amphibian roadkill in a Mediterranean landscape. Biodiversity and Conservation, 32(7), 2513-2537. https://doi.org/10.1007/s10531-023-02616-9

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This chapter maintains the formatting rules of the corresponding journal in which it was published.

2.1. Abstract

Roads can negatively impact ecosystems by fragmenting habitats and affecting animal movements and behaviour. One of the major noticeable effects of roads is animal mortality by vehicle collisions, a paramount threat to wildlife, especially for amphibians. In the context of reduced water availability, particularly in the Mediterranean region, amphibians are projected to be one of the most negatively affected animal groups. In this study, we used 14-year road mortality data collected along 120 km of roads, combined with landscape and remote sensing variables, to identify the drivers of amphibian mortality spatial patterns in a Mediterranean landscape, in Southern Portugal. We recorded 5116 carcasses belonging to five amphibian species. Generalised linear models showed that for most of the species, roadkill numbers increased with the decrease in water availability in water bodies. Also, the distance of water bodies to the roads was important in understanding amphibian roadkill patterns, with a general increase in mortality at reduced distances. Land use variables were also significant drivers for amphibian road mortality, with species-specific responses. Roadkill numbers decreased for the Iberian ribbed newt and the fire salamander in agricultural dominated areas. Our results also show an increase in roadkill numbers for the natterjack toad in areas with higher percentage of olive groves, and an opposite response for the fire salamander. We recognize the importance of long-term studies in assessing roadkill patterns, and their value for amphibian monitoring and conservation.

2.2. Keywords: roadkill mortality, spatial patterns, remote sensing, land surface temperature, conservation.

2.3. Introduction

Roads and highways are one of the most ubiquitous man-made features across the landscape. They aim to facilitate connectivity among humans and economic progress (Meijer et al. 2018), but they also pose a varied range of negative effects, including habitat loss, degradation, and fragmentation; shifts in biophysical flows; spreading of invasive species; changes in the availability of resources such as water and nutrients; and disturbance caused by chemical, light and noise pollution (Coffin 2007; van der Ree et al. 2015). Roads also create a barrier to wildlife movement, isolating populations, and modifying animal behaviour such as migration or dispersal, ultimately jeopardising the long-term viability of populations (Jongsma et al. 2014; Ceia-Hasse et al. 2017; Howell & Seigel 2019). However, one of the major effects of roads on wildlife is the direct mortality by roadkill, whose magnitude depends on ecological requirements and life history traits, making some species more prone than others (Forman et al. 2003, D'Amico et al. 2015, Santos et al. 2016). Among vertebrates, the amphibians are the taxonomic group with the highest mortality rates (Glista et al. 2008) due to their biological traits, such as low vagility, narrow habitat tolerances and seasonal migrations to and from reproduction areas (Carr and Fahrig 2001; Sillero 2008). Higher mortality is often reported on roads close to water bodies used for reproduction (Ascensão and Mira 2005; Orlowski 2007; Santos et al. 2007; Cooke 2011), and on migration routes between high-quality habitats (Sillero 2008). Behavioural traits also make this taxon exceptionally susceptible to death on roads, including immobility when approaching vehicles (Mazerolle et al. 2005); not avoiding roads during their migrations, moving slower on the asphalt, than in surrounding areas (Bouchard et al. 2009); attraction of some species to roads in foraging excursions, to hunt or in search for a potential mate (Speybroeck et al. 2016). In addition, some amphibian species depend on an intricate landscape structure to complete their complex life cycles that allow movement from feeding and aestivation/hibernation to reproduction habitats (Joly 2019). These traits make this group particularly vulnerable to genetic structuring and potential population declines due to roads (Holderegger & Di Giulio 2010).

The Mediterranean Basin is considered the second largest biodiversity hotspot in the world (Médail & Quezel 1999), and is unique because of its long history of coexistence between wildlife and humans. Nevertheless, since the middle of the twentieth-century human pressures have been strongly intensified including the construction of large road networks crossing or close to important natural areas (Blondel et al. 2010). As a result, this proximity could potentially place wildlife at a higher risk (Fahrig et al. 1995), justifying the need for further studies aiming to understand the role of landscape features in promoting or impeding roadkill (e.g. Sillero 2008; Carvalho & Mira, 2011). The Mediterranean is also considered one of the most vulnerable regions in the world to the impacts of global warming (Tuel & Eltahir 2020). During the last century, the air temperature has risen in all regions of the Mediterranean, particularly in the Iberian Peninsula and southern France, with an increase of almost 2ºC (UNEP/MAP 2017). The predictions for this region also include a substantial reduction in rainfall, contributing to desertification (UNEP/MAP 2017; Tuel & Eltahir 2020). This is even more alarming since the Iberian Peninsula comprises almost 50% of European fauna and flora, with more than 30% of endemic species (Araújo et al. 2007) and within these, twenty-eight amphibian species including twelve endemisms (Carretero et al. 2018). Ectotherms are considered especially vulnerable to climate change (Gibbons et al. 2000; Araújo et al. 2006) and for many amphibian species, future predictions point to a contraction of their distribution ranges (Araújo et al. 2006; Carvalho et al. 2010). The increase in temperature and rainfall reduction are expected to decrease the availability of water resources and enhance the spread of infectious diseases (Pounds et al. 2006), two factors that are negatively affecting amphibian populations worldwide. The known water dependency for most amphibian species to complete their life cycles, places this group at exceptional risk, especially in semi-arid areas (Henle et al. 2008; Carvalho & Mira 2011). These effects, coupled with the costs of the roadkill, may produce catastrophic consequences for amphibians, thus, it is urgent to address the drivers of amphibian roadkill in the Mediterranean region to mitigate this threat more efficiently.

Several studies have addressed amphibians' roadkill patterns in conjunction with the use of new technology and methods (Elzanowski et al. 2008; Glista et al. 2008; Sillero 2008; Matos et al. 2012; Mestre et al. 2019); nevertheless, the influence of landscape factors on roadkill patterns, and the extent to which they can be predicted, is yet to be unveiled (Cushman 2006; Beebee 2013; Petrovan & Schmidt 2019). Most studies that address this issue are conducted in short-term periods, with occasional records, and report mostly seasonal variations in roadkill, which could lead to misleading results (Sillero 2008). Long-term studies, however, incorporate data with variations between years, providing more robust inferences that allow to better understand the possible impact on the persistence of populations (Pinto et al. 2020). Coupled with this, the use of free spatial high-resolution remote sensing data that provides consistent long-term Earth observation from local to global scales (Wang et al. 2010), can yield innovative valuable data (such as vegetation productivity and landscape structure) at very small spatial scales and periodicity. Since amphibians respond to small scales, these products are an important tool to assess the landscape and climatic drivers of amphibian roadkill. There are several studies that infer that remote sensing data such as vegetation productivity, water availability and land surface temperature have great potential in mapping land use change trends at small scales (Ehsan & Kazem 2013; Campos et al. 2012; Muro et al. 2018). In combination with other factors that previous research demonstrated to be related to amphibian road mortality (e.g. distance to ponds; habitat quality) (Sillero 2008; Carvalho & Mira 2010; Coelho et al. 2012; Matos et al. 2012; Heigl et al. 2017), these tools should improve the efficiency and feasibility of conservation and mitigation measures, often highly expensive and taxa specific (Ascensão et al. 2019).

In this study, we identify the drivers of amphibian mortality spatial patterns in a Mediterranean landscape in Southern Portugal, by combining landscape and remote sensing predictors. Specifically, we explore water availability drivers across different amphibian species, to better understand the mortality patterns of most roadkilled amphibians. We hypothesise that low water availability in the landscape will be correlated with lower probabilities of amphibian road mortality since these conditions reduce suitable habitats for amphibians (Araújo et al. 2006). Moreover, water bodies closer to roads will be correlated with higher levels of amphibian mortality because these places are common aggregation sites for most amphibian species (D'Amico et al. 2015).

2.4. Methodology

2.4.1. Study Area

The study was conducted in an area of approx. 210.000 ha, located in Alentejo, Southern Portugal (29N 599606E, 4285394N, WGS84) (**Figure 2.1**). The topography is flat (ranging from 100m to 400m a.s.l.), and the landscape is mainly composed of Mediterranean cork (*Quercus suber*)/holm (*Quercus rotundifolia*) forests, with varying tree cover (between 20 to 80 trees per hectare; Pinto-Correia and Mascarenhas 1999) composing the complex agrosilvopastoral system called *montado* (Pinto-Correia 1993), and agricultural areas in equal proportions. Production activities like cereal crops combined with extensive livestock grazing are also present (Pinto-Correia and Mascarenhas 1999), as well as open agricultural areas, orchards and permanently irrigated agricultural areas. This landscape mosaic structure is considered one of the ecosystems with the highest biodiversity in the western Mediterranean Basin (Pinto-Correia et al. 2011). The climate is typically Mediterranean, with hot and dry summers (where temperatures can exceed 40ºC), mild and wet winters (5.8-12.8ºC), and annual precipitation ranging between 500 and 650mm (IPMA 2021). The study area is crossed by the main transportation corridor between Lisbon and Madrid and a network of linear infrastructures. Roadkill surveys were carried out along four National Road sectors (EN4, EN114, EN370 and EN18; > 4000 vehicles/day, IP 2005) and one Municipal Road sector (EM529; < 4000 vehicles/day, IP 2005). All roads are two-lane wide.

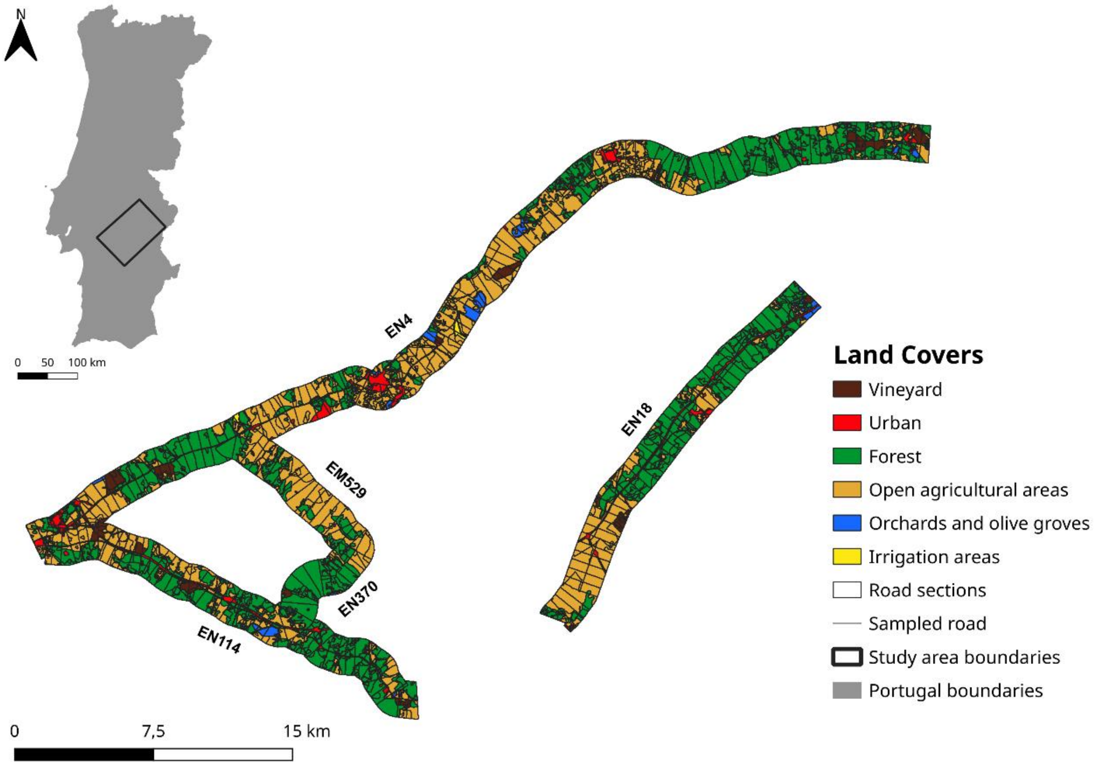


Figure 2.1. Map of the study area in Southern Portugal, with the sampled roads and land cover in the 1000m buffer for each 500m road segment.

2.4.2. Roadkill data

Amphibian roadkill data were compiled from the University of Évora database, for a period of 14 years (2006 to 2020), corresponding to a total of ca. 120 km of sampled roads (distributed across 5 roads; Table S2.1 – Supplementary materials). Over this period, an experienced observer drove a car at 20-40 km/h during the first morning hours (to reduce the impact of traffic and scavengers in carcass removal), checking both sides of the road (including lanes and shoulders), and collecting and registering roadkill animals (for further details on monitoring procedures, see Santos et al. 2011; Pinto et al. 2020). All amphibian carcasses were identified to the lowest possible taxonomic level and removed from the road to avoid double counting. Due to budget constraints over the years, the sampled roads and sampling periodicity varied across the study time frame (STF); some years were sampled daily, others weekly, and others had mixed sampling (daily in Spring and Summer months, and weekly sampling in Winter months; Table S2.1 - Supplementary materials). To standardise carcass persistence errors and maximise temporal and spatial variability, we retrieved only weekly roadkill data. For this study, we selected carcasses that were identified to the species level and retained five species with high numbers of roadkill (> 400): Fire salamander (*Salamandra salamandra*), Iberian ribbed newt (*Pleurodeles waltl*), natterjack toad (*Epidalea calamita*), spiny common toad (*Bufo spinosus*), and Iberian spadefoot toad (*Pelobates cultripes*). We used the taxonomy and nomenclature according to Carretero et al. (2018).

2.4.3. Environmental predictors

For analysis purposes, we split each road into 500 m-length contiguous segments and because species perceive landscape at different scales, sometimes due to a different spatial use (Bennet et al. 2006; Ingham and Samways 1996), we defined four buffer widths at each road section (100 m, 250 m, 500 m, and 1000 m) to characterise landscape traits around each segment. The road segment size was based on the mean distances regularly covered by the most roadkilled amphibian species (Wells 2007; Joly 2019), and the buffer sizes were based on the average road effects on previous studies (Langen et al. 2009; Carvalho & Mira 2011; Joly 2019).

We selected several environmental predictors based on the species ecology and aims of the study (Sillero 2008; Carvalho & Mira 2011; Matos et al. 2012; D'Amico et al. 2015). To provide our models with the most updated information on environmental changes, and variations of local dynamics across the study time frame in our study area, we used remote sensing-derived predictors, revealing a set of local dynamics, together with land cover, topography and distance to nearest highway and water bodies. A total of 19 environmental predictors describing different categories were measured (Table 2.1): land cover (n=6), water availability (n=5), vegetation productivity (n=4), land surface temperature (n=2), road proximity (n=1), and topography (n=1). For the land cover predictors, we used CORINE Land Cover (CLC; EEA) 2006, 2012 and 2018 to characterise the land use during the 2006-2020 period. We merged some land cover classes to reflect the main land uses in the study area (Table S2.2 – Supplementary materials). At each buffer, we extracted the mean land cover class from the three CORINE years to a 30m pixel resolution using QGIS Software (QGIS Development Team 2022).

Water is a critical element for amphibians, regulating their activity and reproduction (Speybroeck et al. 2016); for this reason, we calculated predictors that represent water availability in the landscape. We computed the mean Normalised Difference Moisture Index (M_NDMI; Gao 1995), sensitive to changes in water content on vegetation, and its standard deviation (SD_NDMI), representing the variation throughout the STF (2006-2020); and the mean Normalised Difference Water Index (M_NDWI; McFeeters 1996) and the respective standard deviation (SD_NDWI), to monitor changes related to water content in water bodies and its variability, respectively. These indexes were calculated from LANDSAT images (LANDSAT 5 and LANDSAT 8 image collections (Level 2, Tier 1)) with a 30m spatial resolution (US Geological Survey (USGS) – http://lpdaac.usgs.gov; for a brief description of the LANDSAT program please see Supplementary materials) accounting for the STF. The time frame included full years of the study period, and we only retained high-quality images with 0% cloud cover for the whole study area (WRS-2 scene: path 203, row 33). We also calculated ‘distance to ponds’ predictor by applying Euclidean distance to a 30 m spatial resolution raster (derived from existing cartography) to measure the influence of the water bodies on roadkill (range of water bodies area = 26 – 395 m^2). We performed the calculation of this last predictor using QGIS Software (QGIS Development Team 2022). In addition, we computed several predictors of vegetation productivity that have been successfully used in previous amphibian studies and explain species occurrence (e.g. Qian et al. 2007), namely: mean and SD of the Enhanced Vegetation Index (M_EVI and SD_EVI; Liu and Huete 1995); the mean and SD of the Normalised Difference Vegetation Index (M_NDVI and SD_NDVI; Rouse et al. 1973); and the mean and SD of the Soil-Adjusted Vegetation Index (M_SAVI and SD_SAVI; Huete 1988). All these indexes quantify vegetation greenness and their variability across the STF, however, EVI corrects for some atmospheric conditions and canopy background noise, being more sensitive in dense vegetation areas (Liu and Huete 1995), and SAVI attempts to minimise soil brightness influence using a correction factor (Huete 1988). Since amphibians are ectothermic, the temperature is also a major determinant of their activity (Araújo et al. 2006; Speybroeck et al. 2016). Therefore, we calculated the mean and SD Land Surface Temperature (M_LST and SD_LST; Hulley et al. 2019) and used it as the air temperature surrogate. These indexes (vegetation productivity and land surface temperature) were obtained following the same procedures as the remote sensing water availability predictors (NDMI and NDWI). We combined all the images into composites and calculated the mean (M) and standard deviation (SD) for each index, across the STF. Higher values for the SD denote greater heterogeneity, while lower SD values represent more consistent similar values throughout all years. Overall, we obtained a total of 84 images (Table S2.3 – Supplementary materials), with an average of 5.6 images per year (min= 0 for 2012; max = 11 for 2017; SD = 2.87); the year 2007 contains two images, one for June and another for August, the year 2012 had no quality images available, and therefore was excluded from the analyses. We processed all composite images and calculated all previously mentioned remote sensing indexes in Google Earth Engine (Gorelick et al. 2017).

Roads can exacerbate a barrier effect on amphibians (Fahrig et al. 1995), so we obtained the road proximity predictor (‘distance to highway’) using the same procedure as the ‘distance to ponds’; also using QGIS Software (QGIS Development Team 2022) we extracted elevation from a digital elevation model (DGT 2018) since water typically concentrates in lower topographies, where it can create ponds and water bodies, of extreme importance for amphibians (Santos et al. 2007). We stacked all environmental predictors in a 30 m spatial resolution multi-raster layer (see Table 2.1 for a resume of the predictors) for the entire study time frame using R software (R Core Team 2021).

Table 2.1. List of predictors included in the analyses, with respective code, description, and source.

Predictor class	Name	Code	Description and measure unit	Source	References
Land cover	Irrigation areas	IRRIG	Percentage of irrigated area (%)	CORINE 2006, 2012, 2018	EEA
	Forest areas	FOREST	Percentage of forested area (%)	CORINE 2006, 2012, 2018	EEA
	Olive groves and orchards	OLIVE	Percentage of olive grove and general orchard area (%)	CORINE 2006, 2012, 2018	EEA
	Urban areas	URBAN	Percentage of urban area (%)	CORINE 2006, 2012, 2018	EEA
	Open agricultural areas	AGRIC	Percentage of agricultural area (%)	CORINE 2006, 2012, 2018	EEA
	Vineyards	VINE	Percentage of vineyard area (%)	CORINE 2006, 2012, 2018	EEA
Water availability	Mean Normalized Difference Moisture Index	M_NDMI	Mean water content in vegetation. -1 (indicating no moisture in vegetation) to 1 (indicating high moisture in vegetation)	USGS	Gao, 1995
	Standard deviation of Normalized Difference Moisture Index	SD_NDMI	Inter-annual variation in changes in moisture content of leaves	USGS	Gao, 1995
	Mean Normalized Difference Water Index	M_NDWI	Mean changes in water content of water bodies. -1 (indicating no water) to 1 (indicating water)	USGS	McFeeters, 1996
	Standard deviation of Normalized Difference Water Index	SD_NDWI	Inter-annual variation in changes in water content of water bodies	USGS	McFeeters, 1996
	Distance to ponds	DIST_PNDS	Distance to the nearest water body (meters)	QGIS	N/A
	Mean Enhanced Vegetation Index	M_EVI	Mean changes in primary production content index. -1 (indicating stressed vegetation) to 1 (indicating healthier vegetation)	USGS	Liu and Huete, 1995
Vegetation productivity	Standard Deviation of Enhanced Vegetation Index	SD_EVI	Inter-annual variation in primary production content index	USGS	Liu and Huete, 1995

	Mean Normalized Vegetation Index	M_NDVI	Mean changes in primary production content index. -1 (indicating stressed vegetation) to 1 (indicating healthier vegetation)	USGS	Rouse et al. 1973
	Standard Deviation of Normalized Vegetation Index	SD_NDVI	Inter-annual variation in primary production content index	USGS	Rouse et al. 1973
	Mean Soil-Adjusted Vegetation Index	M_SAVI	Mean changes in primary production content index. -1 (indicating stressed vegetation) to 1 (indicating healthier vegetation)	USGS	Huete, 1988
	Standard Deviation of Soil-Adjusted Vegetation Index	SD_SAVI	Inter-annual variation in primary production content index	USGS	Huete, 1988
Land surface temperature	Mean Land Surface Temperature	M_LST	Mean radiative temperature of land surface (ºC)	Google Earth Engine (source code – Ermida et al., 2020)	Ermida et al., 2020
	Standard Deviation of Land Surface Temperature	SD_LST	Inter-annual variation in radiative temperature of land surface (ºC)	N/A	Ermida et al., 2020
Road proximity	Distance to highway	DIST_HGHW	Distance to highway ‘A6’ (meters)	QGIS	N/A
Topography	Elevation	ELEV	Elevation retrieved from digital elevation model (meters)	Direção Geral do Território (DGT)	DGT, 2008

2.4.4. Data Analysis

We used the number of roadkills per road segment of each species as a response variable. To account for possible bias in our roadkill count data, we included an offset parameter in the models (log scaled). This offset corresponds to the total number of weeks each road segment was sampled (sampling effort) and converts the raw count roadkill data to period standardised rates (Zuur et al. 2007). For each environmental predictor (except for distances) and each species, we selected the best response scale (100 m, 200 m, 500 m, and 1000 m) by applying univariate Poisson Generalised Linear Models (GLM) (Salgueiro et al. 2018) and extracting the predictor scale showing the lowest Akaike's Information Criterion (AIC; Akaike 1974). We then used the selected variables to assemble our models.

Our modelling procedure for each species was based on GLMs with a negative binomial distribution (as our data presented high values of overdispersion, Zuur et al. 2009), and we modelled the count data with all the selected predictors from the four scales, while using 'sampling effort' as an offset parameter. We started our analysis by assessing multicollinearity, using the Variance Inflation Factor (VIF) (Zuur et al. 2010; Dormann et al. 2013), excluding all predictors with a VIF higher than 5 (Dormann et al. 2013; Sillero et al. 2021). The removed predictors included: 'M_NDVI', 'SD_NDVI'; 'M_EVI'; 'M_SAVI' and 'SD_SAVI'. Prior to modelling, we standardised all predictors to zero mean and unit variance, so model coefficients could be comparable (Zuur et al. 2009). We then randomly selected 20% of available zeros for each species dataset as absences, to avoid zero-inflated problems and divided the data into training (70%) and test (30%) data (Field et al. 2012), to evaluate the explanatory power of each model. Following, we performed backwards stepwise selection using AIC to assess the relative likelihood of each model (Akaike 1974) between the stepwise output and the full model (the model constructed with all predictors that we considered after VIF analysis). We selected the models with the lowest AIC, plotted the Pearson residuals to assess normality and evaluated goodness of fit with the percentage of deviance explained by the model. To account for potential autocorrelation in our data, we performed a Moran's I test

(Moran 1950) on each model residuals and calculated a spatial autocovariate as the distance weighted average of neighbouring response values (Dormann et al 2007). While Moran's I measures the global spatial autocorrelation, the autocovariate identifies the local spatial structure of our roadkill data (Anselin, 1995). We then rerun the models with the autocovariate as an additional predictor. We compared the AIC of the previously run models, with the ones accounting for the spatial autocorrelation and selected the models with the lowest AIC values. If ΔAICc < 2, the models were considered equally supported (Burnham and Anderson, 2002), and the one without the autocovariate was retained. Then we performed all the previously mentioned steps for model evaluation. The predictive performance of models was assessed with Pearson correlation between observed and predicted roadkill.

We performed all the analyses using R software packages raster, rgdal, performance, spdep and MASS (R Core Team 2021).

2.5. Results

We recorded a total of 5116 amphibian carcasses, belonging to the five selected species, between 2006 and 2020, most of which belonged to the natterjack toad (47%). The second most roadkilled amphibian species was the fire salamander accounting for 18% of total carcasses (Table 2.2). In total, we registered approximately three amphibian carcasses per road kilometre per year (Table 2.2).

Table 2.2. Amphibian roadkill recorded in the sampled roads during the 14-year study time frame (2006-2020).

Road	***S. Salamandra***	***P. Waltl***	***P. Cultripes***	***E. Calamita***	***B. Spinosus***	**Total**
EN4	150	129	307	678	342	1606
EN18	47	24	17	55	42	185
EN114	262	227	200	1553	270	2512
EN370/EM529	455	118	74	132	34	813
Total	914	498	598	2418	688	5116

2.5.1. Main drivers of amphibian mortality

Overall, models show fair-to-good levels of data fit and predictive performance. The residual plots show randomly scattered residuals around zero, the Pearson correlations averaged 0.37 (0.29-0.58; Table S2.4 – Supplementary materials), and the amount of explained variance averaged 20% (min R2 = 0.112; max R2 = 0.341). Spatial autocorrelation presented minimal values, with Moran's I averaging 0.08 (min = 0; max = 0.16; Table S2.5 – Supplementary materials). Three models improved after inclusion of the autocovariate, although its coefficient was only significant (and positive) for urodeles (fire salamander: coef= 0.149, CI= 0.086, 0.213; Iberian ribbed newt: coef= 0.155, CI= 0.071, 0.239).

Concerning the land cover predictors, the natterjack toad was the only species to show increased mortality in segments with low abundance of irrigation areas ('IRRIG'; coef= -0.484; CI= -0.83, -0.24) and forest ('FOREST'; coef= -0.534; CI= -0.78, -0.29). The mortality of the fire salamander increased in road segments with a lower cover of olive groves and orchards ('OLIVE'; coef = -0.116; CI= -0.23, -0.02) (**Figure 2.2**), while mortality of natterjack toad increased with a higher cover of this land use class ('OLIVE'; coef = 0.168; CI= 0.04, 0.30). Lower proportions of open agricultural areas ('AGRIC') had more roadkills for both the Iberian ribbed newt (coef= -0.197; CI= -0.34, -0.06) (**Figure 2.3**) and the fire salamander (coef= -0.117; CI= -0.23, -0.005), and lower proportions of vineyards ('VINE') also contributed to increased fire salamander roadkill (coef= -0.233; CI= -042., -0.09). In the water availability predictors category, our models show that for all species except the Iberian ribbed newt, roadkill increased in road segments with low mean values of water content in water bodies ('M_NDWI'; Table 2.3). Shorter distances of water bodies to roads ('DIST_PNDS') were associated with higher mortality for the Iberian ribbed newt and for the Iberian spadefoot toad (coef= -0.166; CI= -0.27,-0.06; coef= -0.229; CI= -0.39, -0.07, respectively), but represented less mortality for the natterjack toad (coef= 0.223; CI= 0.05, 0.40) (**Figure 2.4**). Higher variation in moisture content in vegetation was responsible for an increase in the roadkill for the Iberian ribbed newt ('SD_NDMI'; coef= 0.20; CI= 0.06, 0.35). A lower variation in primary production (‘SD_EVI’) caused an increase in the roadkill for the fire salamander (coef= -0.146; CI= -0.24, -0.06) and for the Iberian ribbed newt (coef= -0.157; CI= -0.28, -0.03), and a decrease in the roadkill of the natterjack toad (coef= 0.392; CI= 0.19, 0.61). Road segments with higher mean land surface temperature (‘M_LST’) had higher mortality values for the natterjack toad, the Iberian spadefoot toad, and the Iberian ribbed newt (coef= 0.698; CI= 0.38, 1.04; coef= 0.284; CI= 0.05, 0.52; coef= 0.175; CI= 0.02, 0.33, respectively), and lower mortality values for the spiny common toad (coef= -0.112; CI= -0.22, -0.01) (**Figure 2.5**). A higher variation in the land surface temperature (‘SD_LST’) had the same effect for the Iberian spadefoot toad and the Iberian ribbed newt (coef= 0.347; CI=0.12, 0.58 (**Figure 2.6**); coef= 0.134; CI= 0.01, 0.26, respectively). Roadkills of the Iberian spadefoot toad and the natterjack toad were higher for roads at longer distances to the highway (‘DIST_HGHW’; coef= 0.309; CI= 0.12, 0.50; coef= 0.467; CI= 0.27, 0.67, respectively), while the Iberian ribbed newt had an opposite response (coef= -0.126; CI= -0.24, -0.01). Lastly, lower elevations (‘ELEV’) were associated with higher mortality for the natterjack toad (coef= -0.292; CI= -0.53, -0.06) and the spiny common toad (coef= -0.125, CI= -0.23, -0.02), whereas the fire salamander presented higher road mortality in higher elevations (coef= 0.117, CI= 0.03, 0.21).

In four of the five studied amphibian species, roadkill spatial patterns were driven by water availability and land surface temperature predictors. Land cover predictors, vegetation productivity, road proximity and topography were each significant for three species.

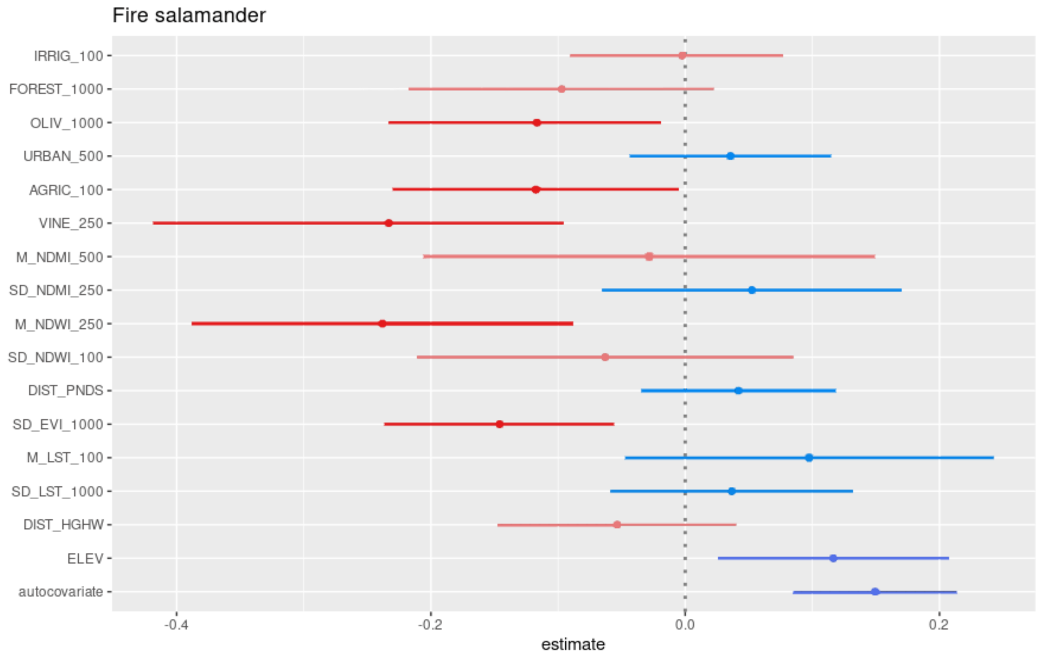


Figure 2.2. Model results for the fire salamander (estimates and 95% confidence intervals). Dark blue lines represent statistically significant positive values; light-blue lines represent non-statistically significant positive values; light-red lines represent non-statistically significant negative values; and red lines represent statistically significant negative values.

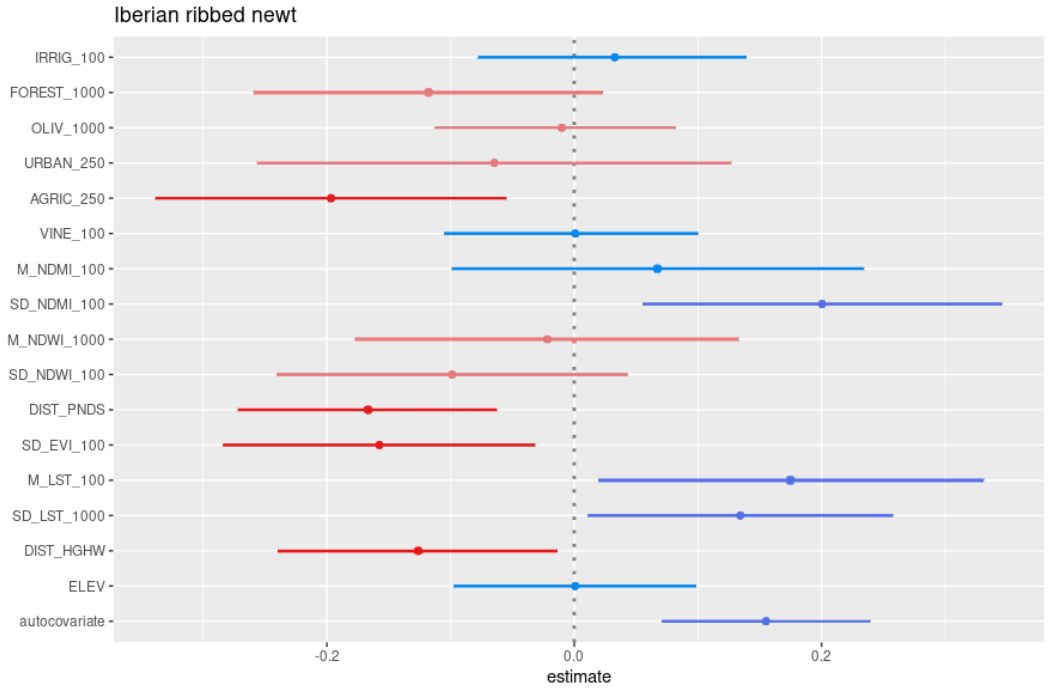


Figure 2.3. Model results for the Iberian ribbed newt (estimates and 95% confidence intervals). Dark blue lines represent statistically significant positive values; light-blue lines represent non-statistically significant positive values; light-red lines represent non-statistically significant negative values; and red lines represent statistically significant negative values.

Table 2.3. Models for the analysed species with coefficients, AIC and R^2. Significant results are in bolt.

	Estimate	Std. Error	t value	p-value	Confidence interval (95%)
Fire salamander (*Salamandra salamandra*)					
IRRIG_100	-0.002	0.042	-0.056	0.955	-0.090; 0.077
FOREST_1000	-0.096	0.060	-1.269	0.205	-0.219; 0.019
OLIVE _1000	-0.116	0.055	-2.128	**0.033**	**-0.233; -0.019**
URBAN_500	0.036	0.039	0.914	0.361	-0.043; 0.114
AGRIC_100	-0.117	0.057	-2.051	**0.041**	**-0.229; -0.005**
VINE_250	-0.233	0.081	-2.878	**0.004**	**-0.418; -0.096**
M_NDMI_500	-0.028	0.089	-0.316	0.752	-0.205; 0.149
SD_NDMI_250	0.052	0.059	0.878	0.381	-0.065; 0.170
M_NDWI_250	-0.238	0.074	-3.201	**0.001**	**-0.387; -0.089**
SD_NDWI_100	-0.063	0.073	-0.860	0.390	-0.211; 0.085
DIST_PNDS	0.042	0.039	1.085	0.278	-0.034; 0.118
SD_EVI_1000	-0.146	0.046	-3.207	**0.001**	**-0.236; -0.056**
M_LST_100	0.097	0.073	1.345	0.179	-0.047; 0.242
SD_LST_1000	0.037	0.047	0.771	0.441	-0.059; 0.132
DIST_HGHW	-0.053	0.047	-1.144	0.253	-0.147; 0.040
ELEV	0.117	0.046	2.550	**0.011**	**0.026; 0.207**
Autocovariate	0.149	0.032	4.672	**<0.001**	**0.086; 0.213**
MODEL AIC	1540				
R^2	0.26871				
Iberian ribbed newt (*Pleurodeles waltl*)					
IRRIG_100	0.033	0.055	0.601	0.548	-0.077; 0.139
FOREST_1000	-0.118	0.071	-1.655	0.099	-0.259; 0.023
OLIVE_1000	-0.010	0.050	-0.200	0.841	-0.112; 0.082
URBAN_250	-0.0646	0.095	-0.683	0.495	-0.256; 0.013
AGRIC_250	-0.197	0.071	-2.751	**0.006**	**-0.339; -0.0557**
VINE_100	0.0007	0.052	0.015	0.988	-0.105; 0.099
M_NDMI_100	0.067	0.083	0.806	0.421	-0.099; 0.234
SD_NDMI_100	0.200	0.074	2.721	**0.007**	**0.056; 0.346**
M_NDWI_1000	-0.022	0.077	-0.280	0.779	-0.177; 0.133
SD_NDWI_100	-0.099	0.072	-1.380	0.169	-0.240; 0.043
DIST_PNDS	-0.166	0.052	-3.150	**0.002**	**-0.272; -0.063**
SD_EVI_100	-0.157	0.063	-2.494	**0.013**	**-0.284; -0.032**
M_LST_100	0.175	0.078	2.252	**0.025**	**0.019; 0.331**
SD_LST_1000	0.134	0.061	2.208	**0.028**	**0.011; 0.258**
DIST HGHW	-0.126	0.053	-2.251	**0.025**	**-0.239; -0.014**
ELEV	0.0007	0.049	0.015	0.989	-0.097; 0.098
Autocovariate	0.155	0.042	3.729	**<0.001**	**0.071; 0.239**
MODEL AIC	880				
R^2	0.15453				
Natterjack toad (*Epidalea calamita*)					
IRRIG_100	-0.483	0.136	-3.565	**<0.001**	**-0.828; -0.236**
FOREST_100	-0.534	0.118	-4.524	**<0.001**	**-0.780; -0.289**
OLIVE _100	0.168	0.065	2.604	**0.009**	**0.040; 0.301**
URBAN_1000	-0.076	0.084	-0.913	0.362	-0.22; 0.095

AGRIC_250	-0.209	0.112	-1.868	0.062	-0.430; 0.011
VINE_1000	-0.179	0.101	-1.768	0.078	-0.422; -0.013
M_NDMI_100	-0.124	0.109	-1.141	0.255	-0.369; 0.121
SD_NDMI_500	-0.265	0.128	-2.076	0.384	-0.586; 0.045
M_NDWI_500	-1.004	0.134	-7.486	**<0.001**	**-1.282; -0.734**
SD_NDWI_500	0.026	0.128	0.200	0.842	-0.255; 0.306
DIST_PNDS	0.2232	0.083	2.677	**0.008**	**0.048; 0.397**
SD_EVI_1000	0.392	0.096	4.076	**<0.001**	**0.182; 0.612**
M_LST_100	0.698	0.137	5.064	**<0.001**	**0.375; 1.035**
SD_LST_500	0.174	0.099	1.763	0.079	-0.049; 0.402
DIST_HGHW	0.467	0.094	4.970	**<0.001**	**0.267; 0.671**
ELEV	-0.292	0.109	-2.667	**0.008**	**-0.525; -0.062**
MODEL AIC	1900				
R^2	0.34087				
Spiny common toad (*Bufo spinosus*)					
IRRIG_100	-0.017	0.058	0.293	0.769	-0.097; 0.129
FOREST_250	-0.075	0.067	-1.121	0.263	-0.209; 0.059
OLIVE _1000	-0.099	0.062	-1.589	0.113	-0.237; -0.017
URBAN_100	0.012	0.046	0.268	0.789	-0.083; 0.107
AGRIC_1000	0.003	0.066	0.044	0.965	-0.129; 0.135
VINE_100	0.049	0.051	0.978	0.329	-0.051; 0.149
M_NDMI_1000	0.029	0.084	0.343	0.732	-0.139; 0.195
SD_NDMI_500	0.068	0.106	0.637	0.524	0.142 0.279
M_NDWI_500	-0.205	0.079	-2.585	**0.010**	**-0.366; -0.051**
SD_NDWI_500	-0.108	0.105	-1.029	0.304	-0.318; 0.101
DIST_PNDS	-0.079	0.052	-1.522	0.129	-0.183; 0.023
SD_EVI_100	-0.031	0.057	-0.543	0.588	-0.144; 0.079
M_LST_1000	-0.112	0.050	-2.230	**0.026**	**-0.216; -0.009**
SD_LST_500	0.015	0.071	0.207	0.836	-0.128; 0.157
DIST_HGHW	-0.036	0.061	-0.600	0.549	-0.160; 0.086
ELEV	-0.125	0.052	-2.389	**0.017**	**-0.229; -0.021**
Autocovariate	0.090	0.046	1.953	0.052	-0.0005; 0.181
MODEL AIC	1258				
R^2	0.11229				
Iberian spadefoot toad (*Pelobates cultripes*)					
IRRIG_100	-0.088	0.095	-0.926	0.358	-0.277; 0.100
FOREST_500	-0.115	0.121	-0.945	0.345	-0.358; 0.127
OLIVE _1000	-0.117	0.092	-1.273	0.203	-0.314; 0.043
URBAN_100	-0.0007	0.079	-0.009	0.993	-0.163; 0.160
AGRIC_1000	-0.041	0.118	-0.350	0.727	-0.282; 0.197
VINE_100	-0.121	0.109	-1.102	0.271	-0.362; 0.074
M_NDMI_1000	0.282	0.167	1.687	0.093	-0.049; 0.615
SD_NDMI_500	-0.052	0.106	-0.489	0.625	-0.262; 0.159
M_NDWI_250	-0.309	0.120	-2.570	**0.011**	**-0.552; -0.069**
SD_NDWI_1000	-0.092	0.111	-0.832	0.406	-0.337; 0.146
DIST_PNDS	-0.229	0.084	-2.704	**0.007**	**-0.393; -0.069**
SD_EVI_100	-.012	0.101	0.121	0.904	-0.197; 0.220
M_LST_100	0.284	0.117	2.405	**0.017**	**0.049; 0.523**
SD_LST_1000	0.347	0.119	2.929	**0.004**	**0.122; 0.577**
DIST_HGHW	0.309	0.094	3.270	**0.001**	**0.122; 0.498**

ELEV	0.035	0.084	0.411	0.681	-0.136; 0.205
MODEL AIC	943				
R^2	0.11011				

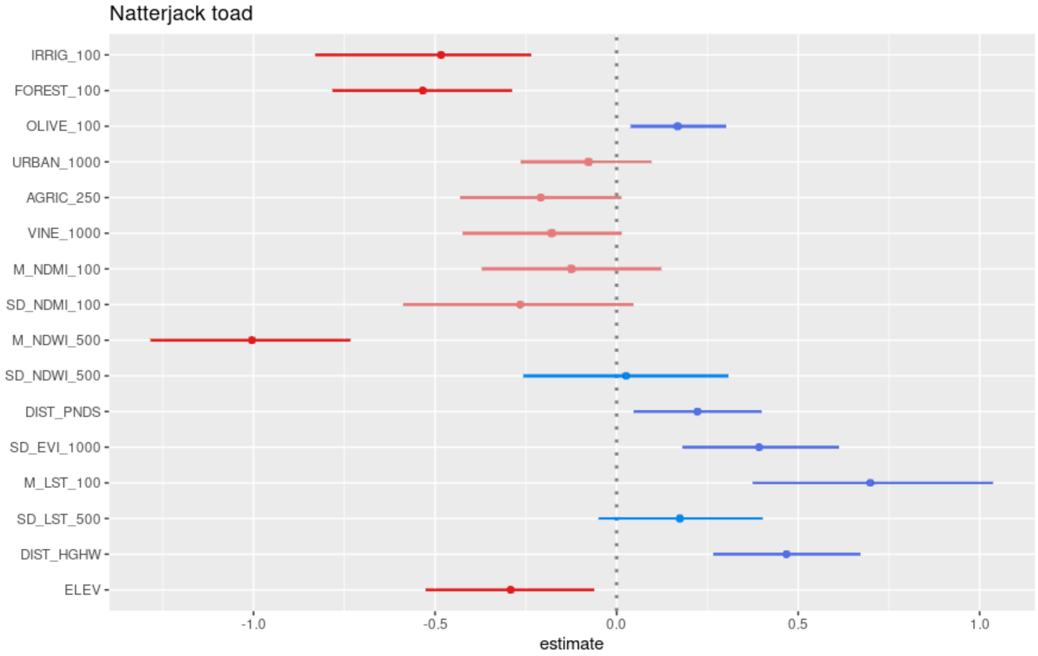


Figure 2.4. Model results for the Natterjack toad (estimates and 95% confidence intervals). Dark blue lines represent statistically significant positive values; light-blue lines represent non-statistically significant positive values; light-red lines represent non-statistically significant negative values; and red lines represent statistically significant negative values.

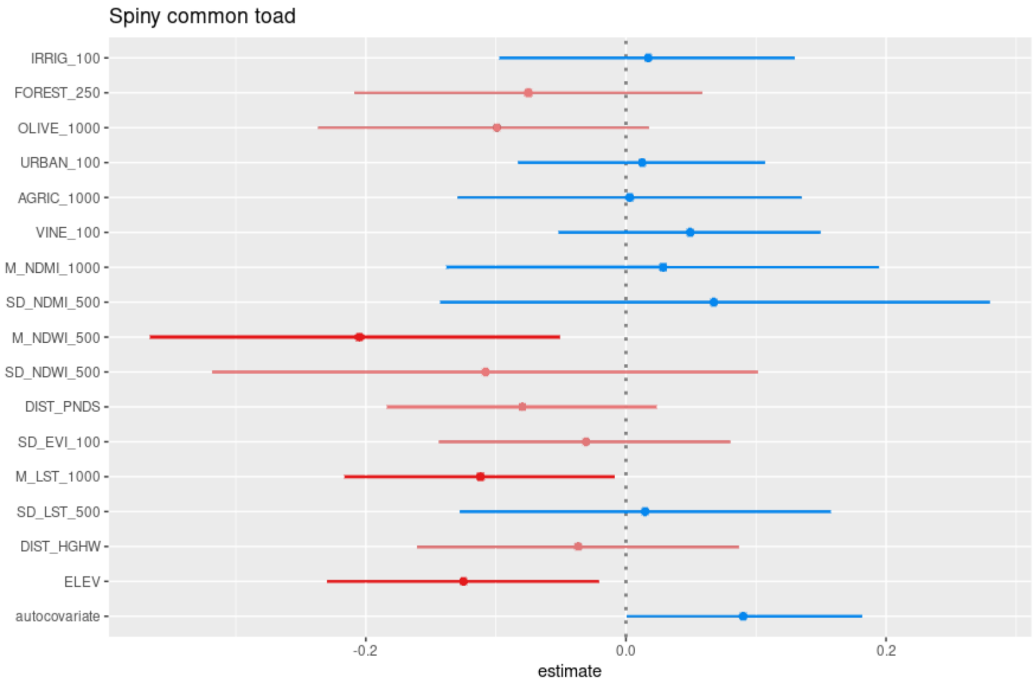


Figure 2.5. Model results for the spiny common toad (estimates and 95% confidence intervals). Dark blue lines represent statistically significant positive values; light-blue lines represent non-statistically significant positive values; light-red lines represent non-statistically significant negative values; and red lines represent statistically significant negative values.

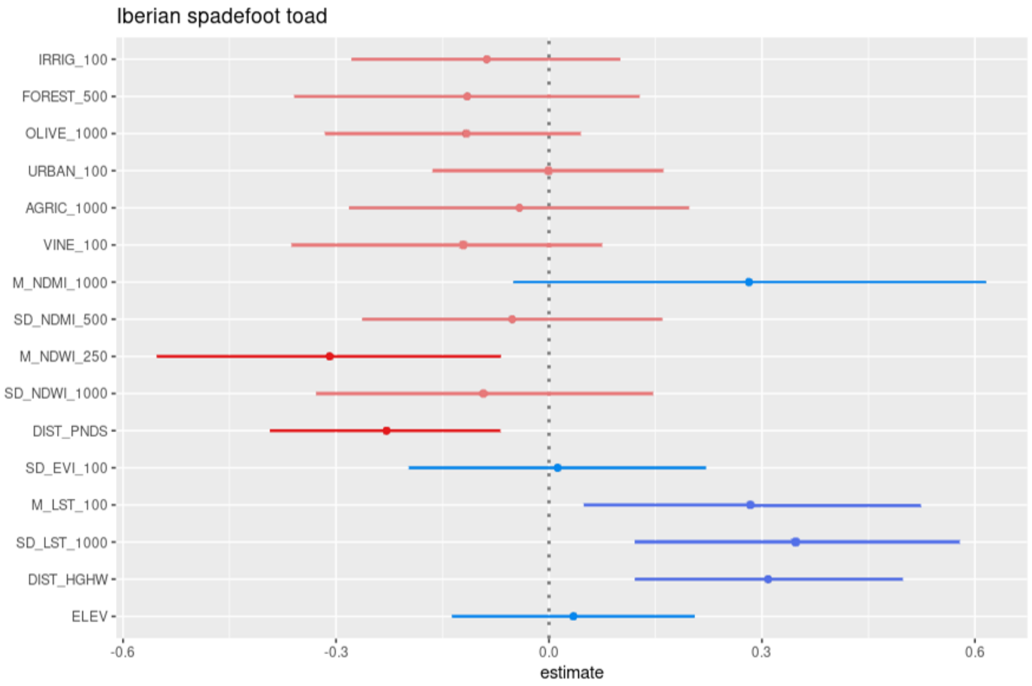


Figure 2.6. Model results for the Iberian spadefoot toad (estimates and 95% confidence intervals). Dark blue lines represent statistically significant positive values; light-blue lines represent non-statistically significant positive values; light-red lines represent non-statistically significant negative values; and red lines represent statistically significant negative values.

2.6. Discussion

Our study analysed whether water-related predictors, together with other important environmental variables for amphibians, would influence their mortality patterns. Our models show contrasting results, supporting the hypothesis that roadkill drivers are species-specific, as previously reported in other studies (e.g. Sillero, 2008; Matos et al. 2012; Mestre et al. 2019). Overall roadkill risk increases when water availability in surrounding water bodies is low; however, our models also show that other predictors also have a significant contribution to amphibian roadkill.

Contrary to other studies (e.g. Sillero 2008; Carvalho & Mira 2011) our models show that few land cover classes were statistically important in explaining amphibians' roadkill, suggesting they may not act singly, but related to other important drivers. We found reduced amphibian mortality associated with agricultural intensification (namely olive groves and orchards, vineyards, and irrigation areas). Beja and Alcazar (2003) registered a decrease in amphibian richness in a protected area in southwest Portugal, mainly due to agricultural intensification. Also, Carpio et al. (2016) addressed the herptile composition in olive groves in Southern Spain and reported a deficit for both amphibians and reptiles in large areas covered by this monoculture. There, fire salamanders were most associated with woodlands and pastures, and less with olive, whereas more generalist species like the natterjack toad, the spiny common toad and the Iberian ribbed newt were more commonly associated with olive groves. Olive groves are generally homogeneous, highly human-intervened (e.g., ploughing) and offer no natural vegetation cover or shelter. The lower roadkill risk in areas with a higher proportion of these land covers may be related to the species' incapacity to reproduce and occur there. In fact, we only detected an increase in road mortality for the natterjack toad in one land cover class associated with agricultural intensification (olive groves). This could be related to the species' generalist characteristics, but also to the water availability in these cultures. Many of the olive groves are drip irrigated, and the presence of puddles could potentially attract this species, since the natterjack toad seeks these shallow temporary waters for reproduction (Gómez-Mestre

2014). Nevertheless, many of these systems are also associated with high levels of agrochemicals, known to be harmful to amphibian survival and growth (Baker et al. 2013). Although there is no abundance data (Loureiro et al. 2010), the natterjack toad is probably the most frequent species in the study area and the one with the highest numbers of roadkill. Orlowski (2007) and D'Amico (2009) found that high levels of amphibian roadkill are related to higher local abundances. Thus, the higher natterjack toad mortality in land use classes where other species are not so frequent could also reveal its higher abundances. Nevertheless, the natterjack toad presents high plasticity and adaptability (Gómez-Mestre 2014), and these results should be interpreted with caution, since other predictors (e.g. dispersal capacity; terrestrial site fidelity; Sanuy et al. 2000; Miaud & Sunay 2005) may exacerbate an effect on the roadkill risk, far more than the land cover. Also, the Iberian ribbed newt and the fire salamander had lower roadkill in segments with a higher cover of open agricultural areas. For the Iberian ribbed newt, this is probably due to its highly aquatic behaviour, which apparently reduces its abundance in agricultural areas with high levels of grazing, as in our study area. Nonetheless, the terrestrial habits of the Iberian ribbed newt are almost unknown (Salvador 2014), which limits our conclusions about this species. The lower abundances of fire salamander roadkill in agricultural areas are probably related to its more specialist habitat preferences, since this species tends to occur in more forested areas (Velo-Antón & Buckley, 2015; Speybroeck, 2016).

Our models highlight that water content in water bodies mediates amphibian roadkill for most of the analysed species (four of them). Although we expected higher quantities of water to attract more amphibians, increasing roadkill risk, our models show the opposite result. All studied species rely on water at least for reproduction and larval development and some of them present high water body fidelity, and aestivation/ hibernation areas often occur in the surroundings of the water bodies (Joly 2019). Years of lower water availability would require individuals to travel greater distances to find suitable sites for reproduction, posing a higher road mortality risk; or even to cease reproduction, which in turn could drastically reduce populations, due to lower recruitment from juveniles

(Cushman, 2006). Segev et al. (2010) found that stable ponds support larger populations of fire salamander (*S. infraimmaculata*) in Israel. Ribeiro et al. (2011) studied the importance of pond networks for amphibian populations in a Mediterranean region and found that the presence of certain species is highly correlated with the structural connectivity of the pond network. These studies highlight the importance of water (ponds) for the stability of amphibian populations in human-intervened landscapes. Future projections predict a strong reduction in water availability for the Mediterranean region (Tuel & Eltahir, 2020). The depletion of suitable water bodies as a consequence of water scarcity, together with the triggered movement in search for potential reproduction areas will increase roadkill. Previous findings describe a positive relationship between water availability and amphibian roadkill (Glista et al. 2008; Sillero et al. 2019). Those studies report to areas with higher rainfall and humidity than ours, where water availability in water bodies should not be a limiting factor for amphibian reproduction, thus reducing their need to move longer distances to complete their life cycle.

The proximity of roads to water bodies seems to exert different responses on our species. The natterjack toad was the only species showing a higher risk of mortality in road segments further away from water bodies. This is expected, as in the study area, this species reproduces mostly in ephemeral ponds with an area lower than 10 m^2 (Speybroeck et al. 2016), too small and too seasonal to be mapped in CORINE land cover products. Thus, these small ponds were not accounted for in the water availability predictor class. Another explanation for this result could be the ability of this species to disperse longer than the others (Sinsch et al. 2012), enabling it to search for water at a larger distance from roads (less disturbed areas). For two other species (Iberian spadefoot toad and Iberian ribbed newt) the roadkill is higher when the distance from water bodies to roads is shorter. This response could be explained by the regular distances covered by these species, reflecting the higher abundance and movements of amphibians near water bodies, since they reproduce on these sites and tend to shelter in surrounding areas, particularly for species with poor capacities for dispersal, such as the Iberian spadefoot toad and the Iberian ribbed newt (Recuero, 2014; Salvador, 2014).

Our models show that for urodeles (the fire salamander and the Iberian ribbed newt), the lower the inter-annual variation in humidity content in vegetation (SD_EVI), the higher the roadkill. Road segments with lower SD_EVI reveal more stable and predictable local conditions across the years, thus being more attractive for amphibians to occur there, increasing their abundance in those places. Moreover, in our study area, these stable sites correspond to less human-intervened dense forests, and these road segments are more prone to amphibian movement, justifying the increase in road mortality for these two species. For the natterjack toad, our models predicted a higher mortality with greater variation of humidity content in vegetation; a response that can be associated with the more generalist behaviour of this species regarding habitat sensitivity and occurrence (Gómez-Mestre 2014). The Iberian ribbed newt roadkill was also associated with road segments that have higher inter-annual variation in moisture content in vegetation (SD_NDMI). This response was not expected since higher moisture levels typically promote amphibian movement (Mestre et al. 2019). As little is known from Iberian ribbed newt movements in their terrestrial phase, it is possible that this species tolerates a wider range of environmental shifts than other amphibian species.

Amphibians are ectothermic and their activity is extremely dependent on the temperature (Speybroeck et al. 2016); our models confirm a decrease in roadkill for the spiny common toad, and an increase in roadkill for two anuran species (the natterjack toad and the Iberian spadefoot toad) and one urodele (the Iberian ribbed newt) in road segments embedded in areas with high land surface temperature. Muro et al. (2018) found that land surface temperature is an indicator of changes in wetlands in Tanzania, with overall temperature increasing with farmland expansion; the same trend has also been observed in changes in hydrological regimes. Although we did not test it, this association could eventually explain the increase in roadkill with higher temperatures (except for the spiny common toad): in the context of future predictions of increased temperatures, the hydrological periods could be shortened, increasing amphibian movements in search for suitable conditions, which would represent potential roadkill risk for these species. Likewise, the inter-annual variation in the temperature produced similar results, with peaks in mortality occurring in road segments with higher variation in temperature for the Iberian ribbed newt and the Iberian spadefoot toad. Higher values of this predictor represent road segments with a greater local variance in temperature, which in turn could force amphibian displacements, increasing the likelihood of roadkill. This positive link between climatic factors such as temperature and roadkill has already been suggested (Puky 2005; Glista et al. 2008).

The proximity of the highway that crosses the study area appears to produce different effects on the roadkill of the studied species. For the natterjack and the Iberian spadefoot toads, roadkill is higher on road segments further away from the highway. This may reveal an avoidance effect due to the disturbance caused by the highway's higher traffic and velocity, or previous roadkill that may have extirpated local populations around this infrastructure (Jackson & Fahrig 2011). Highways are much wider than national roads, and typically concentrate more traffic (the known major determinant of the barrier effect); moreover, amphibians commonly show a reduced tendency for crossing wide roads with these characteristics (Fahrig et al. 1995). Orlowski (2007) also observed higher amphibian mortality rates on roads with low or moderate traffic density, than on roads with higher traffic. Additionally, anurans rely on auditory cues to communicate, which may lead them to avoid noisy locations and concentrate in areas away from them (Nelson et al. 2017). We found the opposite result for the Iberian ribbed newt, with higher mortality close to the highway. Urodeles are poorer dispersers than anurans, known to remain in the same small area for longer periods of time (Segev et al. 2010; Bani et al. 2015; Sinai et al. 2020); also, the noise produced by the highway traffic does not interfere in their communication since they communicate via chemical cues (Speybroeck et al. 2016). This may suggest that other negative effects such as pollutant emission by vehicles, chemical transportation from road runoff, or even herbicides used in road verge maintenance could contribute to the higher mortality of the Iberian ribbed newt close to highways (Forman and Alexander 1998; Colino-

Rabanal & Lizana 2012). However, as stated before, terrestrial movements of the Iberian ribbed newt are poorly studied, which only allows us to speculate on this matter.

For the spiny common toad and the natterjack toad, our results forecast higher mortality in lower altitudes, where the land surface is flatter propitiating water retention. This is in accordance with other studies (Santos et al. 2007; Matos et al. 2012), where the authors suggest that amphibians move towards lower altitudes where breeding sites are more common. However, we detected the opposite relation with the fire salamander, with higher roadkill density at upper elevations. The fire salamander is considered a forest species, with a preference for wet and shaded environments (Velo-Antón and Buckley 2015). Despite the low altitudinal range in our study area, forest patches with high tree density are most concentrated in higher and steeper areas also characterised by high humidity and less human intervention. Nonetheless, our results should be interpreted with caution especially because the altitudinal range in the study area is low (about 300m). Higher amphibian roadkill densities in high altitudinal areas have, however, been observed in other areas of the Iberian Peninsula (e.g., Espinosa et al. 2012; Garriga et al. 2017).

2.7. Conclusions

Our study confirmed that water-related predictors are important drivers of amphibian roadkill. In the context of predicted reduced water availability in the future, amphibians are likely to be severely affected, especially in semi-arid regions such as the Alentejo. Rain irregularity, with possible lower precipitation, may aggravate the scenario of drought, leading to less water availability, necessary for amphibian reproduction and development; this will force the animals to move longer in search of alternative water bodies suitable for reproduction, increasing their roadkill risk. Future studies should combine roadkill data with population data to better access populations' status and evaluate the effect of mortality induced by roads on population trends.

2.8. Acknowledgements

We are truly thankful to all the people who kindly worked hard through all these years to collect road mortality data, namely: Paulo Alves, Denis Medinas, Pedro Costa, Bruno Silva, Sílvia Barreiro, Luís Sousa, Eduardo Ferreira, João Craveiro, André Oliveira, and others. TP is financed by Fundação para a Ciência e Tecnologia (FCT) with a doctoral grant 2020.04581.BD. NS is supported by a CEEC2017 contract (CEECIND/02213/2017) from FCT.

Funding: This work was supported by the European Commission, under the project LIFE LINES – LIFE14-NAT-PT001081. TP is financed by Fundação para a Ciência e Tecnologia (FCT) with a doctoral grant 2020.04581.BD. NS is supported by a CEEC2017 contract (CEECIND/02213/2017) from FCT.

Appendix S2 – Supplementary materials

Table S2.1. Sampling scheme to collect roadkill samples. Description of the total length of each road and the sampling frequency for the different years (2006 to 2020). 'Mixed' refers to daily sampling for some months and weekly sampling for others.

Road	Length (km)	Sampling frequency
EN114	23	2006-2008 (weekly); 2009-2010 (mixed); 2011-2012 (daily); 2013 (mixed); 2014 (weekly); 2015-2019 (daily); 2020 (mixed)
EN4	57	2006-2008 (weekly); 2009-2010 (mixed); 2011-2012 (daily); 2013-2014 (weekly); 2015-2019 (daily); 2020 (mixed)
EN370	6	2006-2008 (weekly); 2009-2010 (mixed); 2011-2012 (daily); 2013 (mixed); 2019 (daily); 2020 (mixed)
EN18	23	2013 (mixed); 2014 (weekly); 2015-2019 (daily)
EM529	9	2006-2008 (weekly); 2009-2010 (mixed); 2011-2012 (daily); 2013 (mixed); 2019 (daily); 2020 (mixed)

S2.1. Studied species

All species included in this study are frequently found in the study area and are subject to high roadkill frequency (Santos et al., 2011; Mestre et al., 2019). The fire salamander is a nocturnal urodele with terrestrial habits, mostly found in high-humidity places, near water bodies where larvae can be deposited. This species has preferences for loose soils with high tree and scrub cover and heterogeneous substrates (Manenti, et al., 2013; Velo-Antón & Buckley, 2015). Active during the first and last hours of the night, however, can be found during the day on rainy days. The Iberian ribbed newt is a highly aquatic urodele. In semi-arid places and with higher temperatures, it can remain in water all-year round, with a preference for larger and deeper water bodies. Little is known about its habits during the terrestrial phase. The species' main threats include the loss of aquatic habitats by drainage, habitat destruction and urban and industrial contamination of waters (Salvador, 2014).

The natterjack toad, the spiny common toad and the Iberian spadefoot toad belong to the anurans and present higher terrestrial phases (Speybroeck et al., 2016). The first is a high generalist species, able to occupy a multitude of habitats, from mountainous to urban areas. However, it shows preferences for open areas with sandy soils, and the adults show greater tolerance to higher temperatures. This species has a reduced length of its larval period, which allows it to reproduce in shallow waters with little or no plant cover. Contrary to urodeles, the natterjack toad presents a wider range of movements, enabling a faster colonization of new habitats (Boomsma and Arntzen, 1985). The biggest threat to this species is the roadkill frequency, especially during the migrations to reproduction areas (Gómez-Mestre, 2014). The spiny common toad is the biggest amphibian in the Iberian Peninsula; with generalist habits, it occurs in all peninsular habitats (Ortiz-Santaliestra, 2014). It is a very terrestrial species, highly tolerant to dehydration, occurring in aquatic environments only during the reproductive periods. It shows a preference for deep, stable water bodies with aquatic vegetation. Predominantly nocturnal, with higher activity during the first night hours, this species is known to travel several kilometres annually from the areas of usual activity to the ponds for reproduction (since this species presents strong site fidelity). Some of the main threats are habitat loss and fragmentation. Roadkill is also a serious threat; in certain areas, the road mortality of this toad is so high that populations from both sides of the road became isolated (Carretero and Rosell, 2000). The third anuran species, the Iberian spadefoot toad is usually found in areas with sandy substrates or less compacted soil at lower altitude areas. It is a nocturnal species with terrestrial habits, moving to water bodies only in reproduction periods. The larval stage is generally higher when compared with other species present in this study. Some of the main threats include changes in land cover, the use of pesticides and the introduction of exotic species. Also, roadkill presents a serious threat to this species (Recuero, 2014).

Table S2.2. Aggregated CORINE 2006, 2012 and 2018 land cover classes into analysed categories

Aggregated classes	Code	% in the study area	CORINE classes
Irrigation areas	IRRIG	3	Rice fields; permanently irrigated land;
Forest areas	FOREST	41	Broad-leaved forest; pine forest; Mediterranean cork and holm woodlands; open woodland; mixed forest
Olive groves and orchards	OLIVE	4.5	Olive groves; orchards
Urban areas	URBAN	3	Urban
Open agricultural areas	AGRIC	46	Non-irrigated arable land; pastures; complex cultivation patterns; annual crops associated with permanent crops
Vineyards	VINE	2.5	Vines

S2.2. LANDSAT program brief description

The LANDSAT program is the longest-running Earth observation monitoring program for acquisition of Earth satellite imagery. The first LANDSAT satellite (Landsat 1) was launched in 1972 (operational until 1978) and the most recent (Landsat 9) has been orbiting Earth since 2021. The current active satellites are Landsat 8 and 9, and these are equipped with optical and thermal bands with spatial resolutions ranging from 15 to 100 m. The revisiting time for the LANDSAT satellites is 16 days and the image acquisition has been used for a multitude of applications, from ecology to geopolitical matters (US Geological Survey (USGS) – http://usgs.gov).

Table S2.3. Number of Landsat satellite images per sampling year. Only 0% cloud cover images were used for the analyses. *2012 was removed from the analysis due to lack of suitable images.

Year	Number of images	Landsat
2006	3	5
2007	2	5
2008	4	5
2009	7	5
2010	6	5
2011	5	5
2012*	0	-
2013	9	8
2014	4	8
2015	9	8
2016	7	8
2017	11	8
2018	5	8
2019	7	8
2020	5	8

Table S2.4. Pearson correlation values (and respective 95% confidence intervals) between observed and predicted roadkill for the five studied species.

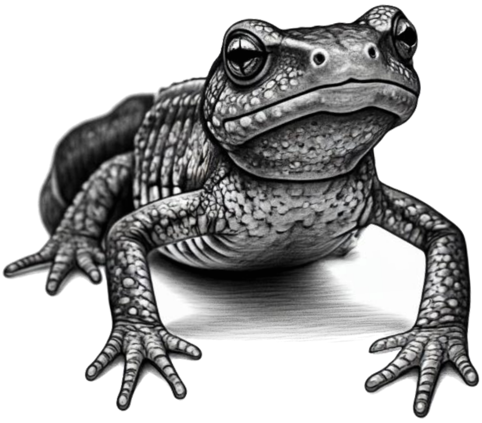
Model species	Pearson correlation [-+ 95% ci]
Fire salamander (*Salamandra salamandra*)	0.3469 [0.27 -+ 0.42]
Iberian ribbed newt (*Pleurodeles waltl*)	0.2919 [0.19 -+ 0.39]
Natterjack toad (*Epidalea calamita*)	0.5838 [0.52 -+ 0.64]
Spiny common toad (*Bufo spinosus*)	0.2884 [0.20 -+ 0.37]
Iberian spadefoot toad (*Pelobates cultripes*)	0.3503 [0.25 -+ 0.44]

Table S2.5. Global Moran's I test values and respective p-values applied to models' residuals for the five studied species.

Model species	Moran's I (p-value)
Fire salamander (*Salamandra salamandra*)	0.0992 (<0.001)
Iberian ribbed newt (*Pleurodeles waltl*)	0.0561 (0.004)
Natterjack toad (*Epidalea calamita*)	0.1619 (<0.001)
Spiny common toad (*Bufo spinosus*)	0.0001 (0.452)
Iberian spadefoot toad (*Pelobates cultripes*)	0.058 (0.005)

Chapter 3

Using the dead to infer about the living: amphibian roadkill spatiotemporal dynamics suggest local populations' reduction



Triturus pygmaeus

Published in *Science of the Total Environment* as:

Pinto, T., Sillero, N., Mira, A., & Santos, S. M. (2024). Using the dead to infer about the living: Amphibian roadkill spatiotemporal dynamics suggest local populations' reduction. Science of the Total Environment, 927, 172356. https://doi.org/10.1016/j.scitotenv.2024.172356

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3.1. Abstract

Roads represent one of the main sources of wildlife mortality, population decline, and isolation, especially for low-vagility animal groups. It is still not clearly understood how wildlife populations respond to these negative effects over space and time. Most studies on wildlife road mortality do not consider the spatial and temporal components simultaneously, or the imperfect roadkill detection, both of which could lead to inaccurate assumptions and unreliable mitigation actions. In this study, we applied a multi-season occupancy model to a 14-year amphibian mortality dataset collected along 120 km of roads, combined with freely available landscape and remote sensing metrics, to identify the spatiotemporal patterns of amphibian roadkill in a Mediterranean landscape in Southern Portugal. Our models showed an explicit general decrease in amphibian roadkill. The Iberian painted frog (*Discoglossus galganoi*) experienced roadkill declines over time of ~70%, while the spiny common toad (*Bufo spinosus*) and the fire salamander (*Salamandra salamandra*) had a loss of nearly 50%, and the Southern marbled newt (*Triturus pygmaeus*) had 40%. Despite the decreasing trend in roadkill, spatial patterns seem to be rather stable from year to year.

Multi-season occupancy models, when combined with relevant landscape and remote sensing predictors, as well as long-term monitoring data, can describe dynamic changes in roadkill over space and time. These patterns are valuable tools for understanding roadkill patterns and drivers in Mediterranean landscapes, enabling the differentiation of road sections with varying roadkill over time. Ultimately, this information may contribute to the development of effective conservation measures.

3.2. Keywords: road mortality; multi-season occupancy modelling; remote sensing; landscape; conservation

3.3. Introduction

Linear infrastructures have shaped the landscape for generations, following human development and economic growth (Forman and Alexander, 1998; Coffin, 2007; Meijer et al., 2018). Roads are one of the most widespread man-made infrastructures on Earth, responsible for fragmenting continuous habitats into smaller and less connected patches and creating barriers to animal movements (Shepard et al., 2008; Ward et al., 2015; Ibisch et al., 2016). Human pressures have intensified the expansion of road networks in natural areas, putting wildlife at a higher risk (Blondel et al., 2010; Garriga et al., 2012). Within this, roadkill (or wildlife-vehicle collisions) is the most visible direct impact of roads and contributes to the decline of species of high conservation importance (Loss et al., 2015; Bencin et al., 2019). These collisions are not randomly distributed across the landscape (Malo et al., 2004; Santos et al., 2017): generally, roadkill locations are located close to high-quality habitats, migration routes between feeding and reproduction sites, or ‘dangerous’ road sections (Carvalho & Mira, 2011; D’Amico et al., 2015). High roadkill aggregation sites (also called roadkill hotspots) are not static over space and time (Medinas et al., 2021) and tend to change their location and extent according to landscape features (Forman et al., 2003; Medinas et al., 2013), traffic density (Fahrig et al., 1995; Zimmermann Teixeira et al., 2017), road type (Clevenger et al., 2003; Medinas et al., 2013) or even weather conditions (Carvalho et al., 2017). The negative roadkill effects can endure over time, affecting the decrease of genetic structuring associated with isolation or reduced population size, and local extinctions for some species (reviewed in: Cushman, 2006; Ceia-Hasse et al., 2017). Thus, it is paramount to understand how roads impact wildlife mortality over time.

Amphibians are the most susceptible vertebrate group to die on roads due to their unique ecological, physiological, and behavioural traits (i.e., lower vagility, narrow habitat tolerances, water dependency and the necessity of seasonal migrations to complete intricate life cycles; Carr and Fahrig, 2001; Mazerolle et al., 2005; Sillero, 2008; Bouchard et al., 2009; Joly, 2009; Pinto et al., 2023). Beebee (2013) analysed amphibian roadkill at a global scale and reported that 1% of amphibian records constituted road casualties, representing 90% of the total vertebrate carcasses. Garriga et al., (2017) have also presented similar results for Iberian Peninsula at a local scale. Numerous studies report high mortality close to water bodies (Ascensão and Mira, 2005; Orlowski, 2007; Santos et al., 2007), near high-quality habitats (Sillero, 2008; Matos et al., 2012) and in specific times of the year related to the higher amphibian activity (Puky, 2005; Glista et al., 2008; Orlowski et al., 2008). According to the IUCN Red List, 20 of the 21 autochthonous amphibian species occurring in Portugal present declining population trends (IUCN, 2022). The persistence of amphibian populations in severely fragmented landscapes is intrinsically linked to population size, landscape types, reproduction ability, location of breeding sites, species' dispersal capacities and road density (Carr and Fahrig, 2001). This prompts the need to understand roadkill tendencies and variations in the long term, for the implementation of more effective mitigation measures (Puky, 2005; Schmidt & Zumbach, 2008; Glista et al., 2009). In addition, studies on the roadkill effects on amphibian populations are still scarce, mainly due to the difficulties in collecting the necessary data to address these issues: a very high manpower and sampling effort are necessary to collect a small amount of populational data (Kays et al., 2011; e.g., telemetry and capture-recapture studies). Roadkill data though can be extremely valuable in allowing researchers to make inferences about the state of populations (Schwartz et al., 2020). Further, if collected over long periods of time, variations between years can provide more robust inferences on the possible impact of roads on population persistence. Temporal studies can be the key to unveiling the influence of landscape factors on roadkill spatiotemporal patterns. Moreover, by collecting data over longer periods of time, researchers increase the probability of gathering information on elusive species. The combination of long-term roadkill data with free high-resolution remote sensing data may assist in capturing environmental changes at local scales (Valerio et al., 2020). This is particularly relevant in the Mediterranean Basin, a region marked by strong seasonal variations with long and dry summers and wet winters (Goubanova & Li, 2007; Pereira et al., 2021), and for amphibians that respond not only to fine scales but are also highly vulnerable to external factors (Puky, 2005; Speybroeck et al., 2016).

In this study, we assess the spatiotemporal patterns of amphibian roadkill and their drivers in a Mediterranean landscape, during a 14-year survey period. Specifically, we implement a multi-season occupancy modelling strategy together with landscape, weather, and remote sensing covariates to explore the long-term trends and patterns of roadkill across amphibian species with different habitat requirements, life-history traits, and roadkill frequency. We expect these patterns to fluctuate over space and time, mainly due to changes in climatic factors and landscape features (Glista et al., 2008; Medinas et al., 2021).

3.4. Material and methods

3.4.1. Study area

The study was performed in the Alentejo region in Southern Portugal, along five selected road sections. The climate is Mediterranean, with mean temperatures ranging from 5.8 ºC to 12.8 ºC in January and from 16.5 ºC to 30.2 ºC in August, and annual rainfall averaging 609.4mm (Évora 1971-2000; IPMA, 2021). The landscape is dominated by Mediterranean forests of cork (*Quercus suber*) and holm (*Quercus rotundifolia*) oaks with variable tree density, mixed with pastures for cattle grazing, composing the complex agro-silvo-pastoral system known as *montado* (Pinto-Correia et al., 2011). The landscape also includes agricultural areas (arable land, olive groves, orchards, and vineyards) in equal proportions to the forest. The topography is mainly flat with gentle slopes, ranging from 100m to 400m a.s.l. This study area has a well-established road network, including the main transportation corridor between Lisbon and Madrid.

3.4.2. Roadkill surveys

The road surveys were carried out for 14 years (from the 1st of January 2006 to the 31st of December 2011 and from the 1st of January 2013 to the 31st of December 2020), along four National Road sections (EN4, EN114, EN370 and

EN18) and one Municipal Road section (EM529), comprising a total of approximately 120 km of sampled roads. All roads are two-lane wide and two (EN4 and EN114) have paved shoulders. The highway A6, although not surveyed, runs parallel to roads EN114 and EN18 (**Figure 3.1**).

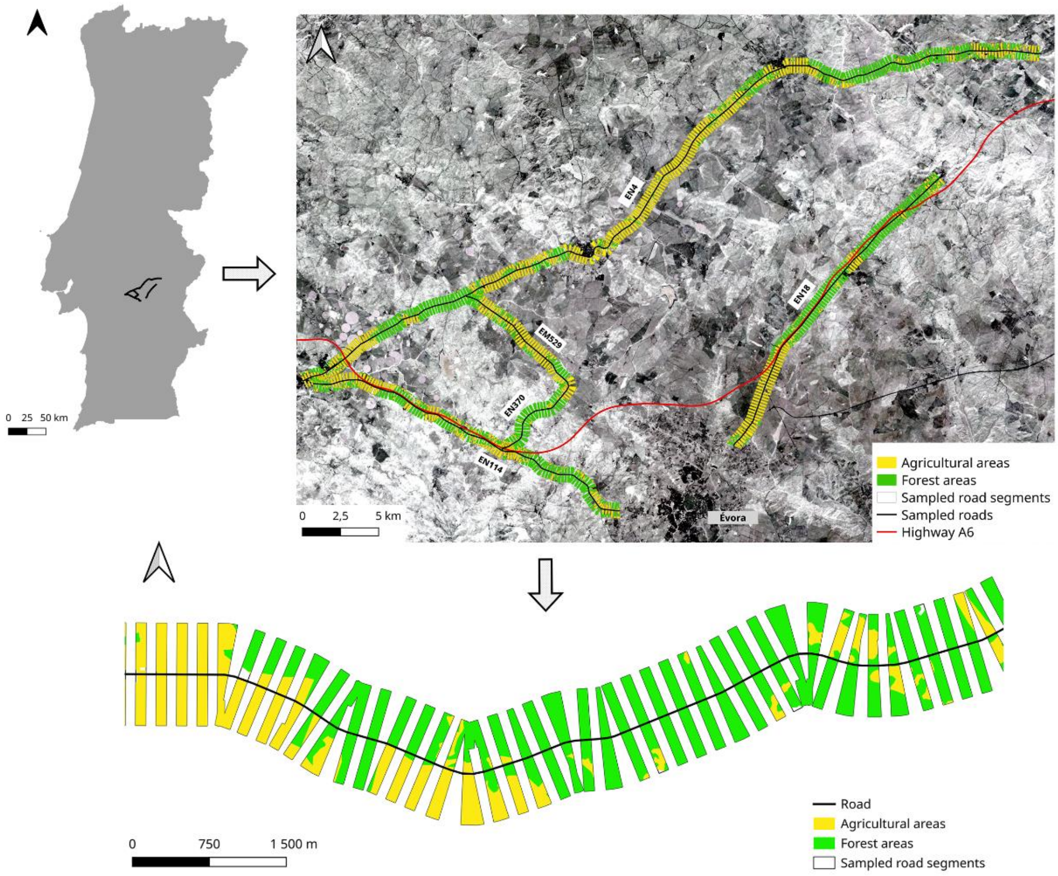

Figure 3.1. Location of the study area in Southern Portugal, with sampled roads and major land cover classes. A detailed zoom highlights the 500m buffer for each 100m sampled road segment.

Surveys followed a standardized protocol (see Santos et al., 2011) and were performed either daily or weekly, by an experienced observer driving a car at 20-40 km/h during the first morning hours to reduce the impact of scavengers in carcass removal. Both sides of the road (including lanes and shoulders) were sampled, and all road-killed animals were collected and registered. All detected amphibian carcasses were identified to the lowest possible taxonomic level, its geographical position was recorded with a handheld GPS device and after this, carcases were removed from the road to avoid double counting during later surveys. Due to budget constraints over the study period, there were three periods where sampling did not occur (April 2013, January to March 2015, and May to September 2017, totalising nine months without surveys) and surveyed length varied over the years (Table S3.1 Supplementary material).

The surveys produced a roadkill dataset of 14062 amphibian carcasses, belonging to 12 species. For the present work, we selected four anuran species: the spiny common toad (*Bufo spinosus*), the natterjack toad (*Epidalea calamita*), the Iberian spadefoot toad (*Pelobates cultripes*), and the Iberian painted frog (*Discoglossus galganoi*); and three urodele species: the fire salamander (*Salamandra salamandra*), the Iberian ribbed newt (*Pleurodeles waltl*), and the Southern marbled newt (*Triturus pygmaeus*). This selection covers species with diverse ecological specialisations (i.e., habitat generalist versus specialist, and open versus forest habitat) and with high roadkill rates (Table 3.1). The IUCN Red List categories for the Iberian spadefoot toad and the fire salamander are Vulnerable, the Southern marbled newt is Near Threatened, while the spiny common toad, the natterjack toad and the Iberian ribbed newt are Least Concern (IUCN, 2022).

Table 3.1. List of analysed species with habitat preference, habitat requirement and the total number and percentage of roadkill in the study area. Mixed habitat represents both open and forested habitat types.

Name	Habitat preference	Habitat requirement	N roadkill	% roadkill
Natterjack toad (E*pidalea calamita*)	Mixed	Generalist	6499	46.6
Fire salamander (*Salamandra salamandra*)	Forest	Specialist	2011	14.4
Spiny common toad (*Bufo spinosus*)	Forest	Generalist	1878	13.5
Iberian ribbed newt (*Pleurodeles waltl*)	Pond	Specialist	1769	12.7
Iberian spadefoot toad (*Pelobates cultripes*)	Open	Generalist	1535	10.9
Iberian painted frog (*Discoglossus galganoi*)	Open	Specialist	182	1.3
Southern marbled newt (*Triturus pygmaeus*)	Mixed	Specialist	87	0.6

3.4.3. Environmental covariates and model definition

Since our selected species cover different movement distances and amphibians typically respond to micro-habitats (Langen et al., 2009), we split each road section into 100 m length contiguous segments (totalising 1182 road segments), to evaluate the spatiotemporal patterns of roadkill occurrences. To reduce spatial autocorrelation among our roadkill data, we used alternate and non-consecutive road segments (thus comprising 591 segments). We used a 500 m buffer around each road segment to extract land use and remote sensing covariates (see below) based on the species ecology (Pinto et al., 2023) and previous studies in nearby areas (Carvalho & Mira, 2011). We performed all the road segmentation and buffer creation in QGIS software (version 3.24.1; QGIS Development Team, 2022).

We modelled the spatiotemporal variation in roadkill with a multi-season occupancy model, a statistical method that estimates the likelihood of a species occurring at a specific site, while accounting for imperfect detection (MacKenzie et al., 2006). Imperfect detection (the inability to sight an individual or species when present) is tightly connected to ecological surveys and particularly to roadkill surveys, especially if a species is cryptic or of small size (Barrientos et al, 2018); when ignored, it can substantially underestimate roadkill numbers (Santos et al., 2016), ultimately leading to the imprecise implementation of mitigation measures (Santos et al., 2018). This model technique requires a repeated sampling design with a detection history during a closed period of time: thus, we considered each model season to be an entire year, where the 591 road segments (sites) were sampled 12 months each year.

This occupancy model contains four main parameters: first-year occupancy (Ψ), extinction (ε), colonisation (γ), and detection probability (ρ) (MacKenzie et al., 2003).

First-year occupancy (Ψ) represents the probability of a road segment having a roadkill in the first year, hereafter treated as the initial roadkill probability. According to Kéry and Chandler (2016), occupancy is modelled as a function of fixed site covariates (covariates that do not vary temporarily during the whole sampling period). We calculated five site covariates regarding land cover and distance to key landscape features. Concerning land cover site covariates, we used CORINE Land Cover (CLC) 2006, 2012 and 2018 to describe the land cover during 2006-2020. We merged some classes to reflect the two dominant land uses in the study area (Forest and Agriculture) and extracted the mean percentage of each land cover class from the three CORINE years for each road buffer. We have also included ‘distance to highway’ and ‘distance to ponds’ (calculated by applying Euclidean distance to a 30 m spatial resolution raster - obtained from pre-existing detailed cartography done in the framework of the LIFE LINES project – LIFE14 NAT/PT/001081) as site covariates because they represent two key features in the study area that we believe have an influence on the presence of amphibian roadkill (Ascensão and Mira, 2005; Pinto et al., 2023). Lastly, we included the percentage of tree density within each road segment, obtained by LANDSAT imagery (with 30 m pixel spatial resolution) to represent the structure of the forest (higher percentages may represent a well-established forest with a substantial canopy and a high number of individuals, meaning high-quality habitat for most amphibians; Matos et al., 2012). We performed all these operations using QGIS software (version 3.24.1; QGIS Development Team, 2022).

The next two model parameters are extinction (ε) and colonisation (γ), which translate the changes of roadkill over space and time. Extinction is the species’ probability of being road-killed in a road segment in a given year, but not the following year, considered therefore as roadkill disappearance. Colonisation specifies the species’ probability of not being road-killed in a specific road segment in a given year, but only in the subsequent year, hence considered as roadkill appearance. Since these two last parameters are related to changes within seasons, that is, between replicates of the same year (MacKenzie et al., 2006), we have used site covariates that change between years and road segments (also known as yearly site covariates). Thus, we used two remote sensing-derived indices proven to reflect determinant drivers for amphibians (Qian et al., 2007; Pinto et al., 2023): 1) mean EVI (Enhanced Vegetation Index; Liu and Huete., 1995) representing local dynamics in primary production, and 2) mean LST (Land Surface Temperature; Hulley et al., 2019) used as an air temperature proxy. For both indices, we computed the standard deviation (SD_EVI and SD_LST) to replicate the variations throughout each year. We calculated these remote sensing indices from LANDSAT images (LANDSAT 5 and LANDSAT 8 image collections - Level 2, Tier 1) with a 30 m spatial resolution (US Geological Survey (USGS) – http://lpdaac.usgs.gov) for each year (2006-2020). We only retained high-quality images with 0% cloud cover for the whole study area (WRS-2 scene: path 203, row 33) and we computed the mean and standard deviation for each set of images representing each year. In total, we obtained 84 images with an average of 5.6 images per year; the year 2012 had no 0% cloud cover images available, so we decided to exclude it from the analyses (Table S3.2, Supplementary material). We processed all composite images and calculated the remote sensing indices in Google Earth Engine (Gorelick et al., 2017). Additionally, we have also used the aggregated total annual precipitation, obtained from monthly climatological newsletters of the Portuguese Institute for Sea and Atmosphere (IPMA, 2021) for the region (Figure S3.1, Supplementary material), as a yearly site covariate since the rainiest years generally result in higher amphibian roadkill numbers (as a consequence of higher amphibian activity; Glista et al., 2008).

The last model parameter is detection probability (ρ) and refers to the probability of detecting a roadkill at a road segment during a monthly survey in a given year, given its occurrence. To account for imperfect detection, we used observation covariates to model detection probability. As our sampling frequency changed (either daily or weekly; Table S3.1 Supplementary material), we used sampling effort (number of sampled days per month) as an observation covariate. We considered two additional observation covariates of relevant interest to detect roadkill amphibians: 1) mean monthly precipitation (IPMA, 2021), since observer visibility may be compromised; and 2) a categorical covariate indicating whether the sampled road section had a paved shoulder or not (0 – no paved shoulder; 1 – paved shoulder). Paved shoulders expand the width of the road, which may affect observer detection performance.

The months where no sampling occurred and the road sections that were not sampled in a given month of a certain year (as the sampled road lengths varied through 2006-2020), were coded in the dataset as missing values (NA), so that estimated parameters of models would not be biased due to the heterogeneity in road surveys.

We performed all statistical analyses using the package '*unmarked*' (Fiske & Chandler, 2011) within R software (version 4.1.2; R Core Team 2021). Table 3.2 lists all used covariates and respective descriptions.

3.4.4. Model selection

We constructed our unique species' detection history matrices with the presence or absence (or 'NA' whenever a road segment was not sampled in a particular month). Before modelling, we assessed collinearity using a Pearson correlation analysis between all continuous covariates and retained all covariates with a correlation higher than |0.7| (Dormann et al. 2013). We did not include highly correlated covariates in the same model ('Forest areas' and 'Agriculture areas', and 'Tree density' and 'Agriculture areas'; Table S3.3, Supplementary material). We standardised all continuous covariates to zero mean and unit variance (Zuur et al. 2009) so that coefficients could be comparable between different types of covariates and used Akaike's Information Criterion with correction for small sample sizes (AICc; Burnham and Anderson, 2002) to identify the most parsimonious models constructed for each of the four parameters.

We started our model selection procedure by modelling detection probability against each covariate in a univariate model. We also constructed a model with all four model parameters constant (null model) to provide a comparison. We discarded all covariates that ranked lower than the null model and tested all the ones that ranked higher in every possible combination.

The detection probability model with the highest rank was then used to model all the remaining three parameters: initial roadkill probability, roadkill disappearance and roadkill appearance, as recommended by MacKenzie et al., (2003). For each one of these, we used a similar procedure (separate selection of covariates for each parameter) while using the best model of the detection probability parameter. After finding the best singular models for each

Table 3.2. List of covariates used in the multi-season occupancy models.

Model parameter	Covariate name and code	Description	Source	References
Initial roadkill probability	Distance to ponds	Distance to nearest water body (meters)	Project LIFE LINES	N/A
	Distance to highway	Distance to highway 'A6' (meters)	Project LIFE LINES	Haklay and Weber, 2008
	Tree density	Percentage of tree density (%)	NASA	Sexton et al., 2013
	Agriculture areas	Percentage of agricultural area (%)	CORINE 2006, 2012, 2018	EEA
	Forest areas	Percentage of forest area (%)	CORINE 2006, 2012, 2018	EEA
Roadkill disappearance/ appearance	Enhanced Vegetation Index (EVI)	Mean changes in primary production content index. -1 (indicating stressed vegetation) to 1 (indicating healthier vegetation)	USGS	Liu and Huete, 1995
	Land Surface Temperature (LST)	Mean radiative temperature of land surface (ºC)	Google Earth Engine (source code – Ermida et al., 2020)	Hulley et al., 2019; Ermida et al., 2020
	Total annual precipitation	Aggregated total annual precipitation for the study area region (mm)	IPMA	IPMA, 2021
	Standard deviation of Enhanced Vegetation Index (SD_EVI)	Annual variation in primary production content index	USGS	Liu and Huete, 1995
	Standard deviation of Land Surface Temperature (SD_LST)	Annual variation in radiative temperature of land surface (ºC)	N/A	Hulley et al., 2019; Ermida et al., 2020
Detection probability	Sampling effort	Total number of days sampled per month	N/A	Santos et al., 2011
	Mean monthly precipitation	Mean monthly precipitation for the study area region (mm)	IPMA	IPMA, 2021
	Road shoulder	Categorical covariate indicating if a road has paved shoulder ('1') or not ('0')	N/A	N/A

parameter, we assigned the respective covariates to a final model. This protocol was applied to each of our seven selected species. We have also performed a Variance Inflation Factor (VIF; Zuur et al., 2010) analysis on all the final models to check for possible collinearity among covariates (Table S3.4, Supplementary materials).

Next, we performed model validation by computing the MacKenzie and Bailey Goodness-of-fit test (MacKenzie & Bailey, 2004) using 1000 bootstrap samples for each full model. We discarded the models with a lack of fit ($p<0.05$) or solid overdispersion (c-hat value > 4). Lastly, we calculated the mean for all model parameters for each species to assess the roadkill risk over time.

3.5. Results

Four models presented good fit: spiny common toad ($\hat{c} = 1.52$, $p = 0.06$), fire salamander ($\hat{c} = 1.03$; $p = 0.16$), Iberian painted frog ($\hat{c} = 0.38$; $p = 0.25$) and the Southern marbled newt ($\hat{c} = 0.37$; $p = 0.22$). The three remaining models were discarded because they presented strong overdispersion and a high lack of fit: Natterjack toad ($\hat{c} = 952.5$; $p = 0$), Iberian spadefoot toad ($\hat{c} = 15.39$; $p = 0$) and the Iberian ribbed newt ($\hat{c} = 8.36$; $p = 0.01$). For this reason, the following results concern only the four species with valid models.

Mean initial roadkill probability was similar across the spiny common toad, the fire salamander, and the Iberian painted frog (0.635 ± 0.09, 0.547 ± 0.07 and 0.543 ± 0.12, respectively), while the Southern marbled newt had the lowest value (0.239 ± 0.08; Table 3.3). Mean roadkill disappearance probability values were low for all species, with the higher values registered for the fire salamander and the Iberian painted frog (0.102 ± 0.02 and 0.111 ± 0.03, respectively; Table 3). Mean roadkill appearance probability values were also low, with the highest values corresponding to the spiny common toad and the fire salamander (0.075 ± 0.003 and 0.043 ± 0.01, respectively; Table 3.3). Mean roadkill detection probability followed the same pattern, with the spiny common toad and the fire salamander presenting higher values (0.023 ± 0.003 and 0.028 ± 0.003 respectively) than the Iberian painted frog and the Southern marbled newt (0.004 ± 0.001 and 0.003 ± 0.001, respectively; Table 3.3).

Table 3.3. Mean initial roadkill, mean roadkill disappearance, mean roadkill appearance and mean roadkill detection probabilities for the four analysed species.

	Spiny common toad	**Fire salamander**	**Iberian painted frog**	**Southern marbled newt**
Mean initial roadkill probability (ψ)	0.635 (±0.09)	0.547 (±0.07)	0.543 (±0.12)	0.239 (±0.08)
Mean roadkill disappearance probability (ε)	0.078 (±0.02)	0.102 (±0.02)	0.111 (±0.03)	0.06 (±0.06)
Mean roadkill appearance probability (γ)	0.075 (±0.03)	0.043 (±0.01)	<0.0001	<0.0001
Mean roadkill detection probability (ρ)	0.023 (±0.003)	0.028 (±0.003)	0.004 (±0.001)	0.003 (±0.001)

We detected a general decrease in the roadkill probability over time for the four species (**Figure 3.2**). The spiny common toad revealed an approximately stable roadkill probability pattern until 2017 but experienced a major decrease in 2018 towards 2020, totalling a reduction of nearly 50% of roadkill probability during the study period. The fire salamander had a descending trajectory in the roadkill probability pattern until 2016, where it recovered to 2009 values, but afterwards experienced a decrease towards 2020, with a total roadkill reduction of nearly 50% as well. The Iberian painted frog and the Southern marbled newt presented a decreasing tendency in the roadkill probability patterns over the entire study period, without a single year with an ascending trajectory. The Iberian painted frog showed a roadkill probability reduction of over 70%, with an accelerated decrease between 2014 and 2016, while the Southern marbled newt registered a more constant decrease throughout all years, totalling a roadkill reduction of 40%.

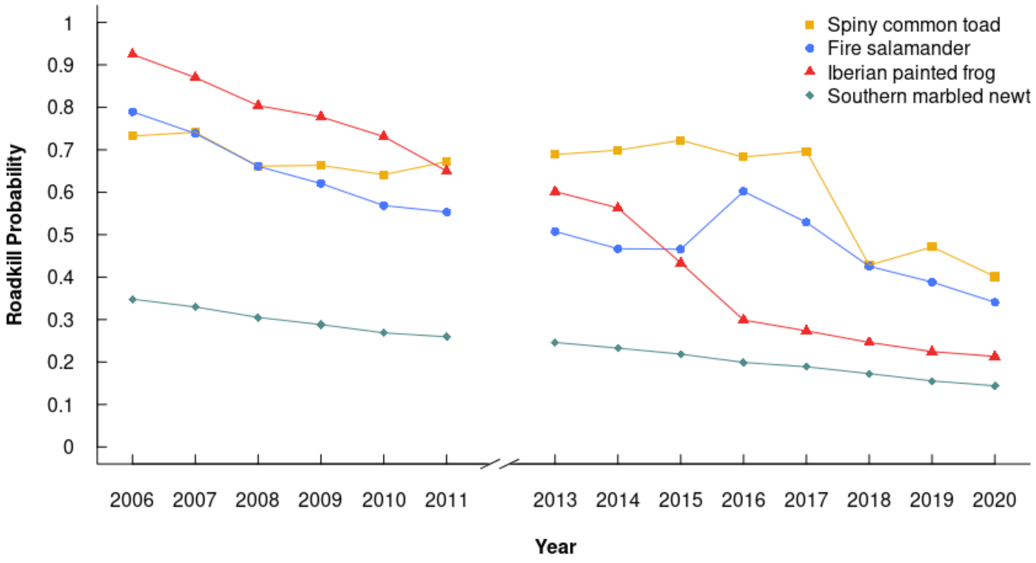

Figure 3.2. Mean roadkill probabilities over the 14 studied years for the four selected species (2012 was not included in the analyses).

We also denoted a peak in roadkill disappearance in 2015 for all species, followed by the years 2017 and 2019 for the spiny common toad, the fire salamander, and the Southern marbled newt (although not so expressive; **Figure 3.3**). The emergence of new roadkill in road segments over time (roadkill appearance) did not follow a general clearer pattern as roadkill disappearance, with the highest values for the spiny common toad and the fire salamander in 2015 (**Figure 3.4**). For all species, mean roadkill disappearance probability was greater than roadkill appearance probability during the studied period (Table 3.3).

Concerning model covariates for the initial roadkill probability, the roadkill probability of the spiny common toad increased in road segments surrounded by forest patches (coef = 6.34; CI =1.62, 11.06) and far away from water bodies (coef = -1.85; CI = -3.34, -0.36). Road segments with higher tree density increased the roadkill probability of the Southern marbled newt (coef =0.716; CI = 0.15, 1.28). The initial roadkill probability for the fire salamander and the Iberian painted frog had no significant covariates.

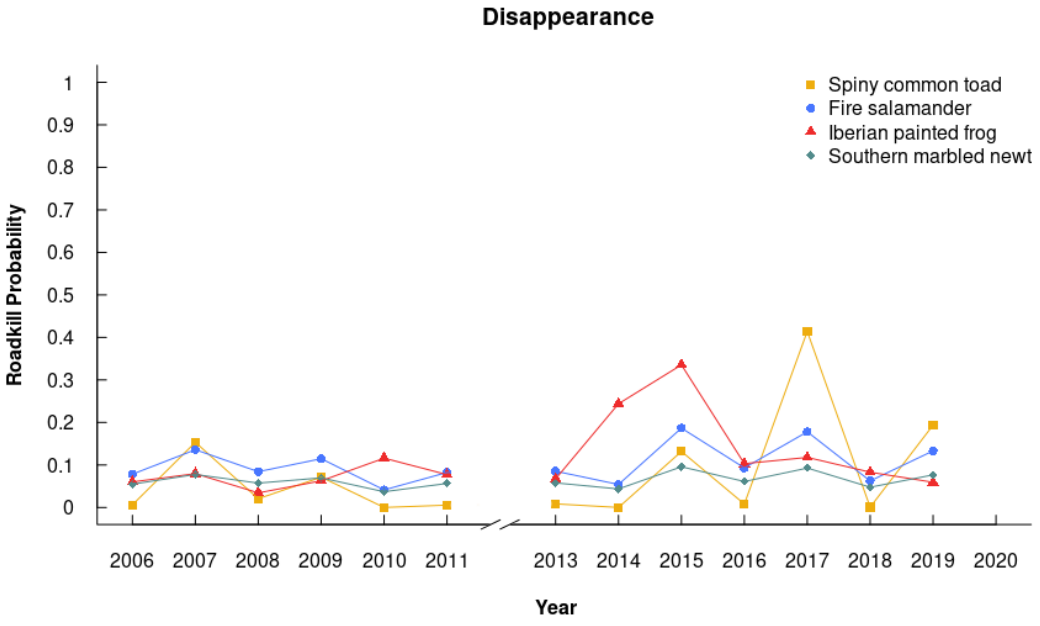

Figure 3.3. Mean roadkill disappearance probabilities over the 14 studied years for the four selected species (2012 was not included in the analyses).

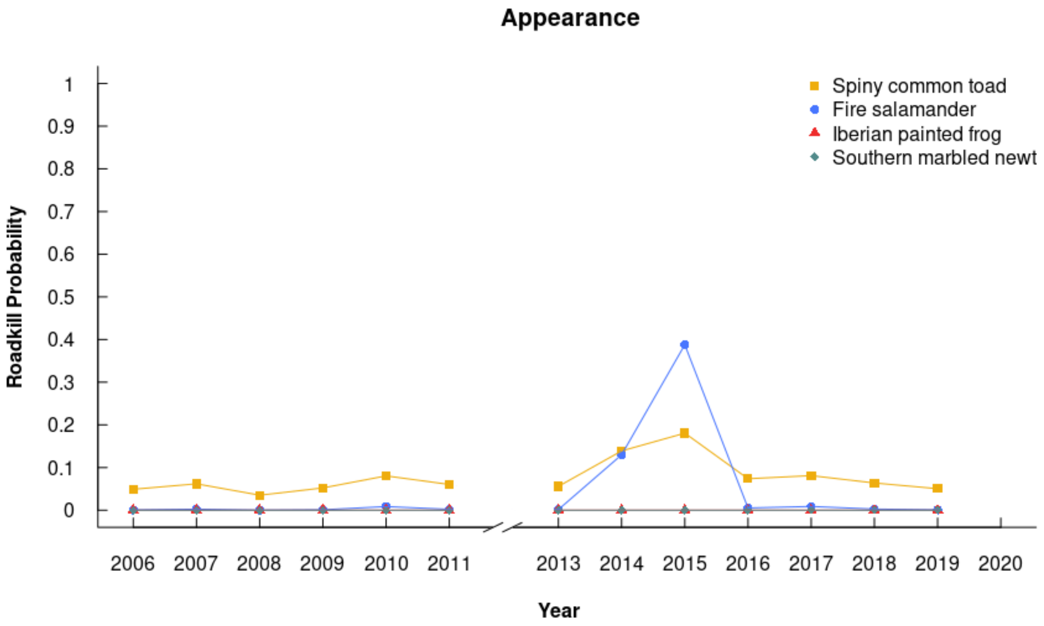

Figure 3.4. Mean roadkill appearance probabilities over the 14 studied years for the four selected species (2012 was not included in the analyses).

Regarding the roadkill disappearance parameter, we found significant results for three of the four analysed species: the variation in the primary production decreased the probability of roadkill disappearance (that is, the roadkill in one year disappears in the following year) for the Iberian painted frog (coef = -0.514; CI = -0.91, -0.12); rain showed the same negative relation for both the spiny common toad (coef = -3.024; CI = -3.99, -2.05) and the fire salamander (coef = -0.479; CI = -0.91, -0.05); high land surface temperature decreased the probability of roadkill disappearance for the Iberian painted frog (coef = -0.758; CI = -1.42, -0.09), but increased the probability of roadkill disappearance for the spiny common toad (coef =1.07; CI = 0.57, 1.56).

For the roadkill appearance parameter, the low land surface temperature increased the probability of new road segments with roadkill for the fire salamander (coef = -2.48; CI = -3.66, -1.30).

Lastly, for the detection parameter, we observed a pattern common to all species, with high sampling effort (total number of sampled days per month) and high precipitation increasing the probability of detecting roadkill (Table 3.4). Also, the probability of detecting a roadkill increased in road segments with paved shoulders for the spiny common toad (coef = 0.812; CI = 0.61, 1.02) and the Iberian painted frog (coef = 0.665; CI = 0.17, 1.16) and decreased the probability for the fire salamander (coef = -0.798; CI = -0.94, -0.66) and the Southern marbled newt (coef = -0.756; CI = -1.36, -0.15). Table 3.4 summarizes these results.

3.6. Discussion

3.6.1. Roadkill trends over time

We detected an overall decrease in roadkill probability over time, for the four analysed species. Four non-exclusive hypotheses can explain this: 1) application of mitigation measures; 2) historical roadkill patterns; 3) increase in traffic intensity; and 4) drought effects.

Concerning the first hypothesis, since 2018, three analysed roads (EM529, EN114 and EN4) have been subject to specific amphibian mitigation measures such as drift fences, underneath tunnels and road warning signs, as a result of a LIFE Nature and Biodiversity Program of the European Commission (LIFE LINES - LIFE14 NAT/PT/001081). Yet, these measures only totalise approximately 5% of the 120km of surveyed roads and were only installed in the last two years of sampling (2018). Even more, our results show a decreasing trend over the entire study period, suggesting this reason might not be a primary source for roadkill reduction.

In relation to the second hypothesis, roadkill might have decreased over time, simply because there are fewer amphibians to be roadkilled, as denoted by Fahrig and Rytwinski (2009) and Matos et al. (2012). Indeed, Cooke (2011) reported a continuous decrease in the roadkill numbers for the European common toad (*Bufo bufo*) during a twenty-year long-term study in England. This decrease was associated with population declines in nearby surveyed ponds, concluding that road mortality has played a significant role in the decline of those populations. Our raw data (roadkill) also indicates a reduction in the roadkill over the sampled years. To our knowledge, there are no studies in our study area reporting reductions in amphibian abundance caused by other factors (e.g., climate change, urbanisation, diseases, persecution, etc). Also, Orlowski (2007) and D'Amico (2009) found that higher numbers of amphibian roadkill are generally associated with higher local abundances, which may suggest that in our study area, amphibian abundances may have been declining over time.

Concerning the third possible hypothesis, and since we have no traffic data for our study area, we gathered the numbers of fuel and new vehicles sold in our region between 2006 and 2020 from the National Statistics Institute (INE, 2021). Although the pattern is cyclical (and likely correlated with past financial crises), the number of new vehicles sold between 2013 and 2020 has nearly tripled (Figure S3.2, Supplementary materials). Also, fuel consumption had an ascending trajectory within the same time period (2013-2020), although not so expressive (Figure S3.2, Supplementary materials). Considering these data as traffic proxies, the hypothesised potential increase in traffic may have exacerbated the barrier effect due to disturbance which may have forced existing populations to move away from roads, leading to the decline of amphibian roadkill. Fahrig et al. (1995) reported a similar response, with a decrease in amphibian road mortality directly correlated with the barrier effect caused by an increase in traffic intensity. Mazerolle (2004) also concluded that even small variations in traffic intensity can trigger distinct responses for

Table 3.4. Coefficients (and SD) of multi-season occupancy models for the four analysed species. Statistically significant coefficients are highlighted in bold; Confidence intervals are in brackets.

	Spiny common toad	**Fire Salamander**	**Iberian painted frog**	**Southern marbled newt**
Initial roadkill probability ψ				
Distance to ponds	**-1.85±0.76** **[-3.34; -0.36]**			-0.583±0.30 [-1.17; 0.01]
Distance to highway		-41.9±59.4 [-158.41;74.53]	-2.24±1.97 [-6.09;1.62]	
Tree density		62.7±90.6 [-114.91;240.25]		**-0.785±0.54** **[0.15;1.28]**
Agricultural areas				
Forest areas	**6.34±2.41** **[1.62;11.06]**			
Roadkill disappearance ε				
EVI		0.06±0.11 [-0.15;0.27]		
LST	**1.07±0.25** **[0.57;1.56]**		**-0.758±0.34** **[-1.42; -0.09]**	
Total annual precipitation	**-3.02±0.49** **[-3.99; -2.05]**	**-0.48±0.22** **[-0.91; -0.05]**		-0.29±0.74 [-2.03;1.45]
SD_EVI	-0.04±0.12 [-0.28; 0.19]		**-0.514±0.20** **[-0.91; -0.12]**	
SD_LST	0.17±0.13 [-0.08;0.42]			
Roadkill appearance γ				
EVI				
LST	-0.511±0.30 [-1.11;0.09]	**-2.48±0.60** **[-3.66; -1.30]**		
Total annual precipitation				
SD_EVI				
SD_LST				
Detection probability ρ				
Sampling effort	**0.911±0.05** **[0.82;1.01]**	**0.644±0.04** **[0.56;0.72]**	**1.140±0.14** **[0.86;1.42]**	**0.868±0.19** **[-0.49;1.24]**
Mean monthly precipitation	**0.627±0.03** **[0.58;0.68]**	**0.819±0.03** **[0.77;0.87]**	**0.562±0.06** **[0.43;0.69]**	**0.708±0.09** **[0.53;0.89]**
Road shoulder	**0.812±0.11** **[0.61;1.02]**	**-0.798±0.07** **[-0.94; -0.66]**	**0.665±0.25** **[0.17;1.16]**	**-0.756±0.31** **[-1.36; -0.15]**
AIC	9518	9106	1657	894

different amphibian species. Alternatively, the potential increase in traffic may have led to a reduction in roadkill, solely due to the lack of animals to be road-killed, as referred in the aforementioned hypothesis.

As for the last hypothesis, the study area is marked by dry weather, with temperature increment and rainfall reduction recorded in the last years (Figure S3.1, Supplementary materials). These weather variations may be less compatible with amphibian activity (Moss et al., 2021), which could also have led to a reduction in roadkill. The effects of droughts have also been linked to amphibian declines in other studies (Rowe et al., 2019; Zylstra et al., 2019; Moss et al., 2021).

We cannot choose a specific reason over the others: most probably the last three aforementioned reasons have contributed to shaping the roadkill patterns. The cumulative effect of road pressures (e.g., traffic intensity; species' historical roadkill patterns) in combination with other factors such as extreme climate events may pose serious threats to amphibian populations and may have helped to shape the local amphibian roadkill dynamics in our study area. This is especially alarming since our models detected that, in 14 years, the probability of the Iberian painted frog (the only amphibian species with the conservation status of Near Threatened in Portugal; Cabral et al., 2005), decreased by approximately 70%. In the case of the spiny common toad and the fire salamander, roadkill probability decreased by nearly 50%, and 40% for the Southern marbled newt. More studies are needed, particularly focusing on amphibian populations inhabiting areas closer to roads, alongside with continuous roadkill monitoring, to understand the underlying factors contributing to the observed declines. Understanding the roadkill cause (e.g., increase in traffic volume, lack of suitable habitat), is pivotal for devising targeted conservation strategies.

Although we have detected peaks in roadkill disappearance and appearance probabilities, these were fairly low (< 0.12), indicating that variation in roadkill spatial patterns from year to year is unlikely, and suggests an apparent stability in roadkill spatial patterns over time.

3.6.2. Influence of covariates on roadkill dynamics

We have found that the drivers of amphibian roadkill spatial patterns are mainly species-specific, as reported in previous studies (e.g., Sillero, 2008; Matos et al., 2012; Pinto et al., 2023). Roadkill of the Southern marbled newt was more likely to occur in road segments crossing areas with higher tree density. Forest areas are considered high-quality habitats for many amphibian species (Sillero, 2008; Carvalho & Mira, 2011; Matos et al. 2012; Heigl et al. 2017). Although Reques (2014) did not find a relation between the occurrence of the Southern marbled newt and tree cover, our model suggests this species might occur in higher tree density areas. The spiny common toad roadkill probability also increased in road segments surrounded by forest areas, a response also reported by Matos et al. (2012) who found higher roadkill for this species in forest habitats. This is expected since the spiny common toads are known to prefer habitats with higher tree cover (Ortiz-Santaliestra, 2014). Additionally, road segments closer to water bodies decreased the roadkill probability for the spiny common toad. Although previous studies reported high mortality concentrations of this species near water bodies (Orlowski, 2007; Santos et al., 2007), the ones closest to our sampled roads are mainly used for irrigation and cattle watering, and thus might not be suitable for this species' reproduction. Spiny common toads prefer deep, permanent, and clean water bodies (Ortiz-Santaliestra, 2014) that in our study area are generally located far from the roads.

Years with less rain had road sections with a higher probability of roadkill disappearance in the next year for the spiny common toad and the fire salamander, meaning that when it rains more, the road sections with roadkill stay as roadkill for the next year. This is expected since rain mediates amphibian activity (Glista et al., 2008): increasing animal movement in turn increases roadkill risk. The low intra-annual variation in primary production (SD_EVI; i.e., humidity content in vegetation) reduced roadkill disappearance probabilities for the Iberian painted frog. Less humidity variability represents stable local conditions for amphibian movement; this is particularly relevant for the Iberian painted frog because it is more dependent on water (Martínez-

Solano, 2014) than other species (e.g., spiny common toad). These road segments with low variation in humidity conditions may represent predictable movement corridors for this species, which in turn decreases the probability of roadkill disappearance at these locations from one year to the next. On the other hand, our models denoted opposite responses for high land surface temperature: a decrease in roadkill disappearance probability for the Iberian painted frog, and an increase for the spiny common toad. Amphibians are ectothermic and temperature governs their activity (Araújo et al., 2006). In fact, this trend can be linked to the patterns of drought in the study area. According to the IPMA (2023), the study area region experienced a drought event every other year between 2014 and 2020, with the years 2015 and 2019 being characterised by moderate droughts, and the year 2017 by a severe drought. The roadkill disappearance probabilities reached higher values in years characterised by drought for the aforementioned species. As stated before, Iberian painted frogs are more water dependent and thus, may be more affected by an increase in the temperature (characterised by a higher probability of roadkill disappearance), since this species typically occurs in moisture areas surrounding water bodies or ponds. However, the spiny common toad is a highly terrestrial species (seeking water bodies only for reproduction; Ortiz-Santaliestra, 2014): therefore, this species may be less exposed to temperature changes and desiccation, leading to a higher probability of roadkill disappearance in the next year.

Concerning the roadkill appearance probabilities, our models only revealed a significant covariate for one species: an increase in the probability of the emergence of new road segments with roadkill with low land surface temperature for the fire salamander. As stated before, amphibians are extremely dependent on the temperature: they typically display seasonal occurrence patterns, with a reduction or complete cessation of their activity at certain times of the year (e.g., winter and summer; Speybroeck et al., 2016). In resemblance with the spiny common toad, the fire salamander is also a highly terrestrial species. Thus, an increase in temperature, associated with increased desiccation risk, may restrain fire salamanders' movement, while lower temperatures may trigger increased movement, in turn increasing the probability of the appearance of new road segments with roadkill.

The Southern marbled newt did not respond to any of the roadkill disappearance and appearance parameters, leading to the perception that perhaps roadkill patterns are less dynamic in this species or there was not enough data to model the temporal parameter of our models. This withstands the importance of continuous sampling and long-term studies, in order to gather data on elusive species to draw better conservation management.

Overall, amphibian detection probabilities were rather low (< 0.1), thus confirming the need to correct it to avoid underestimating roadkill probability. This probability was higher with increased sampling effort. This response was expected since the increase in sampling periodicity will also increase the probability of detecting a roadkill (Santos et al., 2011). Our models also predicted a higher probability of detecting a roadkill in months with higher precipitation. Although previous studies report the opposite relation (Hels and Buchwald, 2001; Santos et al., 2011), amphibian activity is extremely dependent on rainy events in our study area. As amphibian roadkill increases with rain (as demonstrated by our models), the probability of detecting an amphibian carcass on rainy days will likely increase as well. It is true that the observer visibility may be impacted by heavier rainfall during surveys, however, carcass persistence may also increase due to reduced scavenger activity (Sergio, 2003). Concerning the influence of road shoulders on the detection probability, we detected a taxonomic pattern: anurans had higher detection probabilities on roads with paved shoulders, while urodele species had an opposite response. A possible explanation for this result could be related to the site where a given animal is road-killed and to the site's surrounding habitat. For instance, we assessed the roadkill spatial locations for all four species and detected that both the spiny common toad and the Iberian painted frog roadkill are mainly located on roads with paved shoulders (these roads cross a mix of both open forest and agriculture habitats), whereas the majority of the urodele roadkill are located on roads without paved shoulders (surrounded mainly by closed forest). This justifies the somehow order-grouped response, as the probability of detecting a species' roadkill in a given site is directly related to the probability of a carcass being present at that site and the species' preferred habitat. Therefore, caution is needed when extrapolating this result to other study areas.

3.7. Conclusions

In this study, we applied occupancy modelling to unveil the spatiotemporal patterns of amphibian roadkill in species with different habitat requirements. Our models explicitly show a reduction in amphibian roadkill over time for all analysed species. Although with low probabilities, roadkill disappearance dynamics seem to be more related to rainfall events and temperature and were more expressive than roadkill appearance dynamics. Our results seem to point towards an overall reduction in local amphibian populations, with little to no recruitment over time. This might be a serious challenge to the survival and maintenance of amphibian populations in landscapes such as the Mediterranean Basin.

Our study showed that occupancy models can be used to monitor spatiotemporal roadkill patterns, with fair to good results. This technique can be employed for other species and in other regions worldwide to understand the lasting effects of roads on wildlife in ever-changing landscapes. Our results may serve as a starting point to understand amphibian roadkill patterns across space and time. Future studies may investigate further the potential causes.

This work also emphasises the urgency in monitoring amphibian populations occurring near roads, in order to compare the population trends with the roadkill rates. This information would certainly be useful in providing targeted mitigation measures, depending on the cause of the decline (e.g., application of measures to reduce traffic or promote safe crossing structures between both sides of the roads). The acquisition of precise insights into the drivers of roadkill decline is imperative to formulate relevant and impactful conservation measures, particularly in critical places where roadkill is recurrent, and populations are more fragile.

3.8. Acknowledgements

We are sincerely grateful to all the people who gave their best to collect road mortality data over all these years, namely: João Craveiro, Luís Sousa, André Oliveira, Paulo Alves, Erika Almeida, Denis Medinas, Pedro Costa, Eduardo Ferreira, Nelson Fernandes, and others. We are also indebted to David Epple for his assistance in the early stages of this research. TP is financed by Fundação para a Ciência e Tecnologia (FCT) with a doctoral grant 2020.04581.BD. NS is supported by a CEEC2017 contract (CEECIND/02213/2017) from FCT. We also thank to MED (https://doi.org/10.54499/UIDB/05183/2020) and CHANGE (https://doi.org/10.54499/LA/P/0121/2020). This research was supported by project LIFE LINES - LIFE14 NAT/PT/001081 financed by the European Commission, and project POPCONNECT PTDC/AAG-MAA/0372/2014, co-financed by Programa Operacional Competitividade e Internacionalização (POCI) and supported by FEDER (POCI-01-0145-FEDER-016811).

Appendix S3 – Supplementary materials

Table S3.1. Description of the total length sampled of each road and the sampling frequency for the different years (2006 to 2020). 'Mixed' refers to daily sampling for some months and weekly sampling for others.

Road	Length (km)	Sampling frequency
EN114(A)	9	2006-2008 (weekly); 2009-2010 (mixed); 2011-2012 (daily); 2013-2014 (weekly); 2015-2019 (daily); 2020 (mixed)
EN114(B)	14	2007-2008 (weekly); 2009-2010 (mixed); 2011-2012 (daily); 2013-2014 (weekly); 2015-2019 (daily); 2020 (mixed)
EN4(A)	12	2006-2008 (weekly); 2009-2010 (mixed); 2011-2012 (daily); 2013-2014 (weekly); 2015-2019 (daily); 2020 (mixed)
EN4(B)	45	2013-2014 (weekly); 2015-2019 (daily)
EN370	6	2006-2008 (weekly); 2009-2010 (mixed); 2011-2012 (daily); 2013 (mixed); 2019 (daily); 2020 (mixed)
EN18	23	2013 (mixed); 2014 (weekly); 2015-2019 (daily)
EM529	9	2006-2008 (weekly); 2009-2010 (mixed); 2011-2012 (daily); 2013 (mixed); 2019 (daily); 2020 (mixed)

Table S3.2. Number of Landsat satellite images per sampling year. *2012 was removed from the analysis due to lack of suitable images.

Year	Number of images (0% cloud cover)	LANDSAT
2006	3	5
2007	2	5
2008	4	5
2009	7	5
2010	6	5
2011	5	5
2012*	0	-
2013	9	8
2014	4	8
2015	9	8
2016	7	8
2017	11	8
2018	5	8
2019	7	8
2020	5	8

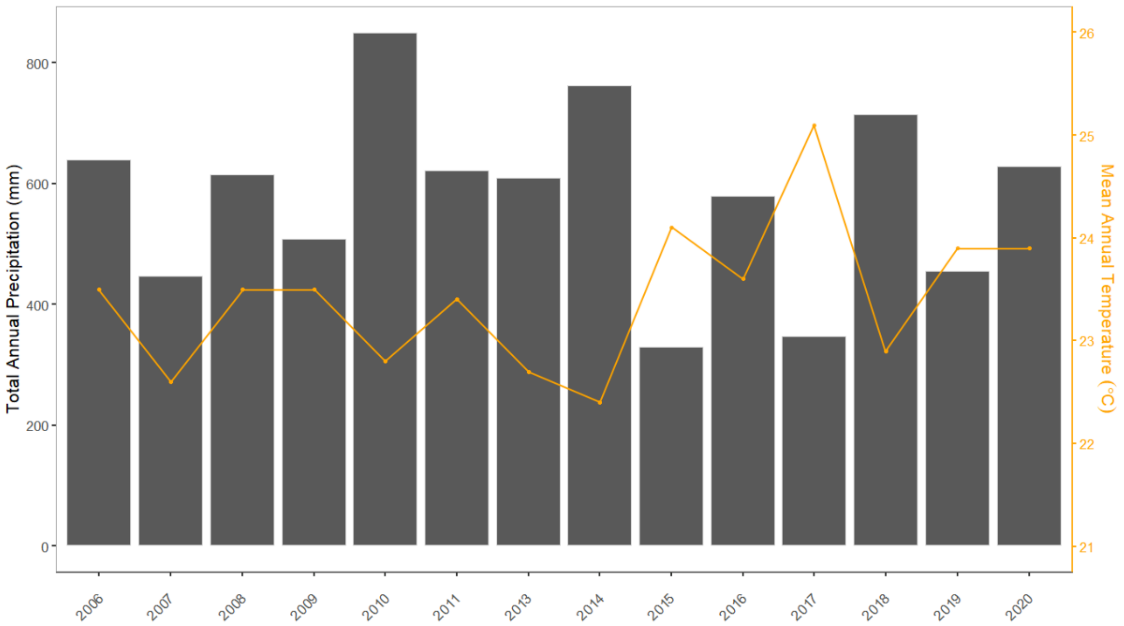

Figure S3.1. Climatogram of Évora region (total annual precipitation and mean annual temperature) between January 2006 and December 2020 (year 2012 was removed from analyses). IPMA data.

Table S3.3. Results of the correlation analysis between the covariates used in the models. High correlated covariates (> 0.7) are highlighted.

	M_LST	SD_LST	M_EVI	SD_EVI	D_ponds	Tree_dens	D_highway	Forest	Agric
M_LST	-	-0.35	-0.66	-0.56	0	-0.67	0.36	-0.37	0.5
SD_LST	-0.35	-	0.27	0.59	-0.08	0.09	-0.14	-0.1	0.06
M_EVI	-0.66	0.27	-	0.48	-0.03	0.68	-0.45	0.36	-0.41
SD_EVI	-0.56	0.59	0.48	-	-0.12	0.24	-0.35	0.04	-0.14
D_ponds	0	-0.08	-0.03	-0.12	-	-0.02	0.2	-0.11	0.08
Tree_dens	-0.67	0.09	0.68	0.24	-0.02	-	-0.31	0.67	**-0.73**
D_highway	0.36	-0.14	-0.45	-0.35	0.2	-0.31	-	-0.18	0.24
Forest	-0.37	-0.1	0.36	0.04	-0.11	0.67	-0.18	-	**-0.92**
Agric	0.5	0.06	-0.41	-0.14	0.08	**-0.73**	0.24	**-0.92**	-

Table S3.4. Results of the VIF analysis applied to the final models. No highly correlated covariates were detected. SCT – spiny common toad; FS – fire salamander; IPF – Iberian painted frog; SMN – Southern marbled newt; NT – natterjack toad; IST – Iberian spadefoot toad; IRN – Iberian ribbed newt.

	SCT	FS	IPF	SMN	NT	IST	IRN
Initial roadkill probability ψ							
Distance to ponds	1.430			1.206			
Distance to highway		1.292					
Tree density		2.192		1.206			
Agricultural areas							
Forest areas	1.430						
Roadkill disappearance ε							
EVI		1					
LST	2.514		1.121				
Total annual precipitation	2.452	1					1.048
SD_EVI	1.157		1.121		1.863		1.049
SD_LST	1.207				1.833		
Roadkill appearance γ							
EVI							
LST						1.06	1.018
Total annual precipitation							1.018
SD_EVI						1.06	
SD_LST							
Detection probability ρ							
Sampling effort	1.018	1.035	1.002	1.005	1.016	1.005	1.023
Mean monthly precipitation	1.019	1.031	1.001	1.003	1.016	1.005	1.023
Road shoulder	1	1.007	1	1.003	1		

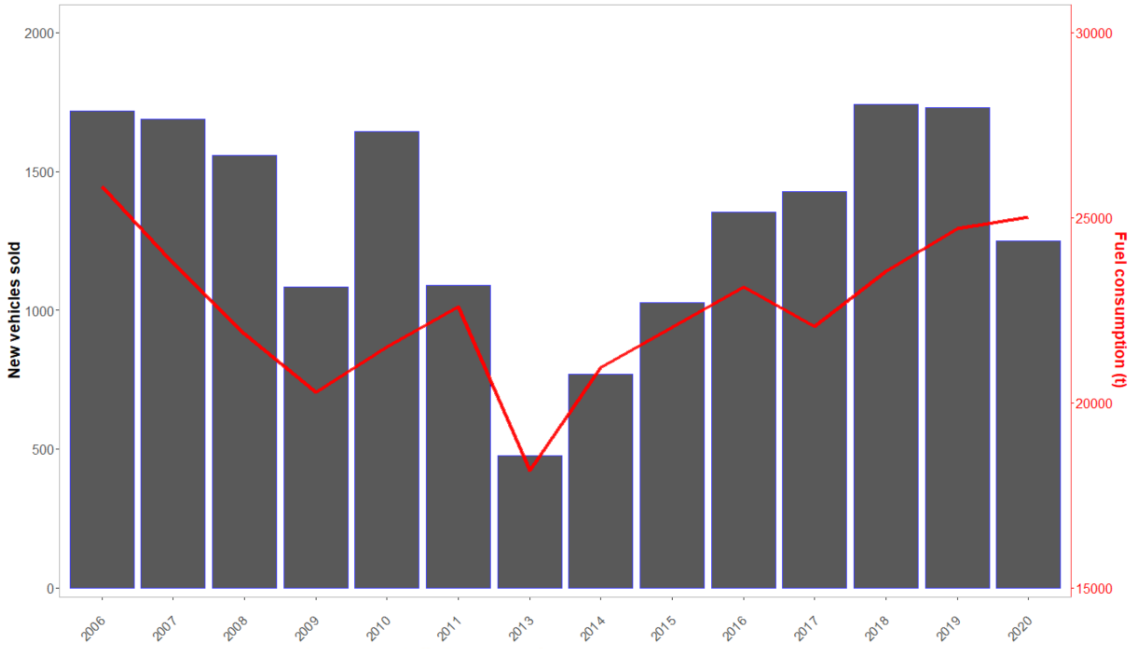

Figure S3.2. New vehicles sold and fuel consumption for Évora region between January 2006 and December 2020 (year 2012 was removed from analyses). INE (2021). Instituto Nacional de Estatística - Anuário Estatístico da Região Alentejo: 2006-2017. Lisboa: INE, 2021. Available at: https://www.ine.pt/xurl/pub/320467879.

Chapter 4

Effectiveness of permanent drift fences in reducing roadkill risk of amphibians

Pelophylax perezi

Published in *Journal of Environmental Management* as:

Pinto, T., Sillero, N., Mira, A., Sousa, L. G., Oliveira, A., & Santos, S. M. (2024). Effectiveness of permanent drift fences in reducing roadkill risk of amphibians. Journal of Environmental Management, 368, 122049. https://doi.org/10.1016/j.jenvman.2024.122049

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This chapter maintains the formatting rules of the corresponding journal in which it was published.

4.1. Abstract

Roads are an important source of human economic progress, but also a threat to wildlife populations and natural habitats. Roads are responsible for the direct mortality of hundreds of millions of animals worldwide, with special negative effects for amphibians. Since the middle of the twentieth century, various types of mitigation measures have been constructed to reduce the negative effects of roads. However, despite the large availability of potential solutions designed for this purpose, there is still a knowledge gap about their effectiveness for amphibians. This study analysed whether permanent concrete drift fences reduced the roadkill risk for amphibians. We applied a before-after-control-impact (BACI) design in two road segments with concrete drift fences for amphibians. We recorded amphibians on these road segments three years before and three years after the fence installation. We further tested whether the presence of these mitigation measures transferred the animals to sites adjacent to the drift fences, creating new potential mortality aggregation sites (fence-end effect). Our results show a significant reduction in the number of amphibians reaching the sites with the drift fences. We were, however, unable to demonstrate the potential movement route transference, as our results were inconclusive. Despite the increase in amphibian numbers at the control sites in the first year after fence installation, the following two years presented similar amphibian numbers as the pre-fence years. We recognise the importance of permanent drift fences in reducing the mortality of amphibian populations; however, we encourage future studies to include tunnel-crossing data as well, to truly unveil the roadkill reduction power of amphibian mitigation measures, while maintaining or increasing connectivity between roadside habitats.

4.2. Keywords: roadkill; concrete drift fences; road impact mitigation; before-after-control-impact; conservation

4.3. Introduction

Every year, hundreds of millions of animals die on the roads, victims of vehicle collisions (Forman and Alexander, 1998; Loss et al., 2015; Hill et al., 2019). This is frequently the primary source of mortality for many species, increasing the risk of extinction in many parts of the world (Forman et al., 2003; Grilo et al., 2021). Currently, amphibians are the most threatened vertebrate group on the planet (Houlahan et al., 2000; Stuart et al., 2004; IUCN, 2023), with roads as one of the major causes of population decline (Carr and Fahrig, 2001; Glista et al., 2008; Beebee, 2013; D'Amico et al., 2015) along with habitat loss and consequent fragmentation (Houlahan and Findlay, 2003; Cushman, 2006). Amphibians are extremely prone to death on roads, especially because of their complex life cycles, with specific movement routes due to distinct terrestrial and aquatic phases (Richter et al., 2001; Joly, 2019). A higher amount of roadkill occurs when roads cross the amphibian movement routes between the terrestrial and aquatic territories (coincident with hibernation/ estivation and reproduction habitats) (Orlowski, 2007; Eigenbrod et al., 2008; Sillero, 2008; Beebee, 2013; Pinto et al., 2023).

Understanding the effectiveness of roadkill mitigation measures in wildlife mortality is crucial to developing new methods to reduce the negative effects of roadkill on populations. The general aim of a roadkill mitigation structure is to impede animals from reaching the road, while (in most cases) providing a safe alternative to cross it: hence, the connectivity between habitats is improved on each side of the road (Forman et al., 2003; Dodd et al., 2004; Hamer et al., 2015). The first roadkill mitigation measures for amphibians were built in Europe in the 1960s (Puky, 2003), and currently, different types of measures are used, including: 1) road signs, used to alert drivers to a particular segment of the road that is frequently crossed by amphibians; 2) temporary road closure, usually during amphibian movement peaks; 3) temporary or permanent drift fences, built of canvas or plastic (temporary) or made from concrete, metal or UV-resistant polymers (permanent) to prevent animals from reaching the road surface; and 4) underpasses or road tunnels, with one entry on each side of the road, to allow animals to move safely between both sides of the road (reviewed in: Jochimsen et al., 2004; Schmidt & Zumbach, 2008). Based on the available data, the use of drift fences together with underneath road tunnels is considered the most favourable solution for reducing mortality in amphibians (Puky, 2003; Schmidt and Zumbach, 2008; Glista et al., 2009; Hamer et al., 2015). However, the susceptibility of animals to bypass the fence ends and access the road, may compromise management efforts to reduce road mortality, potentially undermining the overall effectiveness of the mitigation measure (Rytwinski et al., 2016; Markle et al., 2017).

The construction of these structures frequently has high costs, limiting their implementation in the field (Lesbarrères and Fahrig, 2012); therefore, temporary drift fences may be employed more frequently than permanent ones. However, permanent mitigation measures require less maintenance than temporary ones (Dodd et al., 2004), which ultimately may represent a benefit in the medium/long term (Hamer et al., 2015) and consequently, an increased efficiency. Although several studies attempt to identify the success of these measures, many fail due to insufficient monitoring (van de Ree et al., 2007), or lack the temporal scale with sampling both before and after the installation of the mitigation measure, which may result in uncertain or misleading conclusions (Lesbarrères and Fahrig, 2012).

A before-after-control-impact (BACI) sampling design is often considered the best approach to assess the impact of a stressor in the environment (Underwood, 1991; McDonald et al., 2000), producing useful information in road management studies (Roedenbeck et al., 2007; Lesbarrères & Fahrig, 2012). Therefore, it is necessary to assess mitigation measures’ effectiveness, so that resources can be directed to the most cost-effective ones. The success of allowing amphibians to safely cross roads during their seasonal movements is crucial for ensuring the long-term viability of their populations (Rytwinski and Fahrig, 2012).

In this study, we aim to assess the effectiveness of specific permanent amphibian mitigation measures to reduce road mortality risk in a region with historically high mortality records (Pinto et al., 2023). Specifically, we implemented a BACI design over six years, to test whether permanent concrete drift fences effectively reduce amphibian mortality risk. We also tested whether the presence of these mitigation structures resulted in new roadkill aggregations in adjacent non-mitigated road sections. For this, we established two hypotheses: 1) the concrete drift fences significantly prevent amphibians from reaching the roads, reducing consequentially their roadkill risk; and 2) the presence of amphibians on the road does not increase in road sections adjacent to the drift fences (absence of fence-end effect), not affecting amphibian movement routes.

4.4. Methodology

4.4.1. Study area

This study was conducted in southern Portugal (29N 599606E, 4285394N), in an area with one of the ecosystems with the highest biodiversity in the western Mediterranean Basin, also known as '*montado*' (Pinto-Correira et al., 2011). The area is dominated by a mixture of Mediterranean cork oak (*Quercus suber*) and holm oak (*Quercus rotundifolia*) forests with varying tree density and agricultural areas in equal proportions. The topography is generally flat, ranging between 100 m and 400 m a.s.l. (Pinto-Correia, 1993). The area is characterised by a Mediterranean climate, with mild and wet winters (mean temperatures between 5.8ºC to 12.8ºC) and hot and dry summers (mean temperatures between 16.5ºC to 30.2ºC). The average annual rainfall ranges between 500-650 mm (IPMA, 2021). The study area is intersected by the main transportation corridor connecting Lisbon to Madrid, comprising several roads, including one highway as well as some national and municipal roads. For this study, we surveyed two municipal road segments: EM529 and EM535.

4.4.2. Mitigation measures

Five permanent concrete drift fences were implemented in two road segments of the study area with high amphibian mortality, in late spring (April-May) of 2018: two in road EM529 and three in road EM535 (**Figure 4.1**). The fences were built within a LIFE Nature and Biodiversity Program of the European Commission (LIFE LINES – LIFE14-NAT-PT001081). These structures were developed by a local contractor and were designed considering the characteristics of several models available in the market. The fences are made of 40 cm high concrete blocks with a smooth surface, to prevent most of the species from climbing the top (Conan et al., 2023), and its upper part is (whenever possible) levelled with the road/shoulder surface, to prevent the animals from becoming trapped on the road between fences. They are also “L” shaped, with a slope towards the opposite side of the road (**Figure 4.1A;** more details on supplementary materials Figure S4.1). The drift fences were installed on both sides of the roads and have on average 401 m of length (140 to 1000 m) (Table 4.1).

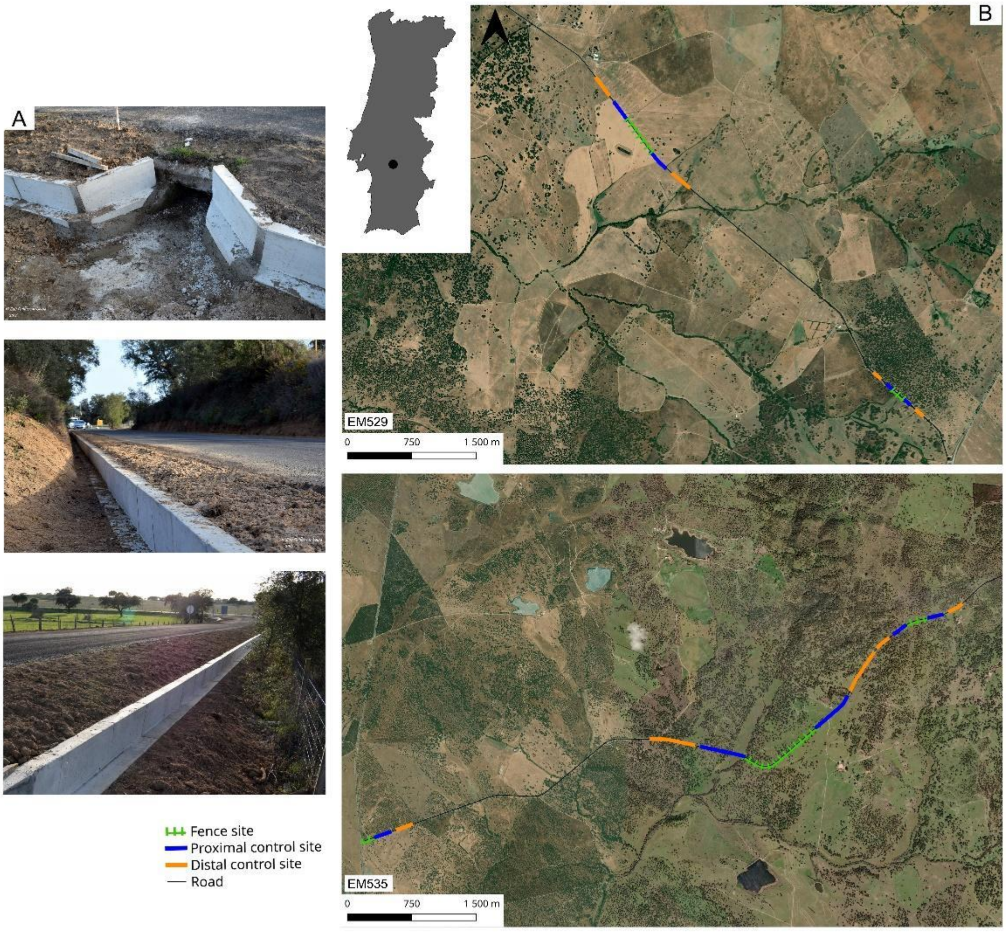


Figure 4.1. Specific amphibian mitigation measures installed on both surveyed road segments. (A) displays the different types of mitigation measures (permanent concrete drift fences and underneath tunnels), while (B) shows the location of roads EM529 and EM535 as well as defined treatment sites (fence, proximal control, and distal control).

Each fence includes at least 2 tunnels: either existing ones (mostly drainage culverts; **Figure 4.1A**) that were adapted to perfectly align their entrance with the drift fences (to prevent animals from reaching the road) or specific amphibian tunnels (ACO, Germany) that were also installed when culverts were absent.

4.4.3. Study design and data collection

The study was carried out using a BACI design (Underwood, 1991; 1994). For each of the five fenced road sections, we assigned four control sites: two ‘proximal control’ and two ‘distal control’ sites with equal lengths (half the length of the respective fence for each side). We defined the proximal control sites immediately after each fence end, and the distal control sites 100 m apart (**Figure 4.2**). We selected this distance for two reasons: 1) to ensure some spatial independence between the two control types while maximizing the number of fences included in the study design; and 2) this distance is typically the maximum length travelled by some species along a drift fence before individuals give-up and turn back if they cannot cross a road (Ottburg and van der Grift, 2019; Brehme et al., 2021). Road section ID 5 presented only one proximal and one distal control site (with the same length as the respective fenced site) as it is located near a crossroad (Table 4.1; **Fig. 4.1B**). To test our hypotheses, we defined a Treatment predictor with three categories: fence, proximal control, and distal control sites. We also defined a Year predictor where the surveys performed in the years 2015, 2016 and 2017 represent the sampling before fence installation, and the ones performed in 2018, 2019 and 2020 the sampling after fence installation. Although our study included data before and after fence installation, it is worth mentioning that, since the fences were installed at sites with high amphibian roadkill, our control sites may still introduce some bias (Soanes et al., 2024).

Table 4.1. Description of sampled treatment sites, the length, road, and percentage of tree density.

Section ID	Site code	Treatment	Length (m)	Road	Tree density (%)
ID 1	F1	Fence	400	EM529	0
	PC1_1	Proximal Control	200	EM529	0
	PC1_2	Proximal Control	200	EM529	0
	DC1_1	Distal Control	200	EM529	0
	DC1_2	Distal Control	200	EM529	1
ID 2	F2	Fence	140	EM529	17
	PC2_1	Proximal Control	70	EM529	11
	PC2_2	Proximal Control	70	EM529	24
	DC2_1	Distal Control	70	EM529	15
	DC2_2	Distal Control	70	EM529	7
ID 3	F3	Fence	300	EM535	0
	PC3_1	Proximal Control	150	EM535	20
	PC3_2	Proximal Control	150	EM535	19
	DC3_1	Distal Control	150	EM535	22
	DC3_2	Distal Control	150	EM535	10
ID 4	F4	Fence	1000	EM535	11
	PC4_1	Proximal Control	500	EM535	3
	PC4_2	Proximal Control	500	EM535	13
	DC4_1	Distal Control	500	EM535	28
	DC4_2	Distal Control	500	EM535	12
ID 5	F5	Fence	165	EM535	0
	PC5_1	Proximal Control	165	EM535	2
	DC5_1	Distal Control	165	EM535	1

Amphibian surveys followed a standardized protocol consisting of night-time surveys in autumn, conducted on rainy nights with minimum wind and average temperature ≥10°C (conditions of maximized amphibian activity – Sillero, 2008; Matos et al., 2012). On each survey, two experienced observers drove a car at a constant speed (20-30 km/h), scouting both road lanes and registering every amphibian encountered on the roads. These procedures aimed to mitigate the potential low detection rates characteristic of roadkill surveys and small-bodied species (Barrientos et al., 2018). All detected amphibians were identified to the lowest possible taxonomic level and the dead animals were removed from the road to avoid double counting during later surveys. Live amphibians found crossing the road were also moved and placed on the road verges in the direction they were heading. The GPS position of each observation was also recorded: observations <1 m apart were considered as a single GPS point with as many observations as animals encountered.

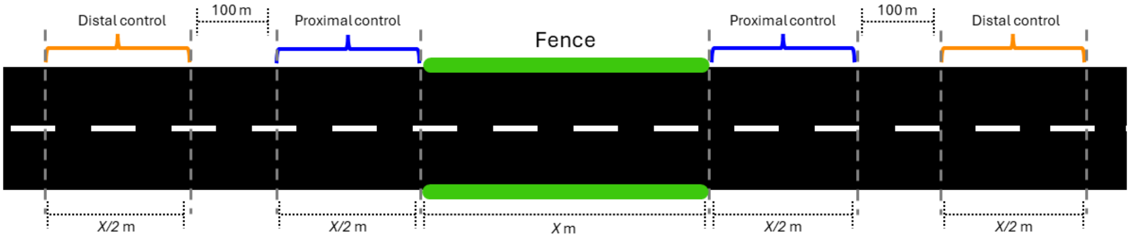


Figure 4.2. Schematic design applied for each drift fence (green line). The proximal (blue) and the distal (orange) control sites extend to both sides of the fence, with half the length of the respective fenced site. Distal control sites are placed 100 m apart from the proximal control sites (Road section ID 5 presented only one proximal and one distal control site).

4.4.4. Data analyses

We used the number of amphibians recorded on the roads (dead or alive) as a response variable and assumed that all live amphibians found on the road were at risk of roadkill. Besides Treatment and Year predictors, we also extracted the percentage of tree density within a buffer of 250 m for each site, from LANDSAT imagery (with 30 m pixel spatial resolution). We used tree density as an indicator of forest structure, where higher percentages represent a proxy of high-quality habitat for most amphibian species.

We built two different models, according to our hypotheses:

- Model H1 assessed the effectiveness of concrete drift fences in reducing the number of amphibians accessing the road (model H1) through a Generalised Linear Mixed Model (GLMM; Bolker et al., 2009) with "Treatment" ('fence' vs. 'distal control'), "Year" (2015 to 2020), and the interaction of "Treatment" and "Year" as main predictors; the percentage of tree density as a predictor to account for habitat differences and the length of each treatment site as an offset parameter (log scaled) to account for possible bias in our data; and the survey ("Visit") and treatment site ("Site code") as random effects to account for unbalanced sampling and possible correlations between successive visits at the same sites (see Table S4.1 on supplementary materials for a resume of the predictors).

- Model H2 assessed whether road sections adjacent to the concrete drift fences were subject to higher roadkill (model H2) through a second GLMM with “Treatment” (‘proximal control’ vs. ‘distal control’), “Year” (2015 to 2020), and the interaction of “Treatment” and “Year” as main predictors. The model structure was identical to the previous one, with tree density as an additional predictor, the length of each treatment site as an offset parameter, and survey and treatment site as random effects (see Table S4.1 on supplementary materials for a resume of the predictors).

We built both models with a negative binomial distribution, as our data presented high values of overdispersion (Zuur et al., 2009). To test for potential autocorrelation in our data, we performed a Moran’s I test (Moran, 1950) for spatial autocorrelation, and a Durbin-Watson test (Durbin and Watson, 1950) for temporal autocorrelation.

To evaluate drift fence impact (model H1), we calculated the BACI effect, representing the differential change between the fenced and the distal control sites, compared in the years before and after fence installation (Schwarz, 2015). We adopted the same procedure for model H2 for both control treatments.

We performed all the statistical analysis using the packages “glmmTMB” (Brooks et al., 2017), “DHARMa” (Hartig et al., 2022), “performance” (Lüdecke et al., 2021), “MuMin” (Barton, 2022) and “lsmeans” (Lenth, 2016) on software R (version 4.1.2; R Core Team, 2021). Tree density was extracted in QGIS software (version 3.24.1; QGIS Development Team, 2022).

4.5. Results

Between 2015 and 2020, we performed 83 surveys in both road segments (35 surveys in road EM529 and 48 surveys in road EM535), with 24 surveys before and 59 after fence installation. These surveys produced a database of 1593 amphibians reaching the road, belonging to 12 species (Table S4.2 – supplementary materials).

The two models showed a good fit to the data despite their R-squared values: model H1 explained 46% of the variance, while the model H2 explained 34% of the variance. Further model evaluation revealed that the residual plots exhibited no patterns, and both spatial and temporal autocorrelations had no significant values (Moran's I – model H1: 0.13, p = 0.25; model H2: 0.29, p = 0.15; and Durbin-Watson – model H1: 1.62, p = 0.17; model H2: 1.98, p = 0.15).

Concerning the effectiveness of the concrete drift fences (model H1), although the "Treatment" showed no significance, the "Year" 2018 was significantly different (Model H1: Z = -0.04, p > 0.05; Z = 1.257, p < 0.01, respectively). The interaction term between "Treatment" and "Year" revealed that all the years representing post-fence installation were significantly different (2018: Z = -1.947, p < 0.01; 2019: Z = -2.438, p < 0.01; 2020: Z = -1.082, p < 0.01). The installed fences significantly decreased the number of amphibians reaching the road. The estimated mean number of amphibians per visit per site on the fenced sites declined from nearly 1 in 2015 to 0.42 in 2020, representing a reduction of more than half the number of amphibians. In contrast, on the proximal control sites, the mean number of amphibians per visit and site increased from nearly 1 in 2015, to 1.6 in 2020 (**Figure 4.3**). The contrast analysis performed to determine the BACI effect estimated from the model H1 was significant: 5.68 ± 1.05 (p < 0.001).

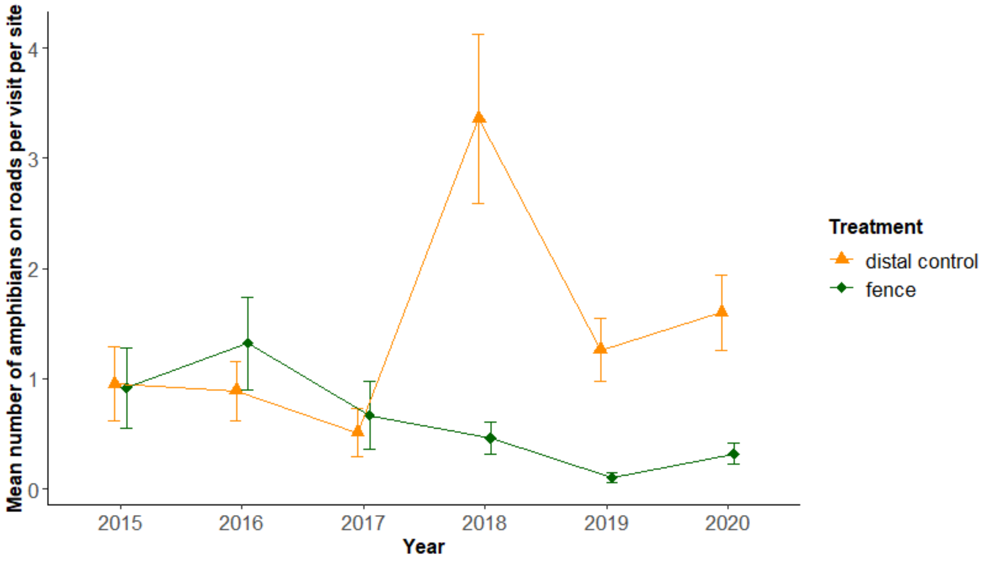


Figure 4.3. Least square means values (and respective SE) of amphibians per visit per site found on both roads in the years before (2015-2017) and after (2018-2020) drift fence installation (all distal control and fence sites). Fences significantly reduced the number of amphibians reaching the roads.

Concerning the potential fence-end effect (model H2), the number of amphibians reaching the road between distal and proximal control sites was not significantly different (Z = 0.271, p > 0.05), but the year 2018 was significantly different (Z = 1.657, p < 0.01). None of the interactions "Treatment" x "Year" was significant (2016: -0.633, p > 0.05; 2017: -0.973, p > 0.05; 2018: Z = -0.439, p > 0.05; 2019: -0.285, p > 0.05; 2020: Z = -0.252, p > 0.05): the number of amphibians is not significantly different between road sections adjacent to the fences and the distal control sites (**Figure 4.4**). The contrast analysis used to determine the BACI effect for the model H2 was also not significant: -0.12 ± 0.84 (p > 0.05).

Tree density was not significant for any of the models (Model H1: Z = 0.02, p > 0.05; Model H2: Z = 0.01, p > 0.05). Table 4.2 summarises model H1 and model H2 results.

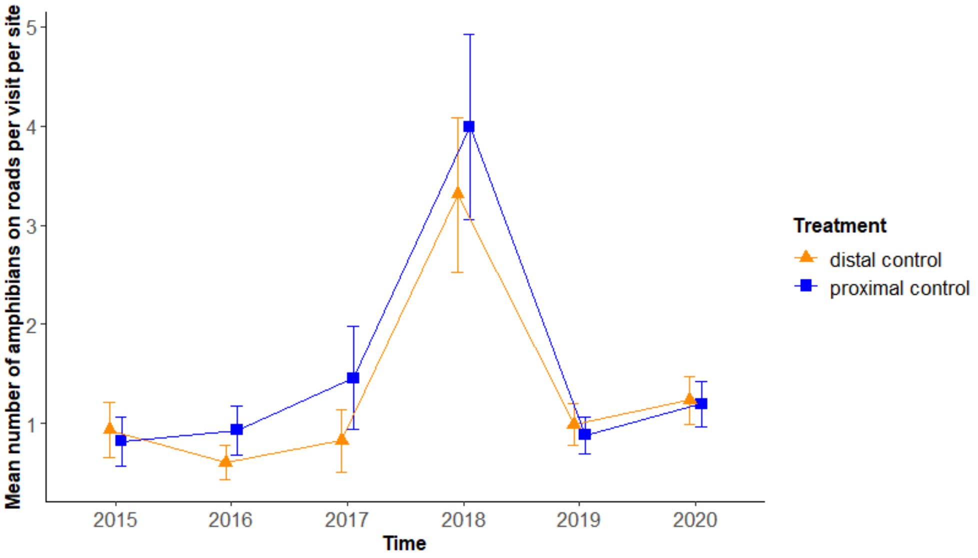


Figure 4.4. Least square means values (and respective SE) of amphibians per visit per site found on roads on both control treatments in the years before (2015-2017) and after (2018-2020) drift fence installation (all distal and proximal control sites). The year 2018 is significantly different in both treatments.

Table 4.2. Models with respective coefficients and R^2. Significant results are in bolt.

	Estimate	Std. Error	z value	p-value	Confidence interval (95%)
Model H1					
Treatment	-0.039	0.404	-0.098	0.922	-0.83; 0.75
Year2016	-0.069	0.442	-0.158	0.875	-0.94; 0.79
Year2017	-0.625	0.538	-1.162	0.245	-1.68; 0.43
Year2018	1.257	0.400	3.142	**0.002**	**0.47; 2.04**
Year2019	0.278	0.398	0.696	0.486	-0.50; 1.06
Year2020	0.516	0.393	1.315	0.188	-0.25; 1.29
Tree density	0.019	0.010	1.903	0.060	-0.001; 0.03
Treatment*Year2016	0.432	0.477	0.907	0.365	-0.50; 1.37
Treatment*Year2017	0.307	0.588	0.522	0.602	-0.84; 1.46
Treatment*Year2018	-1.947	0.461	-4.227	**< 0.001**	**-2.85; -1.04**
Treatment*Year2019	-2.438	0.541	-4.508	**< 0.001**	**-3.49; -1.38**
Treatment*Year2020	-1.082	0.445	-2.432	**0.015**	**-1.95; -0.21**
R^2	0.46				
Model H2					
Treatment	0.271	0.339	0.798	0.425	-0.39; 0.94
Year2016	0.611	0.455	1.343	0.798	-0.28; 1.50
Year2017	0.420	0.531	0.791	0.429	-0.62; 1.46
Year2018	1.657	0.439	3.767	**< 0.001**	**0.80; 2.52**
Year2019	0.758	0.423	1.795	0.073	-0.07; 1.59
Year2020	0.653	0.404	1.618	0.106	-0.14; 1.44
Tree density	0.010	0.005	1.779	0.075	-0.001; 0.02
Treatment*Year2016	-0.633	0.433	-1.464	0.143	-1.48; 0.21
Treatment*Year2017	-0.974	0.521	-1.871	0.061	-1.99; 0.05
Treatment*Year2018	-0.439	0.403	-1.089	0.276	-1.23; 0.35
Treatment*Year2019	-0.285	0.394	-0.724	0.469	-1.06; 0.49
Treatment*Year2020	-0.252	0.377	-0.668	0.504	-0.99; 0.49
R^2	0.34				

4.6. Discussion

Our study measured the effectiveness of roadkill mitigation structures built specifically for amphibians. Our results support our first hypothesis that permanent concrete drift fences are effective in reducing the number of amphibians on the roads. The significance of the interaction terms reveals that the decrease in amphibian numbers was linked to the mitigation, with no reduction observed in the distal control treatment. The concrete drift fences acted as a barrier preventing amphibians from accessing the road, resulting in a roadkill decrease of more than half when compared with the years before fence installation (2015-2017). Several studies reported similar results, with drift fences reducing amphibian roadkill between 40% and 100% (Cunnington et al., 2014; Rytwinski et al., 2016; Helldin & Petrovan, 2019; Boyle et al., 2021).

In fact, Cunnington et al. (2014) stated that fences are a far more efficient road mitigation measure than tunnels in reducing amphibian roadkill and that priority should be given to the installation of fences so that amphibians can be kept away from roads.

Despite our results on fence effectiveness, these were not 100% effective as we still found some amphibians on the roads in the fenced sites after fence installation. There is the possibility that some animals entered the fenced sites either by climbing the drift fences (Dodd et al., 2004), or coming from the adjacent sites (moving along the road). Oppositely to Dodd et al. (2004), who reported a decrease in drift fence effectiveness with tree frogs – overall mortality reduction from 93.5% to 65% - we only found two individuals of a species with climbing abilities (*Hyla meridionalis*) on the roads in fenced sites after fence installation, excluding this as the main reason. Also, a possible lack of maintenance of the vegetation surrounding the fences could have facilitated the access to the road (Hamer et al., 2015; van der Ree et al., 2015). Nevertheless, the concrete drift fences proved to be effective in impeding most of the amphibians from accessing the roads, with a reduction in amphibian sights of nearly 60%. Moreover, in a recent study, Conan et al. (2023) demonstrated – through a series of tests – that a permanent drift fence (e.g., concrete) with a minimum height of 40 cm and an overhang on top, was able to stop most amphibians from reaching the road. Our fences comprise most of the characteristics these authors detected to be essential for the effectiveness of these mitigation measures.

We were, however, unable to demonstrate that the permanent drift fences do not alter amphibian movement routes (second hypothesis), as our results were inconclusive. We did not detect significant differences between the two treatments (distal and proximal control) across the analysed years. Despite the mean number of amphibians on roads at these sites in 2019 and 2020 being equivalent to the years before fence installation, we cannot conclude for sure whether movement routes were modified, as we did not collect tunnel-crossing data. To our knowledge, Helldin & Petrovan (2019) is the only study that reported fence-end effects for amphibians. In this work in Sweden, the authors found that amphibian roadkill increased in an unfenced site adjacent to fences, probably linked with a movement route change and possible mortality transference. We found an increase in amphibians on the roads in 2018 for both proximal and distal control sites; however, this does not necessarily imply a fence-end effect (see below). Other studies have also reported an increase in road mortality at adjacent ends of the mitigation fences (Clevenger et al., 2001; Markle et al., 2017). Yet, these studies were conducted with different taxonomic groups (mammals and reptiles, respectively) and thus, cannot be comparable. Despite the lack of studies reporting fence-end effects for amphibians, potential solutions to reduce this problem can be found in the literature (and some may be adaptable to amphibians), such as: 1) increasing the length of the mitigation drift fences, as some authors reported roadkill reduction at longer fence lengths (Huijser et al., 2016); 2) installation of terminal fence segments – either perpendicular to the road or "V" shaped – to discourage animals from circumventing the fence and accessing the road, and guide them back to the fence and towards a safe underneath passage (Harman et al., 2023); and 3) increasing the number of tunnels along the fence to increase the likelihood of an animal to find and use them (Ottburg and van der Grift, 2019).

According to our models, the year 2018 was significantly different, as we recorded an exceptionally high number of amphibians on both control treatments, with numbers returning to fence pre-construction period in the following years. Amphibian activity is highly dependent on external factors, such as temperature and precipitation (Araújo et al., 2006; Glista et al., 2008), and this increase may have been triggered by environmental conditions present in the study area in that year (and not analysed in this study). For example, the year 2018 was unusually rainy for the region (IPMA, 2023), which could have prompted amphibian activity, justifying these higher numbers. In fact, this year was responsible for 25% of all the collected animals on sampled sites across the entire study period. Nevertheless, it could also mean a possible fence-end effect, with movement transference, as the number of amphibians on the roads increased at sites of both control types in that year. Still, the numbers of amphibian sights in the following years (2019 and 2020) are similar to the ones before fence installation, with even slightly lower numbers in proximal control sites when compared to 2017. This could eventually suggest no fence-end effect, but rather a response to an atypical year, or even a fence construction effect detected in 2018 but not in the following years. We minimised this potential construction effect, by choosing a sampling season (autumn) different from the construction period (late spring), though some effects may still have been detected. Unfortunately, the lack of tunnel-crossing data impedes a clearer conclusion, and the results from our second hypothesis should be interpreted with caution.

As the fences act as a barrier to amphibian movements towards the roads preventing them from being roadkilled, they can also have a counter effect, increasing the barrier effect (Jaeger and Fahrig, 2004). This behaviour has been previously reported by some studies where amphibians gave up and moved back after certain distances travelled along a fence (e.g., Matos et al., 2019; Ottburg and van der Grift, 2019; Brehme et al., 2021), returning to the original habitats without breeding (Schmidt & Zumbach, 2008). Our data does not allow us to measure this behaviour, although we do not discard it may occur. For species dispersing longer distances and subject to higher mortality, drift fences may provide substantial advantages in decreasing road fatalities (Lesbarrerès et al., 2004), but for less mobile species, road crossings may be less frequent (Matos et al., 2019), resulting in genetically isolated populations (Cushman, 2006; Baguette et al., 2013).

Boyle et al. (2021) showed that not only did the fences reduce the number of amphibians on the roads, but underneath tunnels were likely to be used by local species assemblages promoting connectivity at a population level. Jarvis et al. (2019) reported similar results. Although we do not possess this type of data, we expect similar responses from the populations occurring in our study area. Still, more data are needed, especially tunnel-crossing data and data on local populations, to fully understand if these mitigation structures are beneficial for the long-term persistence of amphibian populations. In a 14-year duration study, Pinto et al., (2024) revealed a continuous decrease in roadkill numbers for some amphibian species in the same study area. The authors link this reduction to a possible depletion in local populations (among other reasons). If so, mitigation measures like permanent drift fences together with underneath road tunnels might help to restore connectivity and reduce amphibian road mortality, increasing the likelihood of population recovery.

4.7. Conclusions and Recommendations

Our results highlight the importance of long-term monitoring studies in evaluating the effectiveness of measures to mitigate amphibian roadkill. Roadkill risk has been drastically reduced with the installation of concrete drift fences specifically designed for amphibians, even when environmental conditions are most suitable for this taxonomic group. Since permanent mitigation structures are usually very expensive and demand considerable management (e.g., periodic surrounding vegetation cut), priority should be given to areas with severe historical roadkill patterns, or where existing populations near roads face marked reductions. Within these areas, mitigation measures should be installed in road segments with roadkill hotspots. Drift fence effectiveness should be measured by continuous sampling (both before and after the structure installation). When budgets are constrained, some adaptative measures can be applied: for example, drainage culverts are known to be used by some species; and their adaptation, as was done in our study area (with concrete drift fences towards both culvert sides), may yield similar results for many species.

We also acknowledge that all aspects of the mitigation measures should be accounted for (drift fence and tunnel usage; fence-end sampling; among others) to fully detect possible movement changes and identify what achieves the best results. Most likely there will not be a one-size-fits-all measure but, by understanding what works best, it will be possible to adjust existing structures and implement additional ones that may benefit a wide range of species, at the least possible cost.

4.8. Acknowledgements

We thank all the students and colleagues who joined us on the sometimes-long surveys to collect the data. TP is financed by Fundação para a Ciência e Tecnologia (FCT) with a doctoral grant 2020.04581.BD (https://doi.org/10.54499/2020.04581.BD). NS is supported by a CEEC2017 contract (CEECIND/02213/2017) from FCT. We thank MED (doi:10.54499/UIDB/05183/2020) and CHANGE (doi:10.54499/LA/P/0121/2020). This research was supported by the project LIFE LINES – LIFE14 NAT/PT/001081 financed by the European Commission. We also thank two anonymous reviewers for improving our manuscript.

Appendix S4 – Supplementary materials

Figure S4.1. Installation of permanent concrete drift fences as well as a cross-cut of the "L" shaped piece with a slight inclination of the wall towards the outer side of the road (source: Garcia et al., 2021).

Table S4.1. List of the predictors included in the analyses.

Predictor name	Description and measure unit
Treatment	Treatment category of each specific site (fence; proximal control; distal control)
Time	Whether the survey occurred before (2015-2017) or after (2018-2020) the installation of the concrete drift fences
Tree density	Percentage of tree density (%) obtained by LANDSAT imagery [a]
Length	The length of each treatment category (m)
Visit	Unique identifier for each survey day
Site code	Identification of each surveyed site

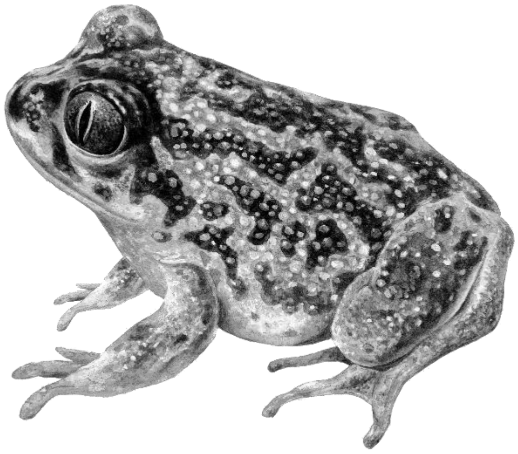
[a] Sexton et al. (2013)

Table S4.2. Information of studied species.

Species	N	Main locomotion	IUCN conservation status
Alytes cisternasii	9	Runner/ crawler	Least Concern
Bufo spinosus	88	Jumper/ crawler	Least Concern
Discoglossus galganoi	46	Jumper	Least Concern
Epidalea calamita	735	Runner	Least Concern
Hyla meridionalis	11	Climber	Least Concern
Lissotriton boscai	32	Crawler/ climber	Least Concern
Pelobates cultripes	193	Jumper	Vulnerable
Pelodytes atlanticus	1	Jumper/ climber	Least Concern
Pelophylax perezi	37	Jumper	Least Concern
Pleurodeles waltl	142	Crawler	Least Concern
Salamandra salamandra	169	Crawler	Vulnerable
Triturus pygmaeus	130	Crawler/ climber	Near Threatened

Chapter 5

Tell me where you go, and I'll tell you where you die: landscape connectivity as a tool to predict amphibian roadkill risk



Pelobates cultripes

Submitted to *Journal of Environmental Management* as:

Pinto, T., Santos, S. M., Mira, A., Sillero, N. (under review). Tell me where you go, and I'll tell you where you die: landscape connectivity as a tool to predict amphibian roadkill risk. Journal of Environmental Management.

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5.1. Abstract

Human overpopulation, development, and consequent activities such as land conversion and linear infrastructure expansion, are currently major threats to biodiversity. Amphibians are especially affected because they depend on both aquatic and terrestrial habitats to complete their life cycles, which can be negatively impacted by roads, due to reduced landscape connectivity. Understanding animal movement across the landscape may improve the prioritisation of sites to implementing mitigation measures. We assessed habitat suitability and landscape connectivity (using ecological niche modelling and circuit theory, respectively) for two amphibian species (the Iberian spadefoot toad and the Iberian ribbed newt) on a Mediterranean landscape in Southern Portugal. We addressed the following question: Can landscape connectivity predict amphibian roadkill risk? Our results showed that both species' distributions shifted between years. In years 2016 and 2019 habitats with low to medium primary production had the highest occurrence probability, while forested habitats revealed the highest occurrence probability during 2017. Our connectivity models assigned higher current movement corridors to heterogeneous habitats composed of sparse forests combined with low-management agricultural areas and good networks of higher-density water bodies. We found a positive correlation between high-connectivity road segments and roadkill for both species, proving that landscape connectivity can help to predict locations with higher roadkill probability.

We acknowledge that maintaining a heterogeneous landscape, with a higher density of short-distance water bodies that connect highly suitable habitats is important for amphibians. The identification of corridors with increased amphibian movement probability provides useful insights for road agencies to implement amphibian-oriented roadkill mitigation measures.

5.2. **Keywords:** habitat suitability; road mortality; movement corridor; spatial analysis; conservation

5.3. Introduction

Habitat loss and fragmentation, as a result of human activities such as land conversion, urban development, and linear infrastructure expansion, are currently among the largest threats to biodiversity (Forman & Alexander, 1998; Baguette et al., 2013). Amphibians are particularly affected as their complex life cycles imply both terrestrial and aquatic habitats, placing this taxonomic group at a higher conservation risk (Blaustein et al., 1994; Beebee and Griffiths, 2005; Matos et al., 2012; Joly, 2019). Amphibians are sensitive to the loss or alteration of these two different habitats, as well as to the connectivity between them (Becker et al., 2010). In terrestrial habitats, linear infrastructures, such as roads, are responsible for habitat loss and fragmentation, and also for introducing a barrier effect that hampers animal movement. This barrier effect directly affects several biological processes such as migration or dispersal, key elements for maintaining population dynamics (Glista et al., 2008; Semlitsch, 2008; Sillero, 2008). Roads are also responsible for chemical, noise, and light pollution, spread of invasive species (Forman et al., 2003; van der Ree et al., 2015), and direct mortality through vehicle collisions - a negative effect to which amphibians are especially vulnerable (Glista et al., 2008; Carvalho and Mira, 2011; Beebee, 2013; Pinto et al., 2023). In aquatic habitats, water bodies are reproduction sites for many amphibian species, and consequently, also aggregation sites (Joly, 2019; Cayuela et al., 2020). When these sites are close to roads, high amphibian mortality rates are commonly reported (Ascensão and Mira, 2005; Santos et al., 2007; Schmidt & Zumbach, 2008; Cooke, 2011; Pinto et al., 2023).

There is broad consensus that preserving these aquatic habitats is of utmost importance, particularly because of the biodiversity they harbour, but also for their contribution to ecosystem services. European legislation (e.g. Habitats Directive and European Water Framework Directive) was implemented to protect these sites across the continent (Biggs et al., 2016; Hill et al., 2018). In the western Mediterranean Basin, many of these water bodies dry up during the summer months, which represents an increased challenge to water-dependent species that naturally remain close to these habitats all year round (Pinto et al., 2023). Other species, less water-dependent, only use these habitats during the breeding season. These movements to and from water bodies (migration and/or dispersal events) involve navigating, on many occasions, through a more or less inhospitable landscape matrix (Joly, 2019; Cayuela et al., 2020).

Landscape connectivity is the degree of landscape that facilitates or hinders movement between resource patches (Taylor et al., 1993). It is of extreme relevance for amphibians breeding in water bodies, whose populational structure is often considered to be organised in metapopulations – distinct populations occurring in the same region exchanging individuals through dispersal (Hanski, 1998; Marsh & Trenham, 2001). The joint effect of roads and landscape fragmentation can increase the isolation of breeding populations, particularly of less vagile species (Carr and Fahrig, 2001), leading to a higher probability of extinction due to lower demographic and genetic input (Zeller et al., 2012; Baguette et al., 2013). However, such effect can be reduced, for example, by: 1) maintaining corridors or small remnant patches (Bodin & Saura, 2010; Saura et al., 2014; Bishop-Taylor et al., 2015) that facilitate movement across the landscape; and 2) installing roadkill mitigation measures to provide safe crossing between well-connected patches (Jarvis et al., 2019; Boyle et al., 2021). This is particularly relevant for amphibian populations since they frequently experience local extinctions, even in non-fragmented landscapes (Trenham et al., 2003). Nevertheless, installing roadkill mitigation measures (usually costly structures) mostly relies on studies that solely consider the association between roadkill records and spatial variables characterising road and landscape features, not considering landscape connectivity. Distinct species (and even individuals; Joly, 2019) make use of the landscape differently, and the understanding of the landscape resistance to animal movement may ultimately help to prioritise sites where roadkill mitigation measures should be implemented or improved. In addition, quantifying landscape connectivity may be useful in highly seasonal dynamic landscapes impacted by anthropogenic and environmental changes, to maintain the viability of populations (Nowakowski et al., 2017).

In this study, we analysed whether roadkill risk increases with landscape permeability by assessing habitat suitability and landscape connectivity for two distinct amphibian species on a Mediterranean landscape in Southern Portugal. We also determined whether road segments with higher connectivity (and, consequently, higher movement probability) are associated with roadkill. If so, connectivity might be used as a valuable tool to identify optimal locations for implementing roadkill mitigation measures, significantly reducing costs when compared to traditional roadkill studies. The ultimate goal is to provide conservation managers and road practitioners with a comprehensive evaluation of functional connectivity among terrestrial and aquatic habitats for amphibians that can be relevant to implement future conservation actions.

5.4. Methodology

5.4.1. Study area

This study was conducted in the Alentejo region, Southern Portugal, in an area covering approximately 210.000 ha (**Figure 5.1**). The landscape is mainly composed of Mediterranean cork (*Quercus suber*) and holm (*Quercus rotundifolia*) forests with varying tree cover and density (Pinto-Correia and Mascarenhas, 1999). It is mixed with agricultural areas in equal proportions, composing the complex agro-silvo-pastoral system known as *montado* (Pinto-Correia et al., 2011). These agricultural areas include mainly pastures for cattle grazing and cereal crops; however, other land uses such as orchards and permanently irrigated crops are also present at lower extents (Pinto-Correia and Mascarenhas, 1999). This landscape mosaic structure is considered one of the highest biodiverse ecosystems in the western Mediterranean Basin (Pinto-Correia et al., 2011). The topography is generally flat with gentle slopes ranging from 100 m to 400 m a.s.l. The climate is typically Mediterranean, with hot and dry summers (with mean temperatures between 16.5°C and 31°C in August) and mild and wet winters (mean temperatures ranging from 5.8°C to 12.8°C in January). The region is also marked by differences in precipitation across the different seasons (mean precipitation - Spring: 174mm; Summer: 34mm; Autumn: 181mm; Winter: 240mm) with mean annual precipitation reaching

650mm (Évora 1871-2008; IPMA, 2021). This region is also crossed by a well-established road network, comprising one highway and several national and municipal roads, including the main transportation corridor connecting Lisbon to Madrid.

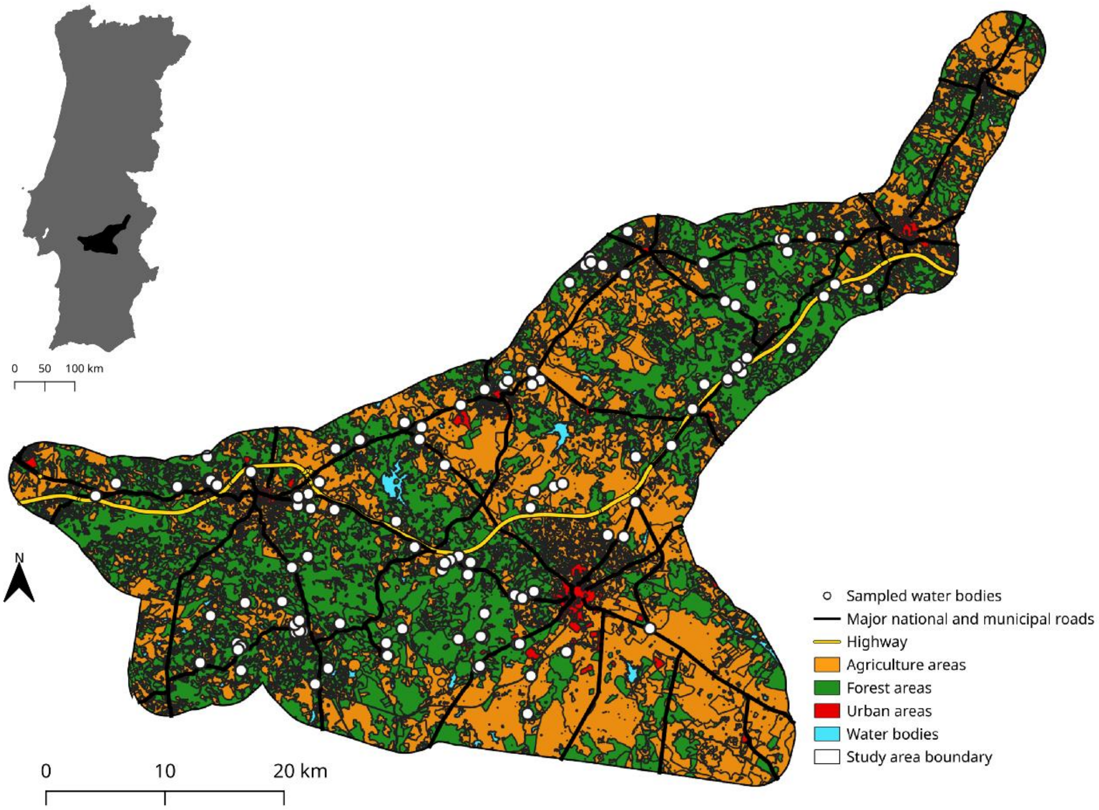


Figure 5.1 – Study area in Southern Portugal, with the main land cover classes, as well as urban areas and larger water bodies. Major national and municipal roads and the highway are also represented. Sampled water bodies (n= 130) are depicted as white circles.

5.4.2. Water body surveys

The amphibian surveys were carried out between March and May (period of higher larvae activity in our study area) over 3 years (2016, 2017 and 2019) across a range of selected water bodies (n = 130, **Figure 5.1**), from temporary ponds to medium-sized water reservoirs (mean = 3236 m^2 ± 9093 m^2). Amphibian larvae sampling was done through dip-net sweeping, each one approximately 1 - 3 metres long. For each of the sampled water bodies, we calculated its area on a GIS software (QGIS); the number of sweeps performed was proportional to the calculated area through categorised classes (Table S5.1, supplementary materials). When possible, we performed the sweeps at different depths, to increase the probability of collecting animals from different species. On each sweep, we counted and identified each larvae to the lowest possible taxonomic level. After the counts, we released the larvae into the water.

For this study we selected two species with different ecological requirements (Pinto et al., 2024) that were present in a sufficient number of water bodies to perform our analysis: the Iberian spadefoot toad (*Pelobates cultripes*) and the Iberian ribbed newt (*Pleurodeles waltl*), this one recognised as a more aquatic species than Iberian spadefoot toads (Recuero, 2014; Salvador, 2014).

5.4.3. Roadkill surveys

We performed roadkill surveys on periods of high adult activity (when most adult amphibians moved to breeding sites) along three National Road sections (EN4, EN114 and EN18) and one Municipal Road section (EM529), comprising approximately 120 km of surveyed roads. Sampling followed a standardised protocol (see Santos et al., 2011) and was performed daily. On each survey, an experienced observer drove a car at 20-40 km/h during the first morning hours (to reduce the impact of traffic and carcass removal by scavengers), scouting both sides of the road, including lanes and shoulders. All encountered roadkilled animals were collected and registered. All amphibian carcasses were identified to the lowest possible taxonomic level, their geographical position recorded with a handheld GPS device (Garmin® eTrex®), and removed from the road to avoid double counting during subsequent surveys. The error in the coordinates was always lower than half of the spatial resolution of the environmental variables (30 m; see below). The data was then filtered according to the two target species and used to relate roadkill mortality to high-connectivity sites.

5.4.4. Environmental predictors

To build the ecological niche models (ENMs), we used the presence of each amphibian species' larvae on each water body as a response variable. Due to the low number of sampled water bodies with species' presence for each sampled year, we chose not to spatially thin the data. However, any potential spatial clustering and sampling bias arising from this decision were addressed using a bias file (see below).

To provide our models with the most updated information on environmental changes at a finer resolution, we used land cover and other remote sensing-derived predictors, such as vegetation indices and topographical data, with a spatial resolution of 30 m (see below). For the calculation of the land cover predictors, we considered the two major classes present in our study area: forest and agriculture (**Figure 5.1**). We used CORINE Land Cover 2012 and 2018 to characterise the land use during the study period and we extracted the mean percentage of each land cover to a 30 m pixel resolution raster. We have also computed the mean Enhanced Vegetation Index (EVI; Liu and Huete., 1995), which represents local dynamics in primary production, as this has been previously used to explain amphibian occurrence (Qian et al., 2007; Pinto et al., 2023; 2024). We calculated EVI from LANDSAT 8 image collections (Level 2, Tier 1; data available from the U.S. Geological Survey) with a 30 m spatial resolution for each sampling season of each year (March to May), retaining high-quality images with 0% cloud cover for the whole study area (WRS-2 scene: path 203, row 33). We processed all composite images and calculations in Google Earth Engine (Gorelick et al., 2017). Lastly, we used a DEM (Digital Elevation Model; DGT, 2018) to derive topographical predictors (elevation and slope) as these often influence amphibian occurrence, for example, by the concentration of water in lower topographies (Santos et al, 2007). Because urban areas and roads are not suitable habitats for amphibians (Van Buskirk, 2012), the pixels from these land cover classes were removed from all predictors before modelling. All these operations were performed using QGIS software (v3.24.1; QGIS Development Team, 2022). Table 5.1 summarises all the used predictors.

Table 5.1. List of predictors used for building the habitat suitability models.

Predictor name and code	Description	Source	References
Agricultural areas (agriculture)	Percentage of agricultural areas (%)	CORINE 2012, 2018	EEA
Forest areas (forest)	Percentage of forest areas (%)	CORINE 2012, 2018	EEA
Enhanced Vegetation Index (EVI)	Mean changes in primary production content index. -1 (indicates stressed vegetation) to 1 (indicates healthier vegetation)	USGS	Liu and Huete, 1995
Elevation	Elevation retrieved from digital elevation model (meters)	Direção Geral do Território (DGT)	DGT, 2018
Slope	Elevation changes across the landscape. Derived from digital elevation model (degrees)	Direção Geral do Território (DGT)	DGT, 2018

5.4.5. Ecological niche models

We calculated correlative ENMs to estimate the species' realised ecological niche (sensu Sillero, 2011) following standard procedures (Sillero et al, 2021; Sillero & Barbosa, 2021), because we were interested in identifying the most suitable sites for the species' presence. Mechanistic algorithms are not suitable for this study as they forecast the fundamental niche not the realised one, which is determined by abiotic, biotic, dispersal, and historical factors (Sillero, 2011). The fundamental niche is only determined by abiotic factors (Sillero, 2011), and the corresponding model would likely identify the whole study area as suitable.

Before model construction, we checked the multicollinearity among predictors through the Variance Inflation Factor (VIF) (Zuur et al., 2010; Dormann et al., 2013). All variables had a correlation below the defined threshold (VIF < 5).

We used the presence-background algorithm Maxent (Phillips et al., 2006; Phillips et al., 2017) to construct the ENMs for each year and each species. Maxent is a machine-learning method particularly flexible, with the ability to capture complex, nonlinear relationships between species occurrence and environmental predictors. This method operates with presence data, random background points and a collection of environmental predictors to generate a continuous probability estimate of habitat suitability. Background points are not equivalent to pseudo-absences as they are a sample of the environmental conditions of the whole study area, including locations where the species occurs (Phillips et al., 2009; Guillera-Arroita et al., 2015; Sillero & Barbosa, 2021). We ran Maxent with the default values (background points (n = 10000); convergence threshold (10^{-5}); number of iterations (n = 500); transformation features (linear, quadratic and hinge)), and we selected the Jackknife method to estimate the environmental predictors' contribution (Philips et al., 2017). We randomly split the data into 70% training and 30% testing and ran each model 15 times. As most of our sampled water bodies were somehow close to existing roads, and because our occurrence data was not spatially thinned due to lower sampling size, we created a bias file for each species according to Barber et al. 2022. This involves creating a two-dimensional kernel density layer from the presence data that is masked with a layer from the study area. This aligns environmental biases in both presence and background data, allowing the model to account for sampling bias effectively.

We assessed the models' predictive performance through the area under the receiver operating characteristic curve (AUC; Hanley and McNeil, 1982), and assumed that models with AUC values < 0.7 had poor predictive performance, AUC between 0.7 and 0.9 moderate to good, and AUC > 0.9 excellent performance (Swets, 1988; Araújo and Guisan, 2006). To analyse uncertainty, Maxent produces an averaged model (mean model of all our 15 runs) for each year, which we considered as our final models (one for each species). Additionally, we calculated 15 null models (for each year and each species) to evaluate the explanatory power of our models, following Raes & ter Steege (2007) methodology. This involves building null models with the same number of points as our empirical models (but randomly selected) and comparing both AUC values to assess model significance (done through Wilcoxon rank sum tests). If the AUC values of the empirical models are significantly higher than the AUC values of the null models, these have a better predictive performance.

We calculated maps of the probability of Iberian spadefoot toad and Iberian ribbed newt occurrence for the sampled water bodies and then projected them over our entire study area (for each sampled year individually). These maps, based on the averaged models, show values close to zero to express a lower habitat suitability, and values close to one to specify a higher habitat suitability. We performed all the statistical analyses using the packages '*usdm*' (Naimi et al., 2014) and 'ks' (Duong, 2007) within software R (v 4.1.2; R Core Team, 2021) and used Maxent (v3.4.4; Phillips et al., 2006) to run the ecological niche models.

5.4.6. Landscape connectivity models

We built the landscape connectivity models for the analysed species with the Circuitscape software (Julia implementation v5.0; Anantharaman et al., 2019). This software relies on electrical circuit theory principles to integrate random walk pathways, where current is injected across a resistance layer to provide an accurate assessment of animal movement and gene flow over the landscape (McRae et al., 2008). This approach aids the assessment of functional connectivity among habitat patches: patches are considered connected if the landscape matrix offers lower resistance, or disconnected if resistance is high (Zeller et al., 2012). The output is the probable movement pathways of varying strengths influenced by the resistance layer, enabling the identification of corridors and other landscape elements connecting habitat patches (McRae et al, 2008).

We averaged the ENMs for the three sampled years into a single model for each species (**Figures 5.2a and 5.2c**) and computed its inverse linear function to represent landscape resistance: the resistance is high when the Habitat Suitability Index (HSI) is low and vice versa (**Figures 5.2b and 5.2d**). We reintegrated the urban areas and roads with the resistance maps and coded them as a high resistance layer. We assigned the highest resistance value concerning the maximum resistance value of our maps (i.e., 20 for the Iberian spadefoot toad, and 141 for the Iberian ribbed newt), as roads are not completely impermeable to movement (Holderegger and Di Giulio, 2010). We used the resistance surface maps as input for the landscape connectivity models. This approach is considered effective, particularly when movement data is not available (Keeley et al., 2016; Valerio et al., 2019; Godet and Clauzel, 2021). We defined our focal nodes as the rasterised centroid of each sampled water body over the three years for each species (91 for the Iberian spadefoot toad and 63 for the Iberian ribbed newt) and unsampled water bodies scattered through the study area. We calculated the third quartile of both the area and the HSI of the sampled water bodies (1952 m^2 and 0.748 for the Iberian spadefoot toad and 1625 m^2 and 0.619 for the Iberian ribbed newt, respectively) and used these as thresholds for the selection of unsampled water bodies as focal nodes (n = 50 for the Iberian spadefoot toad and n = 58 for the Iberian ribbed newt). Water bodies with higher values of HSI may be suitable for amphibians, and the maximum area threshold allows us to exclude water bodies that might have inappropriate dimensions (e.g. large dams for water retention) and therefore not used by these species. We used the pairwise calculation mode, as well as eight neighbour raster cell connections, to calculate the current density between all pairs of focal nodes (McRae et al., 2008). This approach follows the assumption that amphibians randomly move across the landscape through any possible pathways between nodes (Bishop-Taylor et al., 2015; Dickson et al., 2019). After these calculations, the current density was summed into a single cumulative map representing the most permeable pathways for each species' dispersal (McRae et al., 2008).

5.4.7. Landscape connectivity as roadkill probability location predictor

To analyse whether locations with higher connectivity could be associated with roadkill, we compared the mean values of connectivity with roadkill recorded in Autumn (between mid-September and mid-December) for each species throughout the entire study period. We selected this season because it corresponds to the period with the highest amphibian activity in our region when adults are moving towards breeding sites. For this, we divided the roads into 500 m contiguous segments and created a 500 m buffer around each segment. Then, we extracted the presence and absence of each species' roadkill in each road segment, as well as the mean connectivity value of each road segment. Lastly, we performed a regression analysis (Generalised Linear Mixed Model (GLMM) with binomial distribution; Bolker et al., 2009) for each species between the presence and absence of roadkill, and the mean connectivity values (with “Road” as a random factor) within R software (using the packages “glmmTMB” (Brooks et al., 2017) and “DHARMa” (Hartig, 2022), for model construction and validation, respectively).

5.5. Results

During the three-year period, we recorded 2503 Iberian spadefoot toad larvae and 272 Iberian ribbed newt larvae from the water body surveys, and 315 Iberian spadefoot toad and 463 Iberian ribbed newt carcasses from the roadkill surveys.

5.5.1. *Iberian spadefoot toad*

The Maxent models showed moderate-to-good levels of predictive performance, suggesting that the selected predictors effectively characterised the potentially suitable habitats (Table 5.2). The AUC values from the empirical models were significantly higher than the AUC values from the null models (Wilcoxon rank sum test; 2016: $W = 198$, $p < 0.001$; 2017: $W = 223$,$p < 0.001$; 2019: $W = 225$, $p < 0.001$). **Figure 5.2a** shows the spatial output of the (mean) Maxent model for the three years (detailed results of each model can be seen in Figure S5.1, supplementary materials).

We found that mean EVI was the most important predictor for the years 2016 and 2019, while it was the second most important predictor for 2017. The Iberian spadefoot toad’s response was similar across all analysed years: lower to intermediate values of this index were associated with higher probabilities of occurrence. The percentage of forest was the most important predictor in 2017 with a positive response: the probability of occurrence of this species was higher at sites with an increased percentage of forest. The percentage of agricultural areas was the second most important predictor for 2016 and 2019, with a generally flat response across all ranges.

The connectivity model predicted stronger movement corridors between networks of water bodies mostly associated with open forests. This is mostly seen in the North and Northeast part of our study area, where the landscape is dominated by open *montado* forests with varying tree density and traditional agriculture open fields. Isolated water bodies showed less probability of including movement corridors when the surrounding landscape was composed of dense forests in higher altitude areas (**Figure 5.3**). Similarly, sites with low tree abundance and highly agricultural landscapes in the Southeast showed a lower probability of movement.

The generalised linear mixed model analysis revealed a positive correlation between road segments with higher connectivity and roadkill presence of this species. Road segments with roadkill were associated with higher connectivity values, whereas road segments with no roadkill mostly presented lower connectivity values (Z = 0.177, p = 0.02; **Figure 5.4**). Table 5.3 shows this analysis results.

Table 5.2. AUC results for the empirical and null habitat suitability models for the analysed years (2016, 2017 and 2019) for the Iberian spadefoot toad and the Iberian ribbed newt.

Iberian spadefoot toad (*Pelobates cultripes*)		
	Empirical model	**Null model**
Year	AUC	AUC
2016	0.818 (±0.03)	0.759 (±0.04)
2017	0.817 (±0.03)	0.709 (±0.04)
2019	0.822 (±0.03)	0.693 (±0.03)
Iberian ribbed newt (*Pleurodeles waltl*)		
	Empirical model	**Null model**
Year	AUC	AUC
2016	0.897 (±0.03)	0.819 (±0.04)
2017	0.879 (±0.02)	0.742 (±0.04)
2019	0.844 (±0.03)	0.752 (±0.06)

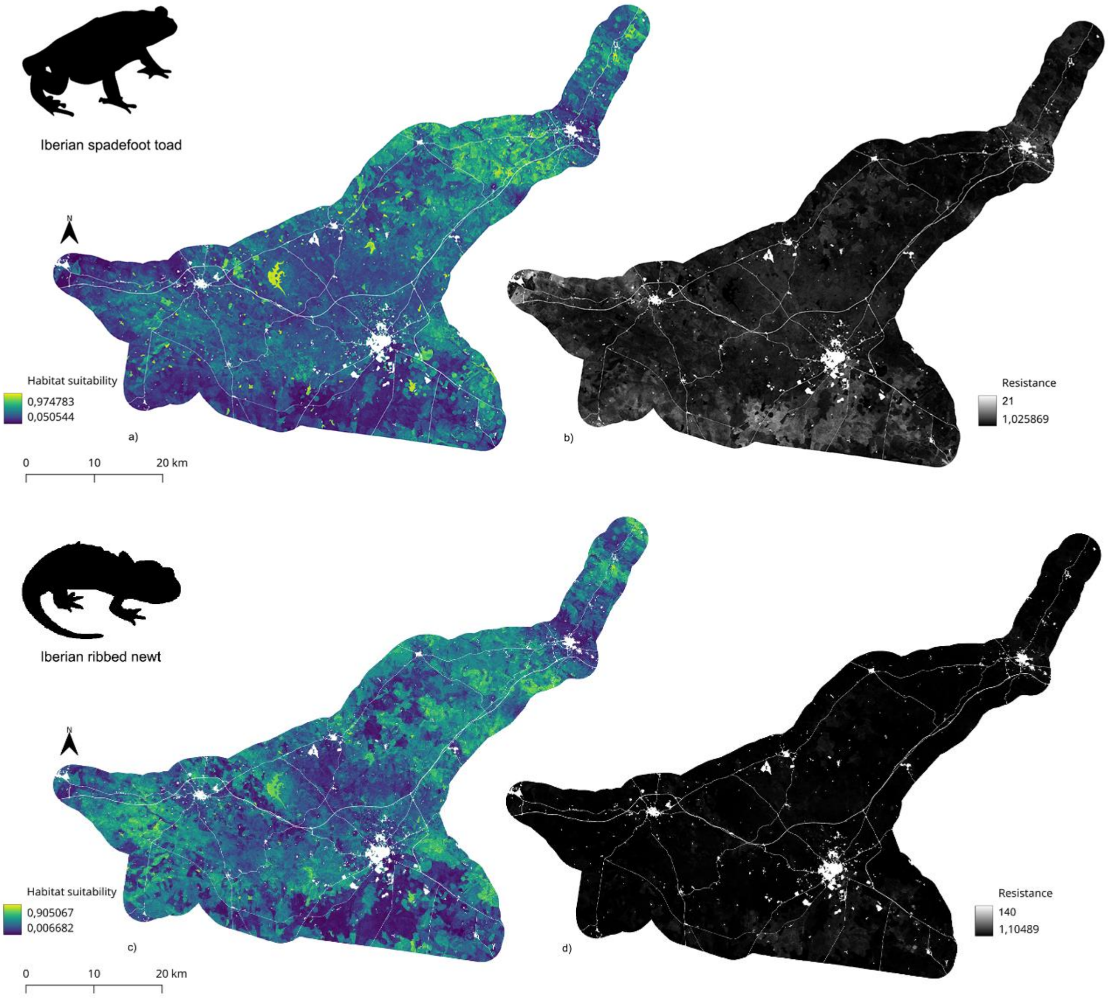


Figure 5.2. Habitat suitability (a and c) averaged map for the three analysed years with lighter-coloured areas representing higher habitat suitability (urban areas and roads were not included in this analysis). Inverted habitat suitability map, representing landscape resistance (b and d) with urban areas and roads as higher resistance surfaces represented in white. Top maps concern the Iberian spadefoot toad, while the bottom ones are for the Iberian ribbed newt.

5.5.2. *Iberian ribbed newt*

Our Maxent models showed moderate-to-good levels of predictive performance for this species as well (Table 5.2). The AUC values from the empirical models were significantly higher than the null models (Wilcoxon rank sum test; 2016: W = 208, $p < 0.001$; 2017: W = 225,$p < 0.001$; 2019: W = 207, $p < 0.001$). **Figure 5.2c** shows the spatial output of the (mean) Maxent model for the three years (detailed results of each model can be seen in Figure S5.2, supplementary materials).

Our models revealed that the response of the Iberian ribbed newt was very similar to that of the Iberian spadefoot toad across the analysed years. Mean EVI was the most important predictor for the years of 2016 and 2019 with a higher probability of occurrence of the Iberian ribbed newt at sites containing lower to intermediate values of this index. The percentage of forest was the most important predictor for 2017 with a positive response: sites with higher percentages of forest showed a higher probability of occurrence. The percentage of agriculture was the second most important predictor for all analysed years but with opposite responses. In 2016 and 2017 the probability of occurrence of the Iberian ribbed newt was higher at sites with a higher percentage of agriculture, while in 2019 we detected an inverse response.

The connectivity model identified areas of increased movement probability for the Iberian ribbed newt mostly in the North and Northeast part of our study area, similar to the Iberian spadefoot toad. Our model also highlights some more evident corridors in the Southern part of our study area. All these sites are characterised by open *montado* forests, disrupted by open fields used for traditional agriculture practices and cattle grazing (**Figure 5.3**) supplied with a good network of short-distanced water bodies.

The regression analysis also revealed a significant relationship between road segments with roadkill and higher connectivity values for this species ($Z = 0.269$, $p = 0.004$; **Figure 5.4**). Table 5.3 presents this analysis results.

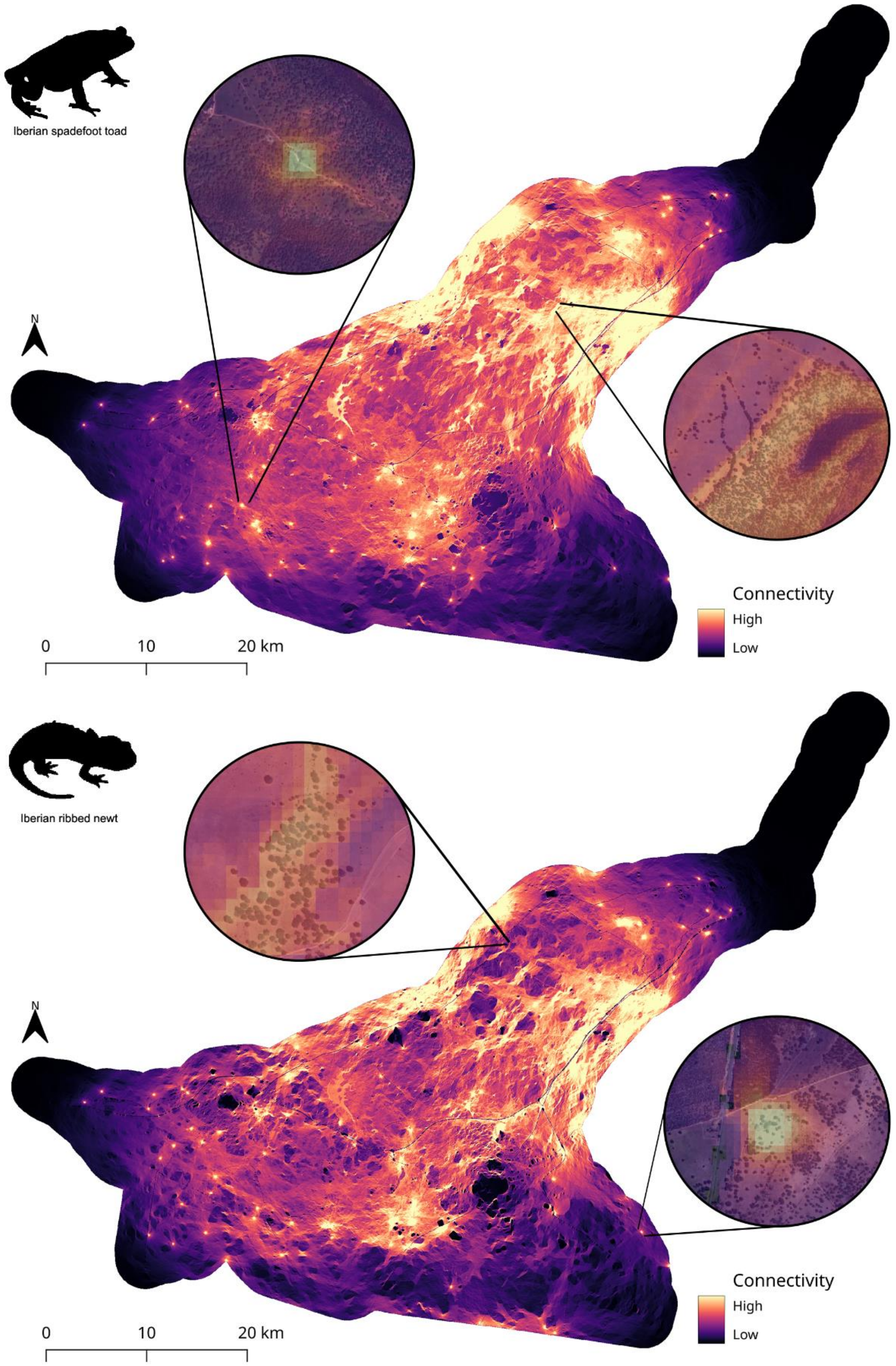


Figure 5.3 – Connectivity maps for the Iberian spadefoot toad (top) and the Iberian ribbed new (bottom). Vivid colours represent sites with higher connectivity and probability of movement. Sites with a higher probability of movement are mainly located in the North/Northeast open *montado* forests. Darker lines crossing the study area correspond to roads. Isolated water bodies are depicted as small lighter dots

closer to the study area borders and in areas with less suitable habitats. In the detailed areas, it is possible to see the difference in the connectivity between transition sites, as well as isolated water bodies with no connectivity corridors, despite the apparent forested (Iberian spadefoot toad) or suitable (Iberian ribbed newt) surrounding habitats.

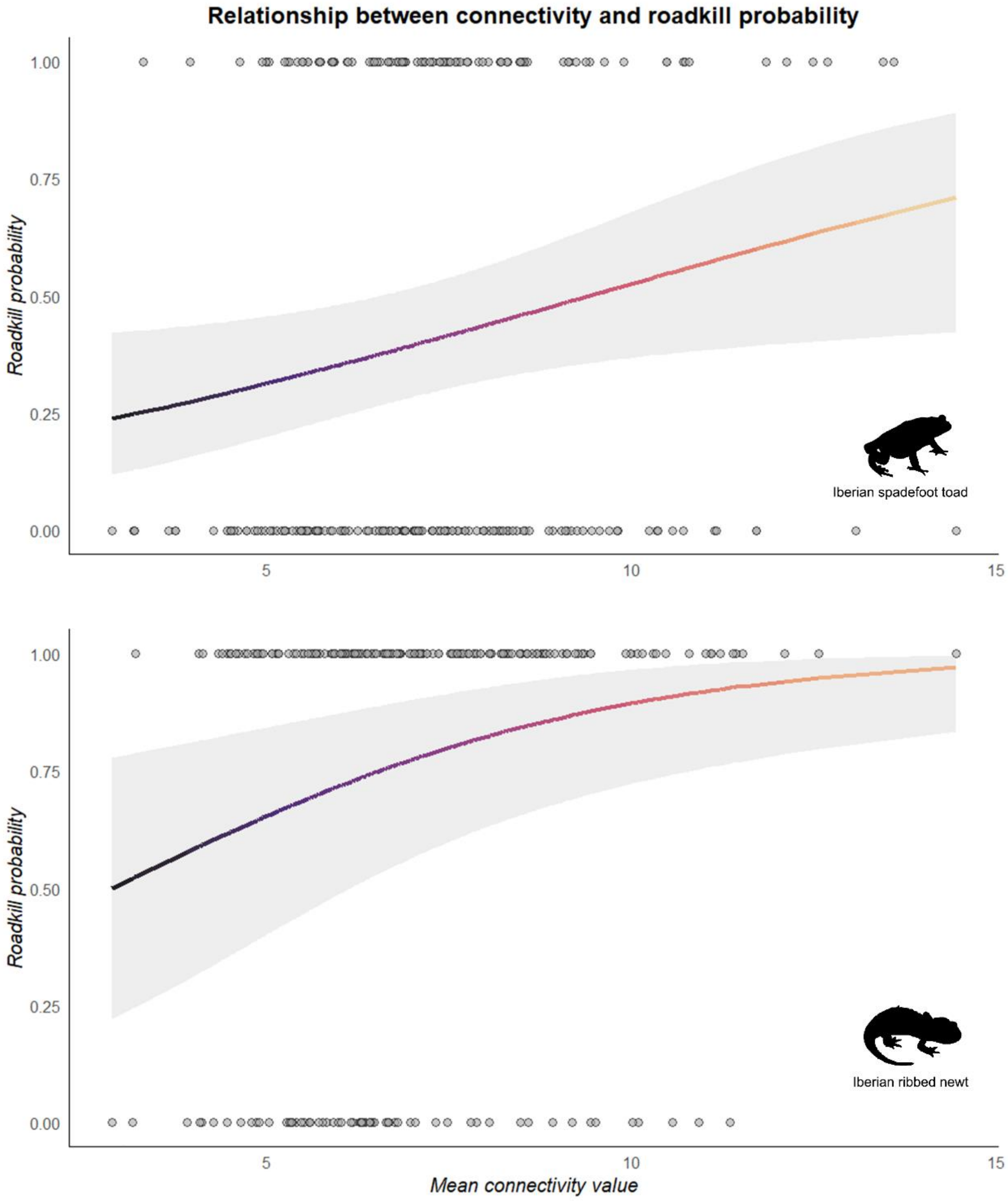


Figure 5.4 –Roadkill probability as function of connectivity for the Iberian spadefoot toad (top) and the Iberian ribbed newt (bottom), according to the results of the regression models. Both species present a positive correlation: road segments with higher connectivity values exhibit higher probabilities of roadkill. Points represent the number of road segments with presences and absences of roadkill.

Table 5.3. Regression models between roadkill and mean landscape connectivity for the Iberian spadefoot toad and Iberian ribbed newt with respective coefficients, AIC and R^2.

	Estimate	Std. Error	z value	p-value	Confidence interval (95%)
Iberian spadefoot toad (*P. cultripes*)					
Mean connectivity	0.177	0.078	2.263	0.023	0.02; 0.33
Model AIC			316		
R^2			0.38		
Iberian ribbed newt (*P. waltl*)					
Mean connectivity	0.269	0.09	2.857	0.004	0.08; 0.45
Model AIC			263		
R^2			0.29		

5.6. Discussion

Our study assessed habitat suitability and landscape connectivity to identify potential movement corridors for two amphibian species with different ecological requirements in a road-dominated Mediterranean landscape. Both the habitat suitability and the connectivity models present convergences, particularly on the response to the different predictors, as well as the location of the most important movement corridors. Our analysis also revealed that landscape connectivity can be used as a tool to identify sites with increased roadkill probability risk, enabling the application of this modelling technique to draw effective conservation measures.

5.6.1. Ecological niche models

The results from the ENMs revealed that both species' distributions were mainly affected by vegetation and land-use predictors. A lower percentage of primary production (EVI) resulted in higher probability of occurrence for both the Iberian spadefoot toad and the Iberian ribbed newt (with EVI as the most important predictor for 2016 and 2019). Although we would expect an opposite response, the observed trend may be related to the species' habitat exploitation associated with environmental changes and variations in rainfall across the different years. According to the IPMA (2023), the year 2016 was a normal rainy year and 2019 a moderate drought year, while 2017 was characterised as a severe drought year. Many amphibians' terrestrial movements are known to be in the vicinity of water bodies (where the humidity is higher), and the change in suitability highlighted by our models between 2016 (normal year) and 2017 (severe drought year) may have reflected the need for cooler, shady areas where both the landscape and the water bodies can retain water for longer periods, explaining the increase in habitat suitability at sites with higher percentages of forest in 2017. This potential habitat use shift associated with water availability may lead to a cessation of reproduction (if no suitable reproduction site is found), further jeopardising populations' viability. Pinto et al. (2023) hypothesised that the higher numbers of amphibian roadkill found at sites with lower water availability could be related to a possible increase in longer distance movements to find alternative reproduction sites. The change in habitat suitability between different years denoted by our models could potentially be indicative of a similar response. The higher probability of occurrence at lower to intermediate EVI levels may also be related to the fact that the ENMs modelled the environmental conditions surrounding the water bodies and were then projected over the entire study area. These surrounding water body areas are prone to less vegetation, as many of these water sources are located in agricultural areas, which could have influenced our results.

Although both species are considered common in our study area with an overlap in their habitat exploitation, their ecological requirements are different (Recuero, 2014; Salvador, 2014). For instance, Iberian ribbed newts present a more aquatic behaviour in their adult stages than Iberian spadefoot toads. This aquatic behaviour may, somehow, be linked to the increase in habitat suitability in agricultural sites during 2017, as most water bodies of our study area are located in more open habitats. Nevertheless, as our study area is mainly comprised of a mixture of forest and open agricultural habitats, these results should be interpreted from a holistic perspective; that is: higher habitat suitability at sites with increased percentages of forest and agriculture may be translated into heterogeneous *montado* habitat. This ecosystem is intrinsically linked to low-intensity cattle grazing and crop production (Pinto-Correia and Mascarenhas, 1999). As such, it is also where most of the water bodies present in the study area are located, which can justify the lower EVI values as well (and the consequent higher probability of occurrence at lower to intermediate levels of this index). The year 2019 was considered a moderate drought year, and the fact that the previous year had normal rain levels (IPMA, 2023), revealed a different response from the analysed species. While both species responded equally to EVI in 2019, the Iberian spadefoot toad occurrence remained practically unchanged throughout different agricultural densities (as in 2016), which makes this response particularly difficult to interpret. Yet, for the same year, the Iberian ribbed newt was mainly present in sites with lower percentages of agriculture. In the same study area, Pinto et al. (2023) detected a decrease in Iberian ribbed newt roadkill in road segments surrounded by open agricultural areas. The authors hypothesise that the lower roadkill rates are linked to the species' lower abundance at those sites. Our ecological niche models also suggest that the probability of occurrence at open agricultural land is lower than in more heterogeneous habitats. However, it is worth noting that the terrestrial movements of the Iberian ribbed newt are practically unknown (Salvador, 2014) which limits our conclusions about this species.

Nevertheless, our results show that water availability on the landscape drives these species' spatial distribution. In fact, Knutson et al. (2004) found that agricultural water bodies can sustain several amphibian species, especially if other wetlands are scarce. In our study site, most of the available water bodies are linked to agricultural practices developed in the area (e.g., livestock watering and irrigation) and represent the primary source of reproduction for the occurring amphibians. Our models emphasise the importance of man-made agricultural water bodies for amphibian populations in Mediterranean habitats as our study area.

5.6.2. Landscape connectivity models

Our connectivity models show the existence of distinct movement corridors for both species, yet some sites present higher movement probability than others. The existence of shared extended movement corridors is particularly evident in the North and Northeast parts of our study area, where two major areas highlight distinct corridors. These largely correspond to heterogeneous *montado* extents (habitats where moderate-density oak forests are mixed with low-intensity agricultural areas). The particular way this distinctive landscape is managed (e.g. scarcely managed forest, with less intensive grazing and agriculture (Díaz, 2009)) allows for the existence of food and shelter, as well as potential stable levels of humidity that may support a greater number of amphibians.

Equally important for the presence of both species, seems to be the proximity of other water bodies, particularly in highly suitable areas. The majority of the higher current density corridors seem to occur in well-established water bodies' network areas on the landscape. Other studies have also reported that higher local water body densities with diversified habitats typically harbour larger amphibian populations (Semlitsch, 2000; Trenham et al., 2003). This is especially relevant since amphibian populations usually experience high turnover (Trenham et al., 2003). Since hydroperiod can be different, even between water bodies located at shorter distances, a well-diversified network of reproduction sites may contribute to population persistence. Ribeiro et al., (2011) reported that the structural connectivity of water body networks is of extreme importance for Mediterranean amphibians. The authors found that the number of species was related to the spatial position of a given water body concerning others nearby in the network, with central ones having higher connectivity. Joly et al. (2001) also highlighted the importance of water body networks on newt occupancy in agricultural areas in France. Yet, it is worth noting that the presence of high connectivity corridors in apparently good networks of water bodies does not guarantee amphibian occurrence. Although the water bodies' surrounding habitat may play a primary role in amphibians reaching those sites, other factors surely influence their occupancy, such as water conductivity and temperature, hydroperiod, and presence of vegetation and predators, among others (Knutson et al., 2004; Denoël & Lehmann, 2006; Fortuna et al., 2006).

Conversely, isolated water bodies seem to be more difficult to access (absence of movement corridors), regardless of the surrounding landscape they are in. Fortuna et al. (2006) stated that water bodies may act as stepping stones in harsher landscapes, providing links for amphibian movement across inhospitable landscapes. This may be visible, at least for smaller distances; however, when water bodies are too distant from others, they are clearly depicted as isolated in our models. Although this is visible in areas with medium connectivity values, it is particularly evident in less connected areas with low water body densities and within less suitable landscapes. This may be especially alarming if the distance to reach other water bodies exceeds the dispersal capacity of the species inhabiting those isolated water bodies. In the medium/long term, this could result in local extinctions for those amphibian populations.

The lack of a solid network of water bodies in densely forested areas (for example, as in the South/Southwest part of our study area) may be the primary reason why the probability of movement (absence of high connectivity corridors) is lower in sites that display less landscape resistance. As stated above, most of the existing water bodies are man-made artificial structures for cattle watering and crop irrigation and densely forested areas are usually less associated with these practices, which could explain the lower densities of these structures at those sites.

5.6.3. Landscape connectivity as roadkill probability location predictor

Our models effectively found a significant positive relationship between road segments with higher landscape connectivity and both species' roadkill, confirming that roadkill occurs predominantly in road segments that have higher movement probability. This relationship is less evident in the Iberian spadefoot toad, perhaps due to its more generalist requirements. Koen et al. (2014) also reported that amphibians are more likely to cross roads in areas of higher connectivity. By applying a high resistance value to roads (and urban areas), our connectivity model allowed us to locate sites where the current is stronger on both sides of a road, which can be interpreted as high probability crossing sites and where roadkill mitigation measures might be more effective. We are, however, aware that connectivity might not be an isolated component in shaping the location of roadkill hotspots (Santos et al., 2013). In fact, sites with increased road mortality are not static over space and time (Medinas et al., 2021), and are known to be influenced by traffic density (Fahrig et al., 1995; Zimmermann Teixeira et al., 2017), road features (Clevenger et al., 2003; Medinas et al., 2013), and weather conditions (Carvalho et al., 2017). Yet, Pinto et al. (2024) highlighted that road segments capable of maintaining stable levels of humidity displayed more consistent amphibian roadkill patterns across various years. This apparent resilience of high mortality road segments may constitute optimal sites for the implementation of mitigation measures (such as underneath road tunnels and drift fences) to maintain or increase landscape connectivity, while reducing the risk of road mortality.

5.7. Conclusions and study limitations

This study provides significant insights into two amphibian species' movements through habitats with varying complexity, on a road-dominated Mediterranean landscape. Our results provide implications for the implementation of roadkill mitigation measures. The use of ENMs and landscape connectivity tools proved to be effective in identifying the most probable sites where these two species may be at higher roadkill probability risk.

It is worth noting that the non-integration of dispersal limits in our landscape connectivity models (due to lack of information about our species' dispersal distances), as well as the exclusion of potentially confounding factors (e.g., traffic volume, local habitat quality) in our regression analysis (also due to data unavailability) may have influenced our results. For instance, this could explain moderate roadkill probabilities that were associated with lower connectivity road segments. While these limitations should be considered when interpreting the results, our approach demonstrates the direct applicability of these modelling tools as viable alternatives to extensive roadkill and population surveys, providing similar precision at considerably reduced costs when compared to more conventional approaches. With the identification of the corridors with the highest likelihood of movement, road agencies and practitioners have guidelines to implement amphibian-oriented roadkill mitigation measures that have already demonstrated effectiveness in reducing amphibian roadkill probability. Therefore, it is possible to provide a connection between suitable habitat patches even in the presence of highly resistant structures such as roads. Nevertheless, the maintenance of heterogeneous landscapes (with equal proportions of both forested and agricultural areas, as well as sufficient shelter), supplied with a good network of water bodies is essential to provide continuous movement corridors for amphibian populations, assuring their persistence.

5.8. Acknowledgements

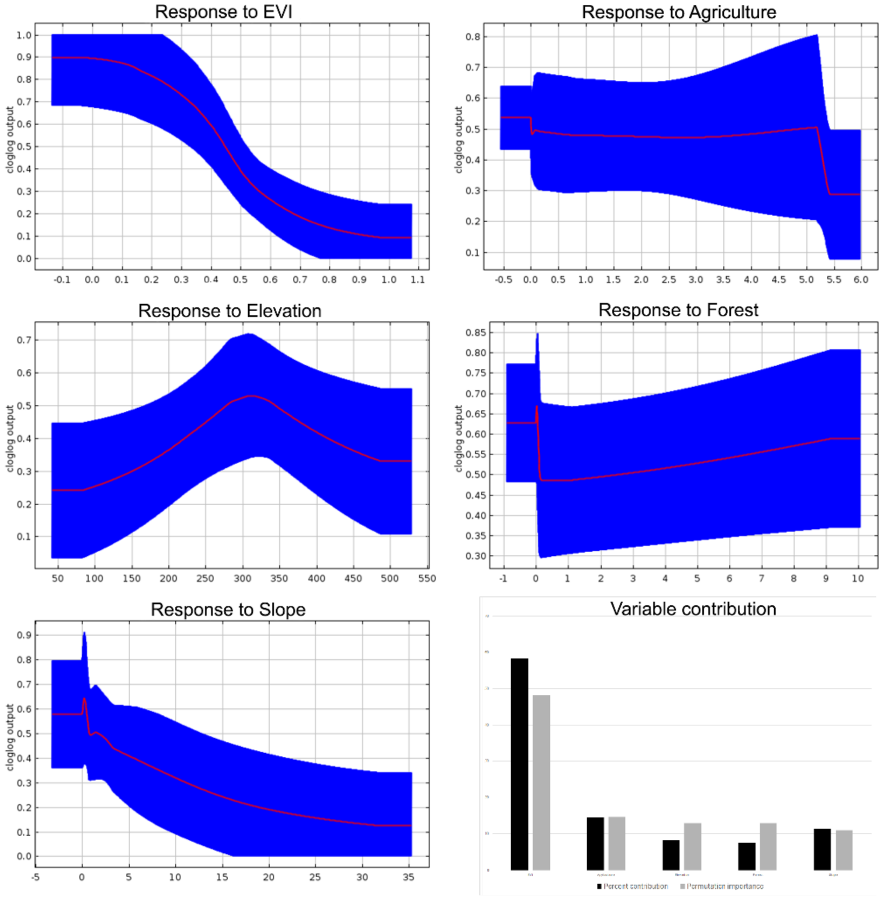
We thank all the colleagues for collecting roadkill data, namely: Luís Sousa, Nelson Fernandes, Sílvia Barreiro, Eduardo Ferreira, and André Oliveira. We also thank to Luís Sousa for the further assistance in the water body surveys. TP is financed by Fundação para a Ciência e Tecnologia (FCT) with a doctoral Grant 2020.04581.BD (doi:10.54499/2020.04581.BD). NS and SMS are supported by a CEEC2017 (CEECIND/02213/2017) and CEEC2023 (CEECIND/07242/2023) contracts from FCT. We also thank MED (doi:10.54499/UIDB/0 5183/2020) and CHANGE (doi:10.54499/LA/P/0121/2020). This research was supported by project LIFE LINES - LIFE14 NAT/PT/001081 financed by the European Commission and project POPCONNECT PTDC/AAG-MAA/0372/2014, co-financed by Programa Operacional Competitividade e Internacionalização (POCI) and supported by FEDER (POCI-01-0145-FEDER-016811).

Appendix S5 – Supplementary materials

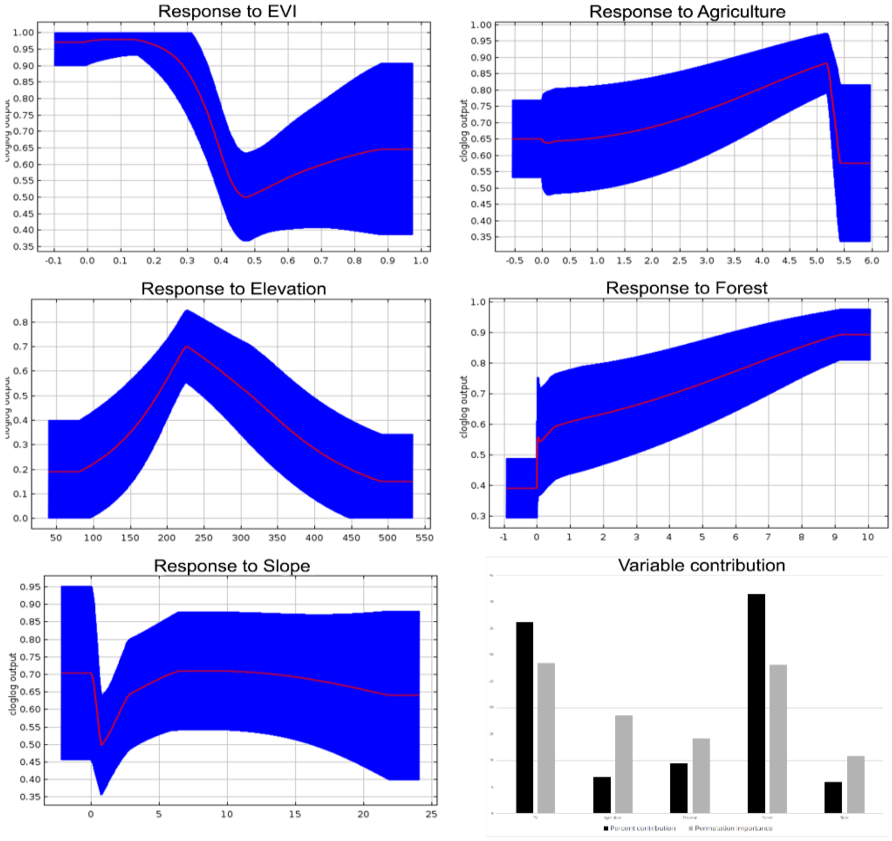
Table S5.1. Number of dip-net sweeps per area and corresponding number of sampled water bodies in each category.

Area (m^2)	Number of dip-net sweeps	Number of sampled water bodies
< 200	1	28
≥ 200 < 500	2	26
≥ 500 < 1000	3	25
≥ 1000 < 1500	4	11
≥ 1500 < 2000	5	15
≥ 2000 < 3000	6	5
≥ 3000 < 4000	7	3
≥ 4000 < 5000	8	3
≥ 5000 < 6000	9	3
≥ 6000	10	11

2016



2017



2019

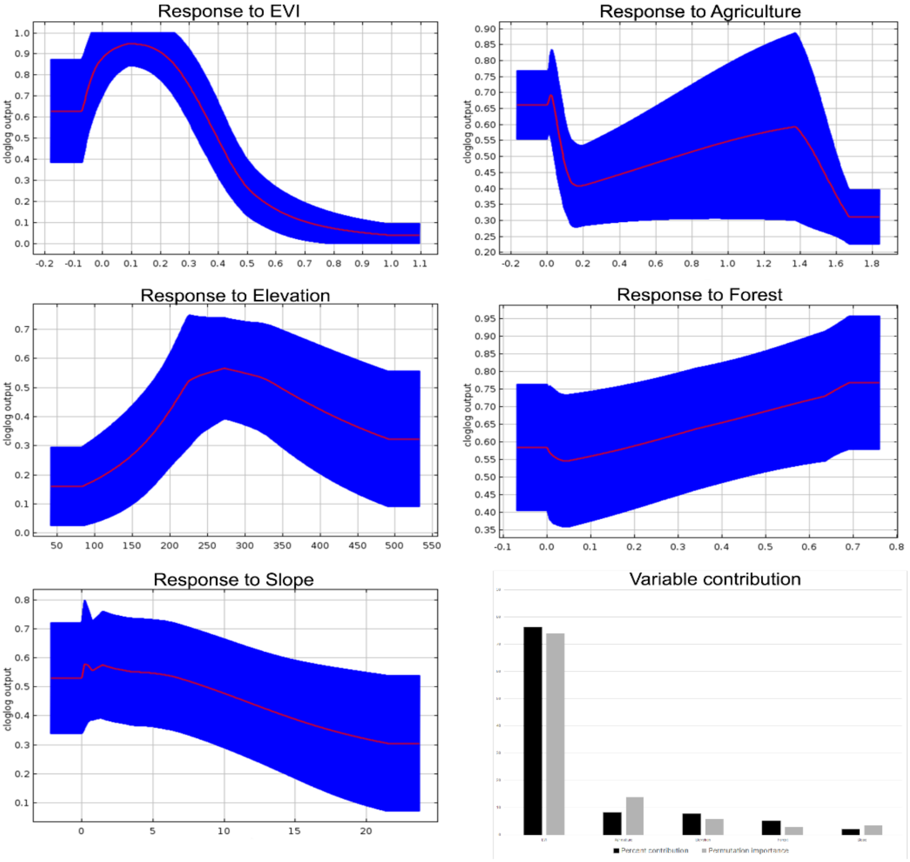
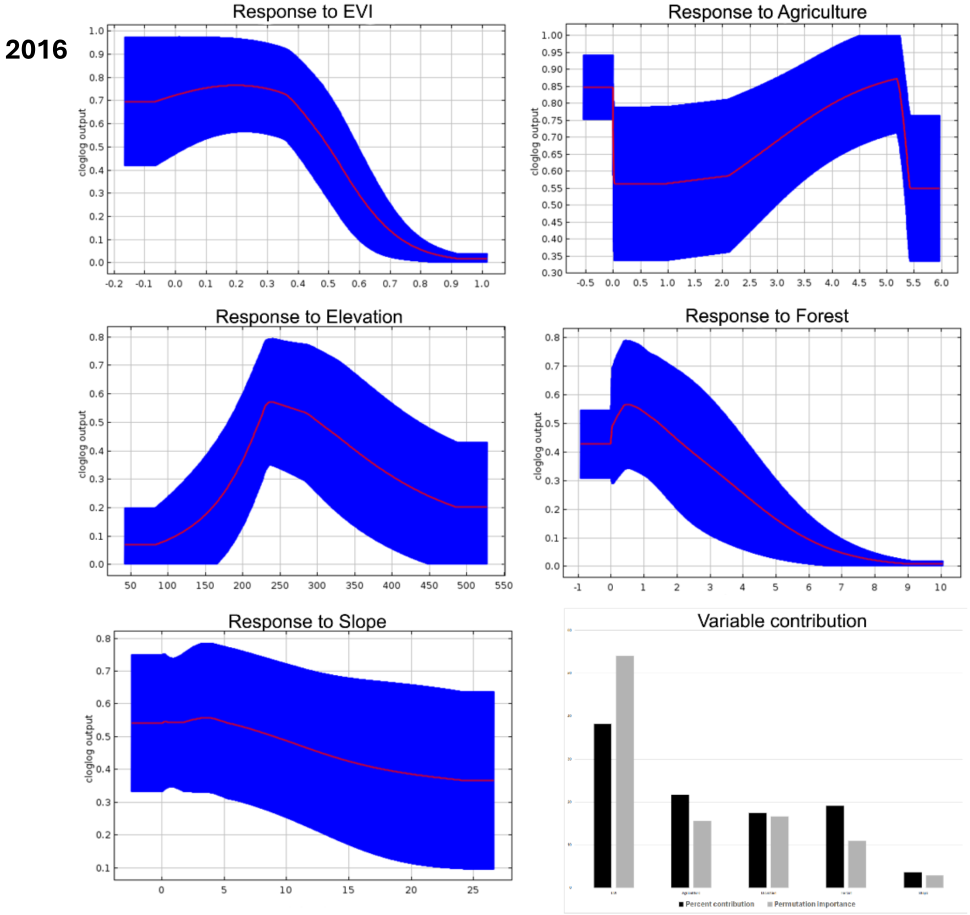
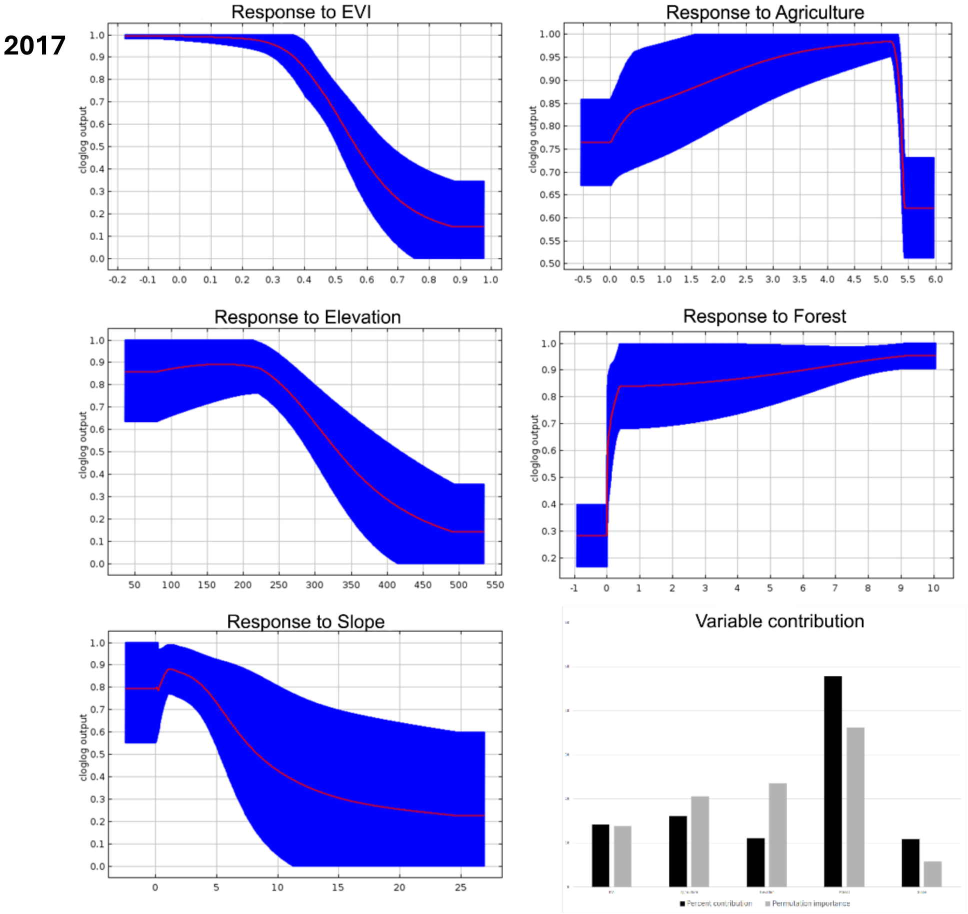


Fig. S5.1. Iberian spadefoot toad habitat suitability models' predictor response, to the three analysed years (2016, 2017 and 2019) and respective predictor contribution.


2016
Response to EVI
cloglog output
Response to Agriculture
cloglog output
Response to Elevation
cloglog output
Response to Forest
cloglog output
Response to Slope
cloglog output
Variable contribution
Percent contribution
Permutation importance


2017
Response to EVI
cloglog output
Response to Agriculture
cloglog output
Response to Elevation
cloglog output
Response to Forest
cloglog output
Response to Slope
cloglog output
Variable contribution
Percent contribution
Permutation importance

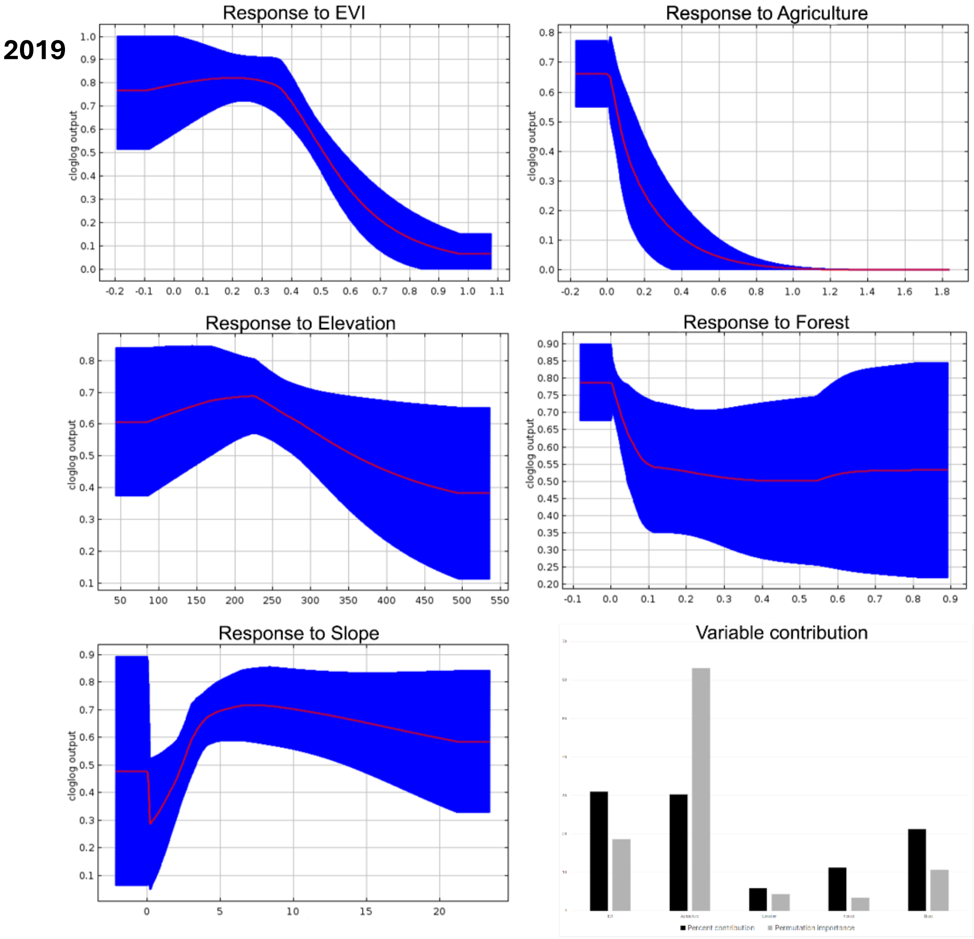


Fig. S5.2. Iberian ribbed newt habitat suitability models’ predictor response, to the three analysed years (2016, 2017 and 2019) and respective predictor contribution.

Chapter 6

General conclusions

Salamandra salamandra

6.1. Synthesis of the main findings

Linear infrastructures are undoubtedly one of the main driving factors behind modern human economy and development. However, the extensive road network poses significant challenges to biodiversity, with extinction rates expected to increase in the near future, which might lead to an environmental collapse (Laurance et al., 2014). To overcome this, careful planning and strategic mitigation actions are imperative, especially with constrained financial resources. However, mitigation must be effective and based on scientifically sound information. Understanding the complex interactions between roads and amphibian communities is pivotal in supporting these goals.

While amphibians are the vertebrate group most negatively impacted by roads (Beebee, 2013; D'Amico et al., 2015), it paradoxically remains one of the most ignored groups, with many ecological questions still needing to be answered. In this thesis, we answered some of those questions, increasing the knowledge on the interactions between roads and amphibians, with special emphasis on the western Mediterranean Basin. The main goal of this work was to understand the influence of landscape and its connectivity on the spatiotemporal patterns of amphibian roadkill, using remote sensing satellite data. To address this, we assessed the spatial drivers (Chapter 2) and the temporal trends (Chapter 3) of amphibian roadkill. We also tested the effectiveness of roadkill mitigation measures specifically developed for this taxonomic group (Chapter 4) and we determined if landscape connectivity could be used as an amphibian roadkill predictor (Chapter 5).

In Chapter 2, most species presented an increase in roadkill numbers in sites with low water availability in water bodies and within reduced distances from water bodies to roads. These findings support our hypothesis that water bodies closer to roads present higher levels of roadkill but contradict the assumption that low water availability in the landscape corresponds to lower probabilities of roadkill. We also detected increased roadkill probabilities in road segments with stable levels of vegetation humidity.

In Chapter 3, our results showed a clear general decrease in amphibian roadkill through time. The Iberian painted frog (*Discoglossus galganoi*) experienced roadkill declines over time of approximately 70%, while the spiny common toad (*Bufo bufo*) and the fire salamander (*Salamandra salamandra*) had a loss of nearly 50%. The roadkill reduction for the Southern marbled newt (*Triturus pygmaeus*) was 40%. Regardless of the decreasing roadkill trend, spatial patterns showed stability from year to year. These results contradict our hypothesis that roadkill patterns fluctuate over space and time according to changes in climatic factors and landscape features.

In Chapter 4, we demonstrated that the permanent drift fences significantly decreased the number of amphibians reaching the roads, which supports our hypothesis. The estimated mean number of amphibians per visit and site on the fenced sites declined from nearly 1 before to 0.42 after fence installation, representing a reduction of approximately 60%. However, our results were inconclusive regarding our hypothesis that the presence of amphibians on roads does not increase in road segments adjacent to the drift fences.

The models presented in Chapter 5 assigned corridors with higher amphibian movement probability to heterogeneous habitats composed of moderate-density *montado* forests combined with low-management agricultural areas and networks of high-density water bodies. We found a positive correlation between high-connectivity road segments and roadkill for the Iberian spadefoot toad (*Pelobates cultripes*) and the Iberian ribbed newt (*Pleurodeles waltl*), supporting our hypothesis that landscape connectivity can be a valuable tool to predict road segments with a higher roadkill risk.

6.2. The importance of water availability for Mediterranean amphibians

The Mediterranean Basin is one of the world's most vulnerable regions to climate change effects (Tuel and Eltahir, 2020), and the Iberian Peninsula has been particularly affected by temperature increase and water scarcity in the last decades (Pereira et al., 2021). In our study region, water mostly concentrates in the form of man-made water bodies used for livestock watering and crop irrigation. Since most of amphibians' natural reproduction sites (temporary ponds) were lost in the last decades (Ferreira & Beja, 2013), the existence of man-made water bodies is particularly relevant as an alternative for amphibian survival (Knutson et al., 2004). We found that networks of close-distanced water bodies located in suitable habitats corresponded to areas of increased movement probability (Chapter 5). Additionally, we also demonstrated that the disruption of these water bodies (due to water scarcity) will likely increase movement in search of alternative sites for reproduction, consequently increasing roadkill risk (Chapter 2). Hendrix and colleagues (2017) revealed that fire salamanders reproducing in permanent water bodies showed less vagility than their conspecifics breeding in temporary ponds. Because water is not a limiting factor for those individuals reproducing in permanent water bodies, the salamanders showed less necessity for large dispersal distances. The high roadkill risk for species with longer dispersal distances, coupled with the probable cessation of reproduction from less mobile ones (in a context of lack of suitable reproduction sites), will likely contribute to the decline of populations.

Considering the predicted reduction in rainfall (Pereira et al., 2021), amphibian populations inhabiting our study area may face new challenges in finding suitable reproduction sites in the near future. To mitigate this, it may be worthwhile to consider complementary measures that could reduce dispersal distances. For instance, constructing new ponds and water bodies, preferably away from roads – as our findings also indicate higher mortality rates when water bodies are closer to these linear infrastructures (Chapter 2) – could provide safer alternatives. Lesbarrères and colleagues (2010) documented the colonization process of an amphibian community across several newly constructed water bodies created to replace other ponds lost in the construction of a highway in western France. The authors revealed that several factors including water body size and respective surrounding landscape influenced species richness and diversity. Although approximately 30% of the species were unable to reproduce in the new water sources, the results suggest that replacement water bodies can provide suitable alternative habitats for most of the analysed species. Despite the different environmental conditions of the referred study, the constructed water bodies showed promising results, which could be beneficial to implement in our study area. For example, constructing additional water bodies in areas of high amphibian habitat suitability but with poorer or disrupted water body networks could reduce the extinction risk for amphibian local communities. At a local scale, the new water bodies would provide alternative reproduction sites, potentially decreasing dispersal distances and thus reducing the roadkill risk. At a broader scale (landscape), these newly constructed water bodies could serve as stepping stones (Fortuna et al., 2006), linking existing networks and enhancing connectivity across habitats (Chapter 5). Indeed, adequate water body management would be necessary, as factors such as hydroperiod and the presence of vegetation and predators significantly influence the maintenance of amphibian population dynamics (Knutson et al., 2004; Fortuna et al., 2006). Nevertheless, the availability of heterogeneous complementary water body networks could potentially enable individual exchange among amphibian populations, increasing gene flow and supporting long-term population viability (Chapter 5).

Since amphibians rely on both aquatic and terrestrial habitats in the course of their life cycles, the structure of the landscape between water bodies is equally essential for amphibian persistence and is dependent on water as well. We detected that the occurrence probabilities of the Iberian spadefoot toad and the Iberian ribbed newt shifted in years with different drought severities (predominantly to forested sites during harsher conditions; Chapter 5). The ability of these species to adapt to existing habitats accounting for the availability of water on the landscape represents an evolutionary trait that probably assists them in persisting in a water-scarce region as our study area. Similarly, road segments with surrounding vegetation less likely to vary in humidity content presented consistent amphibian roadkill numbers across different years (Chapter 3), suggesting the presence of constant movement corridors.

Detailed knowledge of life-history traits and roadkill patterns for many Mediterranean amphibian species still needs to be assessed. A combined approach linking species' ecological needs (such as water dependency) and landscape features accounting for the negative impacts of roads over time may be the key to better understanding how species respond to road stressors and where to act. This information can then be translated into targeted measures to reduce amphibian roadkill risk, while increasing overall landscape connectivity. Providing adequate conditions that support amphibian movement between reproduction sites, while effectively mitigating road impacts in critical locations (high movement corridors that are intersected by roads) is of utmost importance for reducing amphibian roadkill, increasing landscape permeability and population viability.

6.3. The *montado* heterogeneity as a promoter of amphibian movement

Focusing solely on roadkill – and only on the road surface – might not entirely answer the problem of the negative effects of roads, since many of these impacts extend beyond the physical structure and penetrate the surrounding habitats (Forman and Alexander, 1998). Knowledge of amphibian movement through the landscape is needed to increase overall landscape connectivity and gene flow of populations. Part of our results demonstrate the use of roadkill data to predict what landscape features may contribute to increased roadkill aggregation sites. Although we demonstrated that certain roadkill drivers (land-cover) are species-specific, our results suggest that amphibians (mostly urodeles) do not cross roads on random sites, and that roadkill risk increases in road segments with vegetation less likely to vary in humidity content. Oppositely, road segments crossing mainly agricultural areas presented lower roadkill (Chapter 2).

When analysing habitat suitability for the Iberian spadefoot toad and the Iberian ribbed newt, we detected that species' occurrence was tightly connected to sites with both forest and agricultural cover, which in our study area correspond predominantly to heterogeneous *montado* areas (Chapter 5). Other studies have found the same association between this unique agrosilvopastoral system and other taxonomic groups (e.g., mammals: Medinas et al., 2013; birds: Godinho & Rabaça, 2011; reptiles: Godinho et al., 2011). The landscape connectivity analysis also highlighted corridors with the highest movement probability, particularly in heterogeneous habitats, as opposed to agricultural sites associated with more intensive practices (Chapter 5). These results suggest that amphibians move preferentially within *montado* areas, and as a consequence, road segments intersecting these landscape units are more prone to present high roadkill abundances. This is particularly alarming in the study area ecosystem, as a 16-year assessment study of the *montado* in Portugal revealed a degradation of this system associated with intensification of livestock grazing and severe agricultural practices in some areas, along with poor land management. This has impacted natural regeneration and produced larger areas with no trees (Godinho et al., 2016), suggesting a slowly conversion to a more open agricultural landscape. Considering that higher levels of amphibian roadkill are often linked to higher local abundances (Orlowski, 2007; D'Amico, 2009), the observed decrease in roadkill over time (Chapter 3) may suggest that, if this trend prevails, amphibian persistence in the *montado* ecosystem may be at risk.

Evidence suggests that habitat restoration with a reversal of intensive agriculture practices towards more sustainable ones, leads to the recovery of wildlife populations (Donald & Evans, 2006). To this end, the European Union has created various programs and subsidies known as Common Agricultural Policy (CAP), aimed at (but not exclusively) reducing the negative impacts of agriculture and promoting sustainable environmental practices (Kleijn and Sutherland, 2003). However, in *montado* landscapes, these regulations and incentives have failed to guarantee landscape preservation in the long term, due to the lack of strategies that adequately integrate nature conservation into agricultural programmes (Pinto-Correia, 2000; Azeda et al., 2021). Though, the Nature Restoration Law (NRL) approved in 2023 and officially implemented in 2024 (Regulation (EU) 2024/1991) establishes the objective of restoring the degraded ecosystems of all Member States by 2050 (European Union, 2023). The NRL seeks to encourage landowners to integrate surrounding high-diversity natural landscapes into their agricultural practices through a multifunctional management model (e.g., Pinto-Correia and Godinho, 2013), for example, by planting native vegetation patches along their crops. Many of these practices typically occupy a small portion of the agricultural land, representing no competition for farmland use (Benayas and Bullock, 2012), while providing ecosystem services and supporting biodiversity. To further motivate the implementation of such measures in *montado* ecosystems, practices such as “payment for ecosystem services” (PES) – where landowners receive compensation to include certain sustainable management practices on their properties (Benayas and Bullock, 2012) – should be strengthened in our study area. These actions would safeguard the heterogeneity of the *montado*, increasing overall landscape permeability, while also generating added-value products or ecosystem services (Bugalho et al., 2011; Pinto-Correia and Godinho, 2013). Ultimately, these practices would benefit amphibian movement across the landscape. Since the NRL is still in its early stages of implementation and a comprehensive review of the CAP is pending, it is likely that the effects of such measures will require time to become visible.

6.4. Can we mitigate roadkill?

In some world regions, roadkill has surpassed hunting as the main source of human-induced vertebrate wildlife mortality (Forman & Alexander, 1998). The presence of roadkill mitigation structures may reduce the number of roadkill while also increasing road permeability for wildlife, fostering ecological resilience in fragmented landscapes (van der Ree et al., 2015). However, the question of whether roadkill can be mitigated has no straightforward answer, as it involves far more than simply limiting animal movement on road surfaces (although that may be an ultimate goal). Effective mitigation may require a comprehensive approach that addresses the broader ecological impacts of roads, considers the responses of wildlife groups, and ensures that mitigation measures are designed to target all the impacts including roadkill and limitations to movement. Furthermore, true mitigation might demand more extensive social and economic shifts, such as rethinking urban development and limiting resource consumption. Conservation strategies often prioritise local mitigation measures that are easier to implement but might be less effective in the long term. Adopting a broader strategy that reduces urbanisation (e.g., more compact urban centres) and prioritises green infrastructures (Tzoulas et al., 2007), while diminishing transportation dependence could better minimise road impacts on ecosystems at the long term. This, however, would require a paradigm shift toward less resource-intensive economic and infrastructure development - approaches that human societies often find challenging and fail to adopt.

We demonstrated that the presence of amphibian-oriented roadkill mitigation measures significantly reduced the roadkill risk for most of the occurring amphibian species in our study area (Chapter 4). However, the lack of tunnel usage and population data impedes a broader view of the ability of these measures to enhance landscape connectivity and consequent population viability in the long term (Schmidt and Zumbach, 2008). There are still few studies directly linking road mitigation with population trends, and thus effectiveness is often assumed rather than demonstrated (Schmidt and Zumbach, 2008; Jarvis et al., 2019). Since roadkill spatiotemporal dynamics suggest that amphibian local populations are depleting (Chapter 3), the implementation of effective roadkill mitigation measures has never been so urgent. Particularly because populations occurring near roads might receive fewer immigrants from other habitats, which can lead to reduced genetic input and increased risk of inbreeding. These factors further decrease the likelihood of population persistence and increase the risk of local extinctions (Jaeger et al., 2005).

Moreover, since most roadkill mitigation measures are long-lasting and typically expensive structures, their effectiveness should be guaranteed through regular maintenance, and also assessed across all terrestrial amphibian life stages: to determine their ability to reduce roadkill and their capacity to increase landscape connectivity. This includes movements by adults to and from reproduction sites, but also by juveniles after metamorphosis – from water bodies to aestivation sites; or any other type of dispersal movements (Joly, 2019; Cayuela et al., 2020). Such comprehensive information may be crucial to determine the true effectiveness of the mitigation measures. Typically, sites chosen for the implementation of mitigation structures primarily focus on adult amphibian movement. This preference arises because adult movement is usually concentrated in short periods, making it simpler to track, oppositely to juvenile movement which is more dispersed in space and time (Sinsch, 2014; Petrovan and Schmidt, 2019). Moreover, juveniles are smaller than adults, which makes them difficult to spot. Nevertheless, juvenile movement is of great importance, especially because in many amphibian species, they are the dispersal group (Joly et al., 2003; Rothermel, 2004). Consequently, much of the genetic input depends on the successful movements of juveniles (Joly, 2019). Understanding whether amphibians use mitigation measures to cross the roads in different terrestrial life stages is crucial to determining if these measures genuinely contribute to the long-term viability of the populations.

To sum up, answering the question of whether (amphibian) roadkill can be mitigated may still require further research: specifically, assessing the capacity of the roadkill mitigation measures to reduce roadkill across all movement-related life stages, as well as their potential to enhance landscape connectivity over time between suitable habitats crossed by roads. Therefore, efforts to gather this information should be made in future research studies.

6.5. Are amphibian populations declining as a result of roadkill?

Globally, the direct impact of wildlife-vehicle collisions on animal population numbers is substantial, with estimates suggesting that more than one million vertebrates are killed daily in certain world regions (Loss et al., 2014). While understanding critical landscape elements is essential for identifying what road sections need mitigation, insights into how wildlife responds to changes in those elements over time are crucial for developing strategic and effective long-term mitigation actions that may favour populations at local scales. Long-term studies on road effects at population-level remain rare; however, the use of roadkill data may constitute a useful tool for assessing the long-term survival of populations, as roadkill numbers can mirror live population densities (Orlowski, 2007; George et al., 2011). Nevertheless, the effect of imperfect detection (strongly linked to roadkill surveys) can lead to an underestimation of roadkill numbers and influence roadkill patterns (Santos et al., 2016) which is particularly relevant for small-sized species (Barrientos et al., 2018) such as amphibians (Hallisey et al., 2022). Our study allowed a holistic view of the temporal trends of amphibian roadkill and, based on that, the potential status of the populations inhabiting road surrounding areas. Yet, answering the above question requires careful consideration. The main conclusion of Chapter 3 is that the observed decrease in roadkill over time is simply due to fewer animals being present on the roads. Although a reduction in absolute roadkill numbers might seem positive, the lack of information about the proportion of amphibian populations crossing roads, raises a potentially far more concerning question: Why are there fewer amphibian casualties on roads? This trend likely results from factors both related to or independent of roads. For instance, populations inhabiting areas close to roads might be struggling with the possible increase in traffic volume, uncommon events induced by climate change (e.g., extended periods of drought), habitat fragmentation, or inherently smaller population sizes, as suggested by Matos and colleagues (2012). While our study and methodology provide a first insight that amphibian populations may be declining due to roadkill, the true underlying causes require further research. Our findings emphasise the urgent need to conduct population censuses to assess the status of amphibian populations occurring near roads. This should be the focus of future research, as understanding the reasons behind why fewer amphibians are present on roads can only be achieved by monitoring local populations. Only by doing so can we truly measure the impact of roads on local populations' resilience and viability.

6.6. How can remote sensing assist amphibian conservation?

In this study, we used satellite remote sensing data to derive environmental predictors to explain amphibian roadkill trends and habitat suitability. Our findings provide evidence that freely available high-resolution remote sensing satellite data can be used to predict both spatial and temporal dynamics of amphibian roadkill across multiple years. We demonstrated that remote sensing data were essential in tracking environmental changes at local scales, both inter- and intra-annually, due to its narrow spatial and temporal resolutions (Chapters 2, 3 and 5). By using this data in habitat suitability models, we were able to observe, for instance, annual shifts in the distributions of one urodele and one anuran species (Chapter 5). These insights were crucial to understanding how these species respond to variability in their habitats, adding valuable knowledge about their ecological needs.

The fine spatial resolution provided by remote sensing imagery was also important in identifying road surrounding areas where amphibian movement probability is higher and consequently, roadkill risk may also be higher. Identifying road segments with increased roadkill and movement probability is essential for targeting mitigation measures to enhance landscape connectivity and reduce roadkill risk, and remote sensing data can assist road planners in doing so. Furthermore, remote sensing information also assisted us to overcome the limitations of other less accurate products and offered a considerably less expensive alternative than collecting environmental data in the field (Wang et al., 2010; Arenas-Castro & Sillero, 2021), which in our case, would not be possible, given the lack of resources for such extended sampling period. This thesis, therefore, demonstrated the ability of high-resolution remote sensing data as a tool for advancing conservation efforts targeted at the amphibian group.

6.7. Modelling tools contribution

The availability of long-term roadkill datasets enables a variety of possible choices for modelling the negative impact of roads on wildlife. In this work we have applied several modelling tools that, together with remote sensing satellite data aided us in unravelling several roads' negative impacts at high-resolution spatial and temporal scales: for example, the use of generalised linear models on Chapter 2 and multi-season occupancy models on Chapter 3. The methodology of Chapter 3 enabled us to analyse the temporal roadkill trends for species with relatively few roadkill records (e.g., the Southern marbled newt) and for road sections that were not evenly sampled. To our knowledge, Chapter 3 constitutes the first published approach of multi-season occupancy modelling application to roadkill studies. Although this modelling tool requires substantial amounts of data making it unsuitable for many studies (MacKenzie et al., 2006), the growing availability of long-term datasets (e.g., citizen science, large databases of species records) may expand their applicability. The ability of these models to account for imperfect detection (MacKenzie et al., 2003) also allowed us to correct it, avoiding roadkill underestimations that ultimately can lead to imprecise implementation of mitigation measures (Santos et al., 2018).

Testing the effectiveness of the permanent drift fences in Chapter 4 was done using generalised linear mixed models applied to a before-after-control-impact (BACI) framework. The application of this modelling tool (and respective study design) made it possible to track whether differences in amphibian abundances on roads were in fact a consequence of the mitigation structure. Considering the high costs of installing this kind of structure, this approach should become standard practice (Rytwinski et al., 2016), as BACI study design is considered the most effective approach for assessing the impact of a stressor in the environment (Underwood, 1991) and consequently, the effectiveness of the roadkill mitigation measure. Experiments like the one in Chapter 4 provide valuable information and should be used as guides for road agencies willing to implement similar (or other) structures on other roads.

As emphasised throughout this study, landscape connectivity plays a crucial role in amphibian dynamics. The findings of Chapter 5 demonstrated that functional landscape connectivity can be used as a roadkill risk predictor for amphibians. While the application of circuit theory to road ecology is not novel (Dickson et al., 2018), the distinctiveness of our study lies in the fine spatial scale at which it was applied. The approach of Chapter 5 should be replicated, extending the application of the circuit theory to other narrower spatial scales and taxonomic groups. Additionally, spatial analysis at finer scales provides road practitioners with more precise information to guide roadkill mitigation efforts, aiding in reducing uncertainties in implementing mitigation structures.

6.8. Study limitations

While this study provides valuable insight into amphibian trends on road ecology and movement, it is important to acknowledge some identified limitations. First, our inferences on local populations were performed using only roadkill data (Chapter 3). Although roadkill data has been previously used for the same purposes (Schwartz et al., 2020), interpreting these trends requires caution, as a decline in roadkill numbers may not necessarily be indicative of an overall population decline, as the proportions of populations migrating across monitored roads are typically unknown (Beebee, 2013). A way to overcome this would be to validate the roadkill information with data from near-occurring populations. Systematic sampling of nearby water bodies, for instance, could provide valuable insights into the impact of roadkill on local populations. By comparing the quantification of roadkill to the abundance of larvae or adults in these water sources, researchers could assess the population status and more accurately evaluate the impact of roadkill (Cooke, 2011). The lack of knowledge regarding road effects on amphibian populations remains a significant obstacle to effective amphibian conservation.

Second, our conclusions regarding the effectiveness of amphibian-oriented roadkill mitigation measures would benefit from the inclusion of tunnel-usage data. Several approaches could be applied to collect this data, such as capture-mark-recapture, pitfall traps at the entrance of the tunnels, or even camera traps (e.g., Matos et al., 2019; Boyle et al., 2021). With this information we could successfully demonstrate whether amphibians can safely cross roads on both sides and whether these measures, beyond reducing roadkill, also enhance landscape connectivity, contributing to population viability in the long-term.

Lastly, sampling a larger number of water bodies would have allowed us to improve our suitability models and potentially even include additional species in the analysis. Although our results were similar for the Iberian spadefoot toad and the Iberian ribbed newt, the same may not apply to other species. Therefore, future continuous monitoring would provide valuable information on additional species, allowing for similar modelling analyses and comparisons with our findings.

Despite the extensive research in amphibian road ecology, many ecological questions remain unanswered due to limited understanding of the consequences of roads on amphibian populations. This knowledge gap impedes the development and implementation of targeted, multi-species solutions that can effectively reduce costs while broadening mitigation efforts.

6.9. Future prospects

Integrating ecological considerations into road planning and infrastructure development can contribute to minimising the negative impacts of roads on biodiversity (van der Ree et al., 2015). However, it is essential that monitoring and adaptive management practices ensure that the (future) implemented measures effectively address new challenges (e.g., environmental changes; expected traffic volume increase; road network expansion). Collaborative interdisciplinary research involving different practitioners is fundamental to achieving innovative solutions that foster coexistence between humans and wildlife (Schwartz et al., 2020). Moreover, public engagement and awareness initiatives should be prioritised by academia, as they can support conservation efforts, emphasising the intrinsic value of preserving wildlife and their habitats.

The increasing availability of new technologies, including high-performance computation and artificial intelligence (AI), offers new opportunities to enhance ecological research (Lopes et al., 2016; Sillero et al., 2018; Sousa-Guedes et al., 2019). More efficient algorithms and products will be developed, aiding many time-consuming tasks and producing more accurate predictive models (Tuia et al., 2023). Nevertheless, field-based studies will still be necessary. Accurate movement data is particularly relevant to better understand the interactions of amphibians and roads. This could be gathered through the use of tracking devices. However, these require further development to match higher precision and extended battery-life, particularly for small-sized fauna (Kays et al., 2015). Enhanced tracking devices would contribute to increase the current limited knowledge of amphibians' movements and population dynamics. Such advancements are especially urgent in species-rich ecosystems like the *montado*.

Since roads are critical to human society development, the urgency in combining an integrated approach that consolidates ecological considerations into transportation planning has never been greater, especially with the projected expansion of road networks in high-biodiversity regions (Meijer et al., 2018). Reaching a balance between human society's needs and biodiversity conservation is now more vital than ever.

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